ISSN 2088-9771

PROCEEDING

Vol. 1 No. 1 October 2011

ICBB

The International Conference on Bioscience and Biotechnology 2011

Interfacing Biotechnology, Natural Product Chemistry, and Tropical Biodiversity for Sustainable Development

Yogyakarta, October 11th-12th, 2011

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Biology Department
Faculty of Science and Technology
State Islamic University Sunan Kalijaga
Yogyakarta - Indonesia

Supported by

Universiti Kebangsaan Malaysia
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International Conference on Bioscience and Biotechnology (ICBB) 2011

October 11th-12th, 2011, Yogyakarta, Indonesia

PROCEEDING

State Islamic University Sunan Kalijaga Yogyakarta
Faculty of Science and Technology
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PREFACE

Assalamu'alaikum Wr.Wb.

First, let us express our gratitude to Allah SWT for His blessings so that the International Conference on Bioscience & Biotechnology (ICBB) 2011 can successfully be held on 11-12 October 2011 in Yogyakarta, Indonesia.

The event was organized by Department of Biology, State Islamic University Sunan Kalijaga which supported by Indonesian Society for Biodiversity and Universiti Kebangsaan Malaysia.

More than 140 well-known experts, researchers, lecturers, and students have been invited and attend this conference. The high enthusiasm to this conference was in line with one of the ICBB objectives, i.e. to provide an international forum for students, researchers, academics and practicioners to share the most recent research, theory and practice. We further hope that the network built through this conference can be a good foundation for future collaboration research among ICBB 2011 participants.

Ladies and Gentlemen, A large proportion of the world's biodiversity is found in tropical countries, with comprises of more than 70% of world biodiversity. Among them, many have been recognized as the important sources for food, medicine, timbers, and as material supporting for everyday life. The chemical and biological diversity have interrelationship and interdependence as a whole system. Each species, each dimension of the planet, makes its unique contribution to the good of the whole. Our survival is intimately connected to the survival of the other species of the planet.

Finally, I would like to extend my sincere thanks and appreciation to the invited speakers, reviewers, editors, sponsors, people in the Department of Biology, Faculty of Science & Technology, State Islamic University Sunan Kalijaga as organizers, who have dedicated their time and efforts to this conference.

See you in next ICBB conference!

Wassalamu'alaikum Wr. Wb.

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POSTER PAPER

International Conference on Bioscience and Biotechnology (ICBB) 2011

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Abstract from

Keynote Speech

Synergy Research: Approaching a New Generation of Phytopharmaceuticals

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The successful use of **herbal drug combinations** in Traditional Medicine e.g. in European phytotherapy, traditional Chinese medicine or medicines of Ayurveda makes it necessary to find a rational for the pharmacological and therapeutic superiority of many of them in comparison to single constituents.

Which mechanisms my underlying this therapeutic superiority? A first approach towards rationalizing this synergy effect was made by Berenbaum [1]. He developed the **isobol method** as pharmacological tool to differentiate between real synergistic and additional effects of a mixture of two plant constituents or two plant extracts. Among the possible mechanisms underlying these synergy effects, **the multitarget effect** seems to be the most important and interesting one. Since recently molecular biological support for this hypothesis can be obtained from the new genomic microchip array method.

Meanwhile pharmacological evidence for synergy effects has been documented of monoextracts such as *Ginkgo biloba* [2], *Hypericum perforatum* [3], *Cannabis sativa* along with many other herbal extracts and also their combinations [4]. Recently in many publications very effective synergy effects were reported when antibiotics are combined with natural products or plant extracts (essential oils, polyphenols, terpenoids) to combat the worldwide increasing **bacterial multiresistance** (MBR) [5]. Here **polyphenols** e.g. **Epigallocatechingallate** of green tea or the **curcuminoids** of *Curcuma* spec. have found great attention at present as pure compounds or enriched in standardized extracts alone or in combinations with chemotherapeutics.

These polyphenols as monomers or oligomers are able to induce apoptosis, arrest the cell cycle of tumor cells, inhibit insulin – like growth factors, detoxify some enzymes and delay as antioxidants the appearance of several markers of aging and oxidative stress – to offer only a few examples. In combination with anticancer drugs they can inhibit **ABC-cassette transporter proteins** in tumor cells and thereby enhance the efficacy of anticancer drugs. Meanwhile several clinical trials do exist, which show that standardized mono or multiherbal drugs possess therapeutic equivalence with chemotherapeutics at less or lacking side effects.

In summarizing the results available so far, we can conclude that progress in synergy research will not only provide a new legitimation for phytotherapy, but also enhance the possibility to use new phytodrugs alone or in combination with chemotherapeutics for the treatment of diseases which have been treated previously through chemotherapy only.

Natural Products: Chemical and Biological Potential of the Rain Forest

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Abstract – "Why didn't they develop natural product drugs in a sustainable manner at the beginning of this century?" In 2035, when the Earth has a population of at least 10.0 billion, will this be our legacy as the world contemplates the costs and availability of synthetic and gene-based products for primary health care? Acknowledging the recent history of the relationship between humankind and the Earth, it is essential that we consider the health care issues that we are leaving our descendants.

There is a vast health care "gap" in the world, particularly in the area of access to quality drugs, and a vast gap in the development of drugs for major global diseases. For most people in the world, plants, in their various forms, remain a primary source of health care. In the developed countries, natural products derived from plants assume a very minor role as prescription and over-the-counter products, even with the widespread use of phytotherapeutical preparations. Pharmaceutical companies have retrenched significantly on the disease areas of focus. These research areas do not include the prevalent diseases of the middle- and lower-income countries. Natural product extracts from plants are also not a part of large scale, ultrahigh-throughput screening programs in pharmaceutical industry.

What then is the vision for natural product research to maintain the choices of drug discovery and pharmaceutical development for future generations? In this presentation we will examine some facets of how natural products must be involved globally, in a sustainable manner, for improving health care. We will discuss access to the biome, the acquisition, analysis and dissemination of plant knowledge, natural product structure diversification, biotechnology development, strategies for natural product drug discovery, and aspects of multitarget therapy and synergy research. Options for the future are presented which may be significant as countries decide how to approach their own disease burden and the needs of their population for improved access to medicinal agents.

Keywords: natural products; sustainable drug discovery; biotechnology; structure diversification; rain forest resources; strategic implications

Vertebrate Biodiversity and Conservation in Peninsular Malaysia

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ABSTRACT

This presentation aims to highlight the research trends on vertebrate (or wildlife) ecology, biodiversity and conservation in Peninsular Malaysia. Wildlife management and conservation issues in Peninsular Malaysia have changed tremendously in the last 100 years primarily due to changes in land use. Rapid land conversion to plantations, housing areas and other activities since the beginning of 1960's has sacrificed large track of forest areas in Peninsular Malaysia. At present, rapid loss of forest habitat in the past has threatened the survival of many wildlife species (such as rhino) that are sensitive to changing environment (habitat size and quality). As these species have reduced chances to propagate and survive in the wild, they could be at the brink of extinction. Some species (such as monkeys) can easily adapt to the changing environment, propagating and inhabiting the urban setting. Other species may not increase their populations (such as elephant and tapir) but they are displaced outside their normal habitats. Both groups can cause conflicts to human which could result in economic loss and sometime loss of life. Similarly, the vertebrate or wildlife studies have also changed from solely to document the number of wildlife species (diversity), population and distribution in the early 1900's to recently more to answer important questions such as how to control conflict wildlife and how to save the endangered species. In recent years, wildlife studies in Peninsular Malaysia are focusing more on ecology of both wildlife and habitat, and biology especially reproduction although documentation of available species are continuously being done to monitor the species that are still available especially within the Protected Areas. Several strategies have been implemented to control the conflict wildlife species while several captive breeding programs have been developed to save endangered species. Finally, more organized and concerted research efforts are needed for preservation and conservation of vertebrate species in Peninsular Malaysia.

Male Fertility Regulation: Research in Malaysia

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ABSTRACT

Population explosion in some countries gave significant impacts on socio-economy and health in family and the society. Family planning has been promoted to overcome such conditions. The development of antifertility or contraceptive agents from medicinal plants which are safe and effective with minimum side effects is important but remains a big challenge in research. The importance of drugs of plant origin in the pursuit of fertility regulating agents for the male from natural products has long been recognized. Pharmacological data from previous reports showed that some compounds in plants are capable of interfering the production of androgen hormones, affected spermatogenesis process, abortifacients as well as the spermicidal activity. This review includes studies on several plants such *Centella asiatica* L., *Andrographis paniculata* and *Carica papaya* seeds as potential candidate for development of male contraceptive agent. The type of extract, doses, animal model and pharmacological activity of these materials have been reviewed to add impetus to further research and collaboration to resolve the problem of population explosion. Besides screening of potential contraceptive plants, various strategies are being employed to identify the molecules relevant to contraception. Studies to elucidate the mechanism of expression of several novel proteins having a role in sperm production, maturation and fertilization are underway.

International Conference on Bioscience and Biotechnology (ICBB) 2011

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Oral Paper Cluster 1

Analysis Interaction of Glucosyltransferase Inhibitor of Caries from Fatty Acid by Molecular Docking Simulation

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The most common human oral disease is the oral infection dental caries. Dental caries mostly caused by S*treptococcus mutans* that produces extracellular glucosyltransferases (GTFs) that synthesized glucan from sucrose. These glucan is important in determining the permeability properties and adhesiveness of dental plaque. In order to prevent synthesis of glucan formation, inhibitor substances is needed to block the activity of enzyme glucosyltransferase. Nowdays the enzyme inhibitors are used to prevent dental plaque formation are not optimally effective, so the new emerge natural substances is need to develop. In this research, we have conducted *in- silico* study to analysis of Oleic acid, Palmitic acid, linoleic acid and linolenic acid from coconut oil, which has a role as GTFs inhibitor of dental caries. The docking result identified that oleic acid have greater affinity and ability to inhibit of GTFs, this affinity oleic acid complexs is 7.262 µM and energy minimized is -5.6863 Kcal/mol . They have residues contact of OH binding to GTFs are 2 formed hydrogen binding in catalytic site lys228 with score 28.7 % and H distance 2.61 A^o , gly315 with score 35.8 % and H distance 2.77 A^o. The docking result showed that oleic acid has better binding energy and affinity than other bioactive compounds.

Keywords: Glucosyltransferase(GTF), Fatty acid, Caries, Docking simulation

Introduction

Dental caries is the medical term for tooth decay or cavities. It is caused by specific types of bacteria. They produce acid that destroys the tooth's enamel and the layer under it, the dentin. *Streptococcus mutans* has been strongly implicated as a causative organisms of dental caries. The ability of this bacteria to bind to high-molecular-mass glucans synthesized from sucrose is recognized as an important determinant in the formation of dental plaque and tooth decay (Hamada & Slade, 1980)[a]. This organism synthesizes water-insoluble glucan from sucrose with its cell-free and cell-bound glucosyltransferases (GTFs) (Montville, *et al.* 1978). De novo synthesis of glucan is essential for the adherence of *S. Mutans* to tooth surface (Hamada & Slade, 1980)[b]. Many chemical and enzymatic procedures for eliminating *S. Mutans* from tooth surfaces have been explored. However, there are few reports concerning substances that inhibit GTFs activity. Thus, the protein that enable bacteria to bind glucans have attracted interest as potential targets for inhibition of the caries process. Using GTF inhibitors is considered to be a useful means of preventing glucan formation without disturbing the balanced of helpful oral bacteria. In the present study, we developed an agent that can be used either as food or a medicine for safely treating decaying teeth by docking simulation of several fatty acids *In silico*.

In silico is used in the screening process of bioactive compounds or molecular simulation as a drug (Kitchen, *et al.* 2004). Analysis was based on Gibbs energy values, inhibition constants (Gohlke, *et al.* 2000), conformation of the structure, affinity, and hydrogen bonding of enzyme and ligands (Datta, 2002).

Materials and Methods

Materials of this research such as: Protein structures may be downloaded from the site with specific keywords or a PDB alphanumeric filename. The ligands of fatty acid performed using ACD Lab software. MOE software: The docking experiments were performed using the docking software MOE 2008.

Enzyme used in this study is GTFs. GTFs sequences were obtained from complete sequences from *Streptococcus mutans* bacteria. Complete sequences were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/genomes/flu/). Multiple sequence alignment method was based on the ClustalW-program (www.ebi.ac.uk/Tools/clustalw2/index.html website). Homology modeling was performed using the Swiss model which can be accessed through http://swissmodel.expasy.org/SWISS-MODEL.html. A three-dimensional structure was obtained that has the highest similarity with GTFs sequences from PDB (Protein Data Base) with code number 1YRO.

Ligand docking process performed on the fatty acid (linoleic acid, oleic acid, palmitic acid, linolenic acid, oleic methyl, and palmitic methyl) against the receptor. Docking process is performed only on all the amino acids of the receptor. In the process of docking receptors are rigid conditions while the ligand will be conditioned on the state of a flexible so that it can freely move and rotate. The parameters set in the docking process involve setting the scoring function using dG London. Scoring function to measure the biological activity by binding and interactions that occur between the ligand with the target protein (Nylander, 2007).

Molecular docking. For getting the ligands-receptor binding energy procedures of molecular docking were followed. The detailing of the procedure is as follows.
Preparing the ligand and macromolecule files for MOE we prepared the files as follows:
(a) *The Macromolecule file*: The downloaded PDB files were first read in MOE, added waters removed and polar hydrogens were added. And Geometry optimization and minimization receptor Geometry optimization and energy minimization of three-dimensional receptor structures performed using MOE software running on a single computer Intel Pentium Dual Core. The algorithm used is the alpha sphere with a maximum RMS gradient convergence 0.01 kcal / mol Å and molecular mechanics force field parameters AMBER2
(b) *The Ligand file*: In a similar procedure, the ligand files were read in MOL, all hydrogens added, charges added and non-polar hydrogens merged and saved with .mol extension. Geometry optimization and energy minimization of three-dimensional structure fatty acid using ACD Lab software running on a single computer Intel Pentium Dual Core. The algorithm used is the alpha sphere with a maximum RMS gradient convergence 0.01 kcal / mol Å and molecular mechanics force field parameters MMFFx
(c) Preparing the docking parameter file: The docking parameter file, which instructs MOE about the ligand to move. The process begins with preparation docking files are done using a docking program contained in the MOE software. All the molecules fatty acid (to then called ligands) and the enzyme, hydrogen is added to both polar and charge while the hydrogen nonpolarnya MMFX in merge. File ligand and the enzyme is stored in the format Mole for later use in the preparation parameters. Docking calculation algorithm is run with the parameter Alpha sphere with population size 150, as many as 10 million energy evaluations and repetitions (search runs) as much as 100 times. This parameter is saved in Mdb format as a file that will be used to run the docking process.

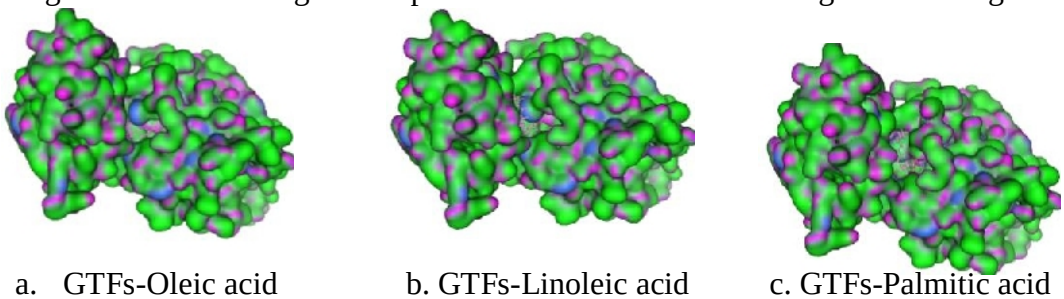
Results

The docking results of the fatty acid ligands result 3 best ligands. Screening based on ΔG and Lipinski's Rule of Five were employed from 3 ligands were resulted 1 of best ligand (Table 1).

Table 1. Docking results of GTFs and ligands

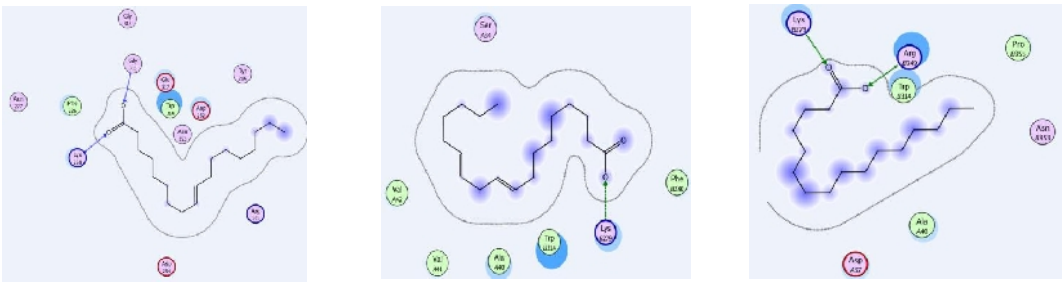
Ligands	ΔG (kcal/mol)	pKi (μM)	MR	H bonds	Log P
Linolenic acid	-3.4550	6.731	279.44	1	4.550
Linoleic acid	-4.2375	5.902	294.48	1	5.973
Oleic acid	-5.6863	7.262	281.460	2	4.774
Oleic metyl	-5.0030	5.447	296.495	-	6.197
Palmitic acid	-3.3276	6.155	255.422	2	4.218
Palmitic methyl	-3.7645	4.808	270.457	-	5.641

The three of best ligands have minimized ΔG and have high of pKi value.
The figure of three best ligandcomplexwith GTFs was shown at Figure 1 and Figure 2.



a. GTFs-Oleic acid b. GTFs-Linoleic acid c. GTFs-Palmitic acid

Figure 1.Complexs 3D of GTFs with Ligands



a. GTFs-Oleic acid b. GTFs-Linoleic acid c. GTFs-Palmitic acid

Figure 2. Complexs 2D of GTFs with Ligands

One of the best ligands were selected based on the number of hydrogen bond as the interaction to the catalytic site of GTFs (Table 2). High score and distance of hydrogen bond shown at Table 2

Tabel 2. Score and distance complex GTFs-Ligands of Hydrogen bond

Ligand	Hydrogen Bond	Score (%)	Distance (Å)
Linolenic acid	Lys279-O	55.2	2.51
Linoleic acid	Lys351-O	32.9	2.53
Oleic acid	Lys228-O	28.7	2.61
	Gly315-O	35.8	2.77
Oleic methyl	-	-	-
Palmitic acid	Lys229-O	14.8	2.42
	Arg349-O	30.9	2.49
Palmitic methyl	-	-	-

Discussion

The docking result of oleic acid has pKi 7.262 μM, this value indicate that the oleic acid has stronger affinity and interaction to form complex compounds with glucosyltransferases (GTFs) compare to another fatty acids. The value of pKi of oleic acid ligand is effective and interact strongly with GTFs (table 1). This means that the larger the value of the pKi, the smaller the Ki of the ligand. So the value of pKi can be used to determine the level of effectiveness in forming the complex enzyme with ligand.
Hydrophobic molecules show better log P (table 1). The ligand oleic acid (4.774), palmitic acid (4.218), and linoleic acid (5.973). Lipinski's rule of five mention that the poor absorption or permeation are more likely when the log P is over 5. Hydrophobic nature is shown green color, hydrogen binding is shown by cyan color and mid polar is shown by blue color (figure 1).

Hydrogen bonding of 3 best docking fatty acid was identified by MOE 2008.10 software on Lig X interaction program. The result of identification of hydrogen bonds between amino acid residues with GTFs ligand can be seen in table 2. Oleic acid has two H-bonds, linoleic acid has one H-bond while palmitic acid has two H-bonds. Hydrogen bonding contribute to the affinity of the ligand in forming the complex with GTF enzyme, which is due to electrostatic interaction between hydrogen atom of oxygen or hydrogen atom of ligand with a residues vice versa. Oleic acid ligand bind to catalytic site into the site of Lys228 and Gly315 which formed hydrogen bond with the active site of two bonds with score Lys (28.7 %) and H distance 2.61 (Å), Gly (35.8 %) and H distance 2.77 (Å). Linoleic acid bind to catalytic site into the site of Lys 351 with the active site of one bond with score (32.9 %) and H distance 2.53 (Å). Palmitic acid bind to catalytic site into the site of Lys 229 and Arg 349 at the active site of the two bonds with Lys (14.8 %) and Arg (30.9 %) and the H distance is 2.42 (Å) and 2.49 (Å).

Conclusion from this study we found the best ligand for docking glucosyltransferase is oleic acid and further study can be performed experiment in laboratory by using natural substances to inhibit glucan forming and prevent dental caries.

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Effect of Nanocomposite-based Packaging on Postharvest Quality of Water Content-treated Coffee Beans during Storage

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Abstract

The objective of this study was to evaluate the physical, chemical, and sensory qualities of green coffeebeans (Coffea arabica L.) during storage in nanopackaging packaging. A novel nanocomposite-based packaging (NCP) was prepared by blending polyethylene (PE) with nano-Ag,chitosan nanoparticle and montmorillonite. The effects of NCP on the quality parameters of water content tretaed coffee beans were investigated during the 21 d of storage at 25 °C. The results showed that adding nanoparticles to the PE significantly decreased the oxygen, water vapor permeability and longitudinal strength . The weight loss, water content , color variation and proximate content of coffee bean were significantly inhibited by 22.67%, 124.84%, 23.46% and 14.42% The results of this analysis demonstrated that this NCP can potentially increase the effectiveness of methods used to preserve and maintain quality in coffe beans during postharvest storage.

key word : NCP, coffe bean, water content

1. Introduction

Coffee is one of the most widely consumed beverages in the world because it contains a wide range of aroma compounds, which is a very important factor in food quality [1].Coffee beans are obtained from the plants Coffea arabica and Coffea canephora (mainly variety robusta). The former is more valuable because its beans produce a better tasting beverage, which is therefore more expensive than the robusta coffee [2] .

Coffee is an agricultural product with a quality-based price. The value of coffee increases significantly with improvements in quality, which are necessary to obtain new markets. During roasting,the taste and aroma of coffee develop from ingredients originall present in raw beans. Taste and aroma are the principal factors affecting beverage quality Storage is one of the stages following production that strongly influences the commercialization of coffee beans. Storage is therefore considered one of the most important factors for maintaining final product quality, meeting between-harvest demand, and securing the best market price for the producer.

Traditionally, green coffee beans have been stored in jute sacks.Jute is most frequently used because it is readily adaptable to smallscale commerce and because it is easily sampled for lot inspections. Elevated operational costs that result from the need for manual handling represent one disadvantage of storage in jute sacks..Another disadvantage is

rapid deterioration in quality when the beans are stored in warehouses without ambient air control. Containers called “big bags” represent another form of storage used in Brazilian warehouses. The ease of mechanized handling, along with operational economies of scale, represent the principal advantages offered by this method of storage. However, big bags, like jute sacks, have the disadvantage of being permeable to water vapor and to gases present in ambient air, affecting the color and the organoleptic properties of the beans[3], and Nobre et al. [4] have stated that storage in hermetically sealed systems that permit atmospheric modification or control represents a viable alternative for preserving coffee bean quality. Certain additional costs are acceptable for the preservation of quality in select coffees of higher value.

Recently, the application of the nanocomposite concept has been proven to be a promising option in order to improve above mentioned properties conveniently [5] It is worth emphasizing many diverse characteristics existed in nanocomposites including composite reinforcement, barrier properties, flame resistance, electro-optical properties, cosmetic applications and bactericidal properties.

Relatively little research has been conducted to the food packaging involving in nanotechnology, such as material development of biodegradable starch/clay [6] whey protein isolate/ clay[7] polylactides/nanoclay composite films [8] and their application in Chinese jujube [9] green asparagus [10], orange juice [11] and Chinese bayberries [12]. Microbial growth rate inorange juice were significantly reduced as a result of using packaging material containing Ag and ZnO nanoparticles, which prolonged the shelf life of fresh orange juice up to 28 days without any negative effects on sensorial parameters [11]. Our previous study also showed that the nano-packing had quite beneficial effect on sensory, physicochemical, and physiological quality of fresh strawberry than polyethylene bags [13] .To the best of our knowledge, there are no published reports on the effect of nanocomposite-based packaging on preservation of kiwifruit. Therefore, the objective of the present work was to develop a preferable nanocomposite-based packaging to preserve coffee bean and to investigate the effect of NCP on maturity of coffe bean induced by watwr vapour during cold storage. To well understand the effect of this novel nanocomposite-based packaging material, the morphological characterization, physical properties and antibacterial effect of the nanocomposite were conducted as well.

Better understanding of storage factors and the advent of new forms of packaging permit extension of coffee storage times. These developments are of immense importance for preserving product quality. Preservation of product quality over longer periods of storage

secures a longer sales period for growers and guarantees better prices. To achieve these goals, the present study proposes and evaluates a new storage system that preserves the physical, chemical, and sensory qualities of stored green coffee beans on a commercial scale using nanopackaging.To reduce these effects of microbiological, chemical and physical events, it is possible to act on food processing or, more usually, on packaging. However, toughness and other properties such as thermal stability, medium gas barrier, low solvent resistance (e.g., against water) and antibacterial properties of pure polymer are often insufficient for food packing applications [14]

2. Materials and methods

material The low-density polyethylene (PE) was used as matrix material (Translucency, Melt flow index 2.2 g/10 min, density 0.92 kg/m3, softening point 95 °C, A. The nanopowders (35 wt.% of nano-silver, 40 wt.% of nanoparticle chitosan and 25 wt.% of Na^+ montmorillonite) in the range of 40–80 nm were obtained from a analytical chemistry laboratory, state universiti of Jakarta.

2.1. Preparation and characterization of nanocomposite based packaging

Firstly a PE-nanocomposite masterbatch containing 30 wt.% of the nano-powder, 56 wt.% of PE granule and 14 wt.% of cross-link reagent KH-570 were immingled in uniformity through a high-speed mixer for 1 h. After air cooling, they were extruded to PE nanocomposite masterbatch using a twin-screw extruder with a screw diameter of 22 mm, a screw length/diameter ratio of 42 and a screw speed of 600 rpm.

In the second extrusion step, 0.15 kg of masterbatch and 3.85 kg of PE granule were immingled for 30 min. Subsequently the compounds were blown into a film of 50 μm thickness via a plastic extruder After cooling, films of 50 μm thickness were used to make bags of 20×22 cm2 using a heat sealer (Polyethylene bags of the same thickness and size without nanocomposite masterbatch nano-powder served as controls.

2.2 Surface area analysis

The specific surface area and average pore size of the nanopackaging film were determined by nitrogen adsorption at 77 K (Quantachrome Autosorb 1).The nanopackaging film were outgassed at 300 °C for 12 h and 150 °C for 8 h, respectively. The BET method was used for the corresponding calculation.

2.3. Physical properties analysis of NCP

Measurements of the water vapor permeability (WVP), oxygen permeability (OP) and longitudinal strength were carried out by thesheet-cup method, differential-pressure method

and mechanical determination respectively according to National Standard of Indonesia(SNI . The test film was sealed to a permeation cell with a 50% relative humidity gradient across the film at 23 °C. Five measurements were performed for each sample.

2.4. Plant material

The experiment was conducted in a warehouse of the Coffe Society PT in Sukmajaya Bogor, Eas Java, Indonesia. The coffee used in the experiment was obtained from a lot taken from the 2008 (Coffea arabica L.) crop and passed through 17 and 18 screens. The beverage made from this lot had a minimum score of 80 points, classifying it as good-quality coffeeon the Specialty Coffee Association of America (SCAA) scale. Coffee was bagged in 1 kg quantities in nanopackaging pastic sack and LDPE plastic.

2.5. Treatment

The coffee samples were randomly collected using a grain sampler in the nanopackaging For all package, 500 g of coffee beans were collected at 3-months intervals at 0, 1, 2, 3 and 4 months of storage. The samples were analyzed to determine water content, color, and content of sugars. Sensory analysis was also performed at these sampling times. The analyses were performed at the Laboratory of Agricultural Products, Department of Food and Technology, Institute of Agiculture Bogor. All analyses were made using bean samples passed through 17 and 18 screens to guarantee uniformity during roasting.

2. 6. water content

Water content was determined by oven-heating at 105^0C for 16 h. Bean color was determined on a Minolta model CR300 colorimeter by direct reading of the coordinates (L), (a), (b) and according to the method described by Nobre (2005).

2.7. Color measurement

Color was measured using a digital imaging method that used a combination of a digital camera (Panasonic, Japan), a computer, and a graphics software. A Petri dish containing 25 ml of coffe bean was placed into the lighting system that consisted of two CIE source D65 lamps 45.0 cm long, mounted on the two sides of a frame installed on either side of the Petri dish, 30.5 cm above and at an angle of 45° to the coffee bean sample plane. Images of the bottom surface of the coffe bean were taken and saved using the digital camera that was placed 30.5 cm above the sample with its lens facing down wards towards the orange juice. The color was analyzed using the Photoshop software. By turning on the grid feature in Photoshop, a grid was superimposed on the sample. As the computer pointer was placed at a grid point along the x or y axis, L, a, and b values corresponding to the pixels of that grid

point were obtained from the Info Palette. The total color difference (ΔE=[(ΔL)2+(Δa)2+(Δb)2)]1/2) was determined in duplicate using CIE L, a, and b values .

2.8. total sugars and reducing sugars

Tissue (50 g) from twenty coffe bean was immediately homogenized using a blender (HR2864, Philip) for the determination of total soluble sugar and reducing sugars. Twenty-five gram aliquots of the homogenates were transferred to a beaker and 150 ml of distilledwater were added. The mixture was heated in a water bath of80 _C for 0.5 h and then filtered into a 250 ml volumetric flask. The contents were made up to 250 ml with distilled water after the addition of about 3 ml of 1 M zinc acetate and 0.25 M potassium ferrocyanide mixture. Aliquots of 10 ml were titrated to measure the contents of total soluble sugars and redureducing sugars by the Fehling's method, using glucose as reference.

2.9. Sensory evaluation

A sensorial test was run to determine packed fruit star whole quality as determined by its appearance. A panel of seven judges assessed the sensory characteristics of the investigated fresh-cut produce during the entire observation period, according to the procedure reported in the literature. Freshcut produce was used as control (score=5). The products were presented on coded plastic dishes. The intensity of the evaluated general appearance was indicated on a scale from 1 to 5, where 1–2=very poor, 3–4=fair, and 5=excellent. The sensory evaluation was used to determine the shelf life of packed produce. Scores below 3 for any of the attributes assessedwere considered as an indication of food product unacceptability. During the test sessions, the sample presentation order was randomized.

3.Result and discussion

3.1 Surface area analysis

Fig. 1 shows the nitrogen adsorption–desorption isotherms measured on nanopackaging and PE samples. The specific BETsurface areas of PE packaging and nanopackaging were 49 m2/g and 24 m2/g, respectively. The lower surface area of nanopackaging was attributed to the compact packing of the chitosan molecules in the interlayer space, resulting in pore blocking that inhibited the passage of nitrogen molecules. The average pore diameter of nanopackaging was 15.6 nm compared to 14.8 nm of PE..

3.2. Physical properties analysis of nanopackaging

Mechanical strength is generally required to maintain the structural integrity and barrier properties of films. Therefore, to provide more understanding on the physical and mechanical characteristics of this prepared film, WVP, OP and longitudinal strength were performed as a function of nanoparticles. As shown in Table 1, the WVP and OP of the pure PE films were 2768.35 cm^3m^{-2} 24 h^{-1} $(0.1\ MPa)^{-1}$ and 6.85 gm^{-2} $(24\ h)^{-1}$; as for nanocomposite films, the values decreased by 1802.13 and 4.98 respectively.

Table 1 Physical properties of nano packaging and normal packaging films.

Films	Oxygen permeability cm^3m^{-2} 24 h^{-1} (0.1 MPa)$^{-1}$	Water vapor permeability gm^{-2} (24 h)$^{-1}$	Longitudinal strength (MPa)
nanopackaging	6.85	2768.35	29.68
PE packaging	4.98	1802.13	31.96

Longitudinal strength of NCP film was 31.69% higher than the normal film. Recently the application of the nanocomposite concept had been proven to be a promising option in order to improve mechanical and barrier properties [6]. It could be inferred that the nanoparticles could affect the WVP, OP and longitudinal strength of films by the exfoliated montmorillonite which could yield significant mechanical property advantages as a modification of polymeric systems.Generally, this layered filled polymer composites exhibited extraordinary enhancement of mechanical and physicochemical properties at a low level of filler concentration in comparison to pure polymer.

3.3 water content

Coffee beans in jute sacks showed an elevated water content until the sixth month of storage. The value of water content increased, on average, between 9.80% and 11.40%. The water content remained in equilibrium with the temperature and the humidity, relative to the ambient air. The water content in the impermeable packaging remained stable at approximately 10% throughout the storage period (Fig. 2).According to Harris and Miller (2008) the water content required for secure storage is between 10% and 11%. These authors have verified that coffee beans stored in GrainPro effectively maintained a stablewater content level for four months. The results of the present study demonstrate that a stable water content level can be maintained in hermetic big bags or GrainPro for 12 months. An elevation of water content in processed beans in jute sacks can compromise quality. According to Vilela et al. (2000), the increase in water content in the green coffee during storage produces undesirable changes in the physical-chemical composition of the beans.

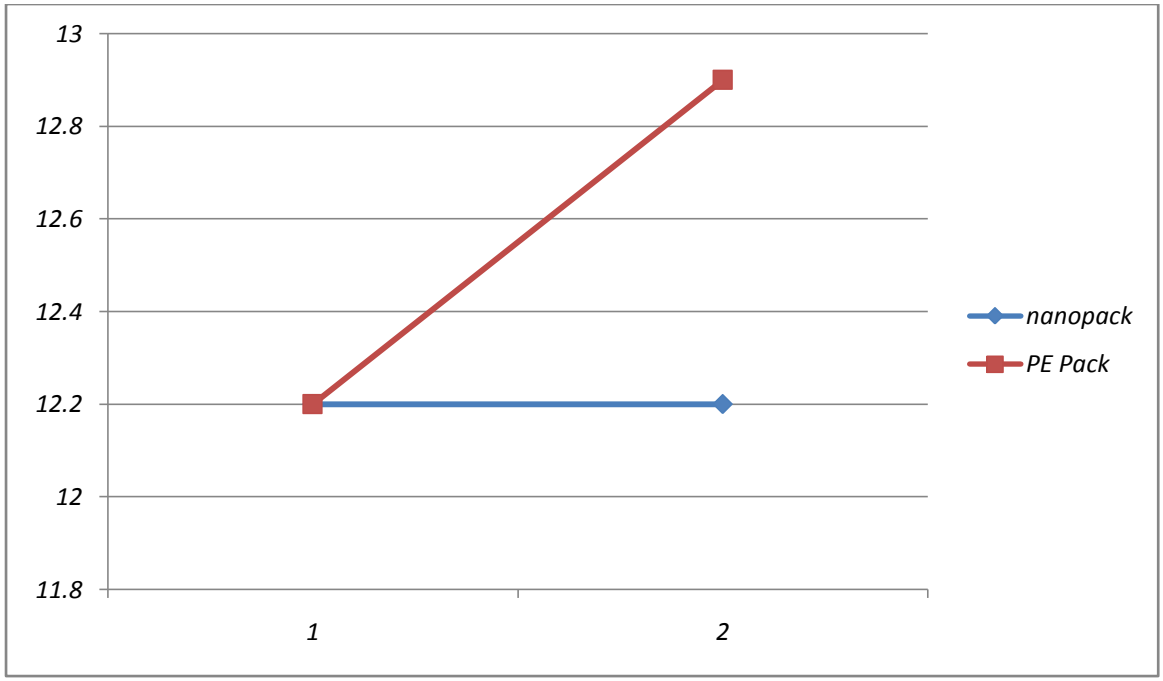


Fig. 2. Average values of water content for storage of green coffee. Experimental treatments: nanopackaging and Pepacaking film

3.4 Colour

Colour parameters for crude beans are presented in Table 2. Table 2 shows the changes in total color differences (ΔE=[(ΔL)2+(Δa)2+(Δb)2)]1/2) for nanpackaging compared with pure LDPE. Statistical results show significant differences ($p<0.05$) in ΔE after 7 days of storage, indicating that storage time is an important factor influencing color value and ΔE It can be observed all the nanopackages tested had a significant difference in their ΔE values after 28 days compared with pure LDPE. It is clear that ΔE values are lower for nanopackages.

Table 2 Effect of packaging on total color differences (ΔE) during 21 days storage at 25 °C.

No	Storage time (day)	ΔE	
		nanopackaging	LDPE packaging
1	0	0	0
2	7	5.62	4.62
3	14	6.14	5.12
4	21	7.93	6.88

3.4. Total soluble sugars and reducing sugars

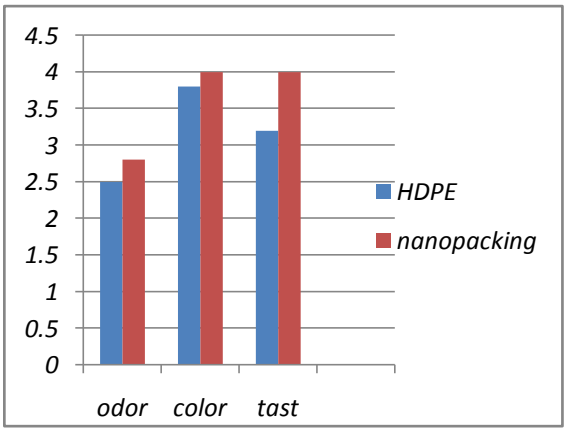
The sugar content was related to flavour quality for a variety of fruits and it determines the optimum time for harvesting. As shown in Fig. 3a, the total soluble sugars of Coffe bean

with different packings continued to increase throughout the 21-day storage at room temperature. Nano-packing could significantly inhibitthe increase of total soluble sugar content compared with the control. On day 21, the total soluble sugars of the nano-packinggroup reached 28.4%, which was significantly lower than the controlof 30.0% ($P < 0.05$).Compared with the total soluble sugar content, similar trends inthe reducing sugars content were observed during room temperaturestorage (Fig. 3b). The reducing sugars content of the nanopacking group was lower than that of the control, suggesting that the coffe bean with nano-packing synthesized reducing sugars at alower rate than did the control. These results indicated that theapplication of nano-packing might be able to slow the metabolismto give prolonged storage life to the fruit.

3.7 Sensory

Fig. 5 shows the change of sensory attributes of star fruit packed in different packages. The high similarity observed in color attribute scores of the packages after 28 days of cold storage ($p<0.05$) indicates that the change in the color of the samples is still invisible.

These results correlated well with the values of browning index. Odor attribute is greatly influenced by microbial growth and may lead to fermentation in orange juice during storageAfter 15 days of storage, a significant difference is observed between the odor of star fruit packed in the test packages and that in pure package except for the onecontaining 1% nanoZnO. Changes in the taste of packed star fruit during 15 days of storage show the positive effect of nanoantimicrobial packaging. It is obvious that there is a significant difference between nano-packaging and HDPE.The sensory panelists recognized as the best packaging material in terms of overall acceptability. It is noteworthy that changing orange juice flavor during storage is not only due to thegrowth of microorganisms but also to heating, storage time, and the common chemical interactions that occur in stored juices Souza et al. [14] reported that lower storage temperatures of unpasteurized orange juice gave rise to a higher sensory acceptance than the higher temperatures for 72 h. Leizerson and Shimoni[13] reported that the sensorial shelf life of orange juice is equal to half its microbial and 2/3 its chemical shelf life.



Conclusion

In this study, a novel nano-packing material with higher barrier and mechanical properties was successfully synthesized and then applied to the preservation of star fruit during room temperature storage. The results showed that the nano-packing material had quite beneficial effects on physicochemical and physiological quality compared with normal packing material. Furthermore, these nano-packing materials have the advantages of simple processing and industrial feasibility in contrast with other storages, some of which are time-consuming, costly and alter colour and flavour.

Therefore, the nano-packing may provide an attractive alternative to improve the preservation qualities of star fruit during extended storage. Moreover, further research will be needed to explore the exact nano-packing mechanism during storage to facilitate the application of nano-technology over a broader range in the future.

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The Modification of Coffee Leaves Beverage (Air Kawa) Processing Through Enzymatic Oxidation

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Air kawa is a traditional beverage from coffee leaves in West Sumatera. Processed by dried and boiled the leaves. One method of processing to improved beverage flavor by performing enzymatic oxidation coffee leaves. Coffee leaves were processed by modification method to get Air Kawa beverage by oxidation enzymatic. The process includes the series of withering, milling / rolling, enzymatic oxidation, drying and brewing. In the process, coffee leaves were divided to two types, leaves in the top and first leaves from the top (p+1) and the second and third of leaves from the top (2+3). The dryer were divided to three types are cabinet dryer, roaster, and cabinet smoker. And brewed was performed with varied the drink to three concentrations, 0.5%, 1.0% and 1.5% (w/v). This study also determined the effect of type of leaves, type of dryer, and variation of concentrations to consumer acceptance. And also to evaluate tannin content in astringent taste and brown color, caffeine content in bitter taste, and pH in flavor of Air Kawa beverage that most favored by consumer. Air kawa most favored by consumers with high intensity of flavor, brown color and freshness and with low intensity of bitter and astringent taste. Leaves p + 1 are a leaves that can be accepted by consumers in the manufacture Air kawa. Roaster is a most acceptable to consumers. Concentration 0.5% (w/v) is acceptable consumers. Intensity the astringent taste and brown color is not only influenced by the tannin content. Intensity the bitter taste is not only influenced by caffeine content. Intensity of flavor is not only influenced by pH. PH ranged from 5.08 – 5.47. Caffeine content ranged from 94.50 – 139.22 ppm, tannins content of Air Kawa ranged from 106.80 – 385.73 ppm.

(Key words: Air Kawa, Coffee Leaves, Enzymatic Oxidation)

1. Introduction

Air Kawa is a beverage of coffee leaves. The drink is most popular in West Sumatera, Indonesia, as a traditional beverage. Made by drying the leaves over an open flame up to twelve hour and then boiled for two hours. According to the anonymous (2011), the color of Air Kawa is more similar to tea, but still has a coffee flavor with slightly astringent – bitter taste.

Bioactive compounds in water and ethanol extract of coffee leaves form of phenolics and tannins group. Whereas in the chloroform extract form of phenol and alkaloid group especially caffeine. Coffee leaves contain of alkaloids, saponins, flavonoids and polyphenols. Phenolic chlorogenic, caffeic acid, caffeine alkaloid, and some derivates are present in coffee leaves (magalhaes, et al, 2008). While the caffeine content in the *C. canephora* is 1417.32 ppm. Caffeine is synthesized in young coffee leaves which remain sequestered in the vacuole, but not entirely biosynthesis occurs in leaves (Aerts & Baumann, 1994; Fujimori & Ashihara, 1994; Ashihara, 2006). Only caffeine, theobromine, and xanthine, present in detected amounts when the leaves and top extract were analyzed by HPLC. Shoots and young coffee leaves contain the highest concentrations of caffeine with about as much as one-third were detected. According to Johnson and Peterson (1974), caffeine in a pure state has the form of white powder and hexagonal prism-shaped crystal. Caffeine is a compound with no smell,

taste bitter, and have toxic properties (Sievets and Desrosier, 1979). Presence of tannin in the diet can determine its taste. Astringent taste of the food is usually caused by tannins.

One method of processing to improved beverage flavor by performing enzymatic oxidation coffee leaves. According to Spiller (1988), oxidation process kept under a temperature 30 ^{o}C. At a temperature 15 – 20^{o}C, both to improve flavor.

The purposes of this study are to get Air Kawa processed through enzymatic oxidation, determined the effect of type of leaves, type of dryer, and variation of concentrations to consumer acceptance. And also to evaluate tannin content in astringent taste and brown color, caffeine content in bitter taste, and pH in flavor of Air Kawa beverage that most favored by consumer.

2. Material and Methods

a. *Materials*

Coffee leaves were divided into two types. Type p+1, leaves with on tops and first leaves from tops position. And the other type 2+3, leaves with on second and third position from tops. Coffee leaves picked from coffee plantation in the Pentingsari village, Cangkringan, Sleman. Preliminary research conducted to obtain the optimal process of withering and oxidation enzymatic for Air Kawa processing. Coffee leaves through a series of process. The series are withering, rolling, oxidation enzymatic processing, drying, and brewing.

b. *Experimental Design*

Selected three factor for this experiment are type of leaves (p+1 and 2+3), type of drier (cabinet drier, roaster, and cabinet smoker), and steeping concentration of Air Kawa (0.5%, 1.0% and 1.5% w/v).

Table 1. Variation of Air Kawa processing method

Type of leaves	*Type of drier*	*Steeping concentration of Air Kawa*
Type P+1	*Cabinet drier*	*0.5 % (w/v)*
		1.0 % (w/v)
		1.5 % (w/v)
	Roaster	*0.5 % (w/v)*
		1.0 % (w/v)
		1.5 % (w/v)
	Smoker	*0.5 % (w/v)*
		1.0 % (w/v)
		1.5 % (w/v)
Type 2+3	*Cabinet drier*	*0.5 % (w/v)*
		1.0 % (w/v)
		1.5 % (w/v)
	Roaster	*0.5 % (w/v)*
		1.0 % (w/v)
		1.5 % (w/v)
	Smoker	*0.5 % (w/v)*
		1.0 % (w/v)
		1.5 % (w/v)

Products were analyzed by sensory analysis, tannin, caffeine, pH analysis. Analysis of tannin use Rangana (1986) method and analysis of caffeine use AOAC.Official Method 962.13 – 1999, *Caffeine in Non Alcoholic Beverages.*

c. *Withering*

The temperature was at 29^{o}C, RH 76 %. The process was done during 20 h on one layer of leaves spread.

d. *Rolling*

Out of withering, the leaves will be rolled and use manual rolling machine (with screw grinder). Leaves rolled into the grinder. The rotation of milling is 40 rpm. Rolling performed at room temperature (28 – 30^{o}C).

e. *Enzymatic oxidation*

The condition of enzymatic oxidation process was at 23.5^{o}C, RH >85% during 30 min.

f. *Drying*

Drying performed until the condition of water content reaches 2.5 – 3.5 %. Leaves were not over burn, not occure case hardening and dried whole indicate completing drying.

Drying use cabinet drier performed at temperature 70 – 100^{o}C. Roaster run at 90 – 150 ^{o}C, rotate in 20 rpm. The smoker run at 100^{o}C. Smoke generated from wood.

g. *Brewing*

Use boiled water (100^{o}C) during 15 min to brewing dried leaves to make Air Kawa

3. Result and Discussion

a. Preference Level of Air Kawa Beverage in Differents Variations of Concentrations

Table 2. The most preferred concentration of Air Kawa by panelists

Treatment	Steeping concentration of Air Kawa % (w/v)
Leaves P+1, cabinet drier	0.5
Leaves P+1, roaster	0.5
Leaves P+1, smoker	0.5
Leaves 2+3, cabinet drier	1.0
Leaves 2+3, roaster	0.5
Leaves 2+3, smoker	1.0

Table 2. shows that in concentration 0.5 % (w/v), sample from leaves p+1 have been the most preferred Air Kawa. As for the leaves 2+3 required higher concentration to be the most preferred concentration (1.0 % w/v), except on the sample leaves 2+3,roaster. In 0.5 % (w/v), sample leaves 2+3, roaster has been the most preferred Air Kawa.

b. The Sensories Parameter Value on Variety of Treatments.

Oxidized polyphenol compounds may influence the sensory properties. Different leaves position may affect the different intensity of sensory properties. In his research, Rodrigues, et al (2008) states that the amount of phenols in young leaves of fruit crops is 174.0 mg / g and that no production plant is 186.5 mg / g, greater than the amount found in mature leaves of the observations made by Oliveira & Romeiro (1991) in Rodrigues, et al (2008), who also found a greater concentration of phenols in young leaves.

Tabel 3. The preferance Level and Intensity Value of Air Kawa on Variety of Treatments

Treatment	Preference Level					Intensity Value				
	Flavor *	Bitter taste*	Astringent taste*	Freshness *	Brown color*	Flavor**	Bitter taste**	Astringent taste**	Freshness**	Brown color**
Leaves p+1, cabinet drier	3.1^{ab}	2.35^{ab}	2.75^{a}	2.85^{a}	2.0^{a}	3.2^{a}	3.35^{ab}	3.0^{ab}	2.2^{ab}	2.05^{b}
Leaves p+1, roaster	3.45^{b}	2.65^{ab}	2.8^{a}	2.95^{a}	2.9^{b}	3.35^{ab}	2.85^{ab}	2.65^{a}	2.3^{ab}	2.1^{b}
Leaves p+1, smoker	3.4^{b}	2.7^{b}	2.8^{a}	3.0^{a}	1.85^{a}	3.0^{a}	3.05^{ab}	2.65^{a}	2.2^{ab}	1.4^{a}
Leaves 2+3, cabinet drier	3.0^{ab}	2.0^{a}	2.7^{a}	2.75^{a}	3.5^{c}	3.3^{a}	3.85^{c}	3.05^{ab}	2.0^{a}	3.2^{c}
Leaves 2+3, roaster	3.45^{b}	2.6^{ab}	2.95^{a}	3.05^{a}	1.9^{a}	3.5^{a}	2.6^{a}	2.8^{ab}	2.65^{b}	1.45^{a}
Leaves 2+3, smoker	2.6^{a}	2.15^{ab}	2.4^{a}	2.75^{a}	3.25^{c}	2.95^{a}	3.5^{bc}	3.5^{b}	1.85^{a}	2.55^{b}

Note: The superscript sign with the same letters are not on the same column indicate values significantly different (α = 5%)
Symbol "*" : samples were tested with hedonic test
Symbol "**" : samples were tested with scoring test
Preference level : 1. Dislike 2. Less like 3. Like 4. Very like 5. Very very like
Intensity Value
Flavor : 1. Not tasty, 2. Less tasty, 3. Tasty, 4. Very tasty, 5. Very very tasty
Bitter taste : 1. Not bitter, 2. Less bitter, 3. Bitter, 4, Very bitter, 5. Very very bitter
Astringent taste : 1. Not astringent, 2. Less astringent, 3. Astringent, 4. Very astringent, 5. Very very astringent
Freshness : 1. Not fresh, 2. Less fresh, 3. Fresh, 4. Very fresh, 5. Very very fresh
Brown color : 1. Very weak brown, 2. Weak brown, 3. Brown, 4. Strong brown, 5. Very strong brown

The higest intensity of flavor is the highest preference level of Air Kawa. The highest intensity of bitter taste is not the highest preference level of Air Kawa. The highest intensity of astringent taste is not the highest preference level of Air Kawa. The highest freshness intensity is the highest preference level of Air Kawa. The highest intensity of brown color is the highest preference level of Air Kawa.

c. Tannin, Caffein, pH Analysis

Table 4. Tannin content of intensity value of brown color and astringent taste, caffeine content of Intensity value of bitter taste and pH value of intensity value of flavor

Treatment	Intensity Value of		Tannin content (ppm)	Intensity Value of bitter taste	Caffeine content (ppm)	Intensity Value of Flavor	pH Value
	Brown color	Astringent taste					
Leaves p+1, cabinet drier	2.05^{b}	3.0^{ab}	385.73	3.35^{ab}	112.44	3.2^{a}	5.43
Leaves p+1, roaster	2.1^{b}	2.65^{a}	308.14	2.85^{ab}	139.22	3.35^{ab}	5.42
Leaves p+1, smoker	1.4^{a}	2.65^{a}	140.52	3.05^{ab}	94.50	3.0^{a}	5.18
Leaves 2+3, cabinet drier	3.2^{c}	3.05^{ab}	383.81	3.85^{c}	131.83	3.3^{a}	5.47
Leaves 2+3, roaster	1.45^{a}	2.8^{ab}	106.80	2.6^{a}	103.08	3.5^{a}	5.33
Leaves 2+3, smoker	2.55^{b}	3.5^{b}	263.60	3.5^{bc}	135.97	2.95^{a}	5.08

Note: The superscript sign with the same letters are not on the same column indicate values significantly different (α = 5%)

Shown in table 4. tannin content can not be attributed to the intensity of brown color and astringent taste of Air Kawa. Tannin content in Air Kawa ranged from 106.80 – 385.73 ppm. The lowest concentration of tannin varied to create astringent taste, depending on the solvent. In the water, tannins with concentration of 20 mg per 100 ml had aroused astringent taste. Burdock (2010) states that the threshold to feel the tannin is unknown.

One of the brown color-forming compound is tannin. However, the color brown can also be formed from the treatment process, such as the Maillard reaction. Maillard reaction is a reaction that occurs between the amino group of a free amino acid, peptide or protein chain residues, the carbonyl group of a carbohydrate when both are heated or stored for a long time. There are 3 lines melanoidin brown color formation in the Maillard reaction. First, through Amadori compounds are converted to 1,2- eneaminol and 2,3-enediol. Second, aldol condensation, which is an alternative path. Third, Strecker degradation is not directly form the pigment but provides a reducing compound essential for the formation of brown color (Eskin 1990).

The brown color is affected by the loss of chlorophyll in the leaves. The main reaction is the replacement of Mg^{2+} atom in chlorophyll by hydrogen under acidic conditions to form a peofitin. Next piropeofitin a and b as a result of degradation peofitin a and b can cause a brown color (Eskin 1990). Kim et al. (2003), examined changes in chlorophyll content of the flour dough containing spinach powder (Spinacea oleracea) are fried in soybean oil at a temperature of 160^{0C} for 1 minute and stored in glass bottles. After incubated at 60^{0C} in the dark for 12 days, there was a decrease of chlorophyll, while the content increased and then decreased peofitin. Besides peofitinitation, chlorophyllase endogenous enzymes capable transforming of chlorophyll to chlorophylide with the loss phytol group. The combination of work chlorophyllase and acid cause the loss of Mg 2 + and phytol group, thus forming peoforbida. It should be noted that all changes in chlorophyll reactions can take place by heat (Eskin 1990 in Francis 1996).

Burdock (2010) states that the trheshold for detection caffeine in water is 0.0095% (or 95 ppm) , in the liquid diet 0.0184% (or 184 ppm). In one study, the panelists could distinguish the solution with caffeine content 0.0058% (or 58 ppm) of control. Threshold to distinguish the caffeine solution with the control was 0005% and to differentiate bitterness is 0011%.

Bitter taste is not only influenced by caffeine compounds. These compounds contribute to forming a bitter taste, which is benzaldehid, hydrogen cyanide, triasetin, 2 - tridicenal, trietil citrate, thiamin hydrochloride (Burdock, 2010), picroretine.

Thus, caffeine content could not be attributed to the intensity of bitter taste in the Air Kawa. Caffeine content in the Air Kawa ranged from 94.50 - 139.22 ppm.

PH in the Air Kawa ranged from 5.08 – 5.47. Acid compounds can create the flavor of the foods. Acids in Air Kawa processing formed during withering. In withering, the protein will break down into volatile amino acids and enhance flavor.

4. Acknowladge

Product favored by consumers have a high intensity of brown color, freshness and flavor and also low intensity of bitter and astringent taste. With the lower concentration of 0.5% (w/v), Leaves p+1 and roaster already accepted by consumers.

Intensity the astringent taste and brown color is not only influenced by the tannin content. Intensity the bitter taste is not only influenced by caffeine content. Intensity of flavor is not only influenced by pH. PH ranged from 5.08 – 5.47. Caffeine content ranged from 94.50 – 139.22 ppm, tannins content of Air Kawa ranged from 106.80 – 385.73 ppm.

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Utilization of Ligninolityc Enzyme in Biobleaching of Pulp from Empty Fruit Bunches of Oil Palm

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Abstract

A study was conducted to assess the possibility of using white rot fungi in bleaching of pulp from empty fruit bunches of oil palm (EFBOP) in laboratory scale. Bleaching of EFBOP-pulp was done by inoculating Omphalina *sp. and* Pleurotus ostreatus or their ligninolityc enzyme *and incubated for 20 days.*

The results showed that the opacity of the paper obtained from EFBOP qualify as newspaper. However the physical properties of paper sheets such as tensile index, water absorption, formation, and the degree of white is not yet qualify as a newspaper based on ISO standards. The use of enzyme (100%) for EFBOP bleaching process produces sheets of paper that was not fullfil standard of newsprint. It seem likely that a combination between chemical and biological processes still to be considered for the manufacturing of paper from EFBOP.

Key words: biobleaching, pulp-EFBOP, paper of newspaper

Introduction

At this time most of the pulp produced using chemicals such as alkali to liberate the cellulose fibers from other components (hemicellulose and lignin) and bleaching such as chlorine to reduce the color because of the remaining lignin. Scarcity of raw materials, chemicals and high energy prices and environmental concerns are increasingly pushing the research to produce high-quality paper that behavior is marketed using biotechnology. In addition to saving energy use, use of enzymes can improve the mechanical properties of paper. The results showed that in the process biopulping for two weeks can reduce electric energy more than 30% and improve the quality of the paper on the safe side for the environment. So it can dikataan use of enzymes in pulp and paper industry is an efficient technology and environmentally friendly.

JPP some species produce enzymes that break down lignin. Lignin is a compound found in the middle lamela combined with cellulose and hemicellulose in the wall of the second layer. With the degradation of lignin, the cellulose which is a carbon compound that is needed in the manufacture of paper can increase its concentration. Nevertheless there are problems using JPP as a producer of enzymes ligninolitik ie one organism can produce a mixture of enzymes and vary widely between organsime with one another. However,

theoretically it is possible to use enzymes from the JPP as a biocatalyst in the two processes of pulping and bleaching in paper industry.

Empty fruit bunches of oil palm is the biggest waste generated in oil palm cultivation. Several alternatives have been offered in the utilization of this waste even so the diversity of utilization of this waste will provide stability in oil palm cultivation as a whole. However, paper pulp can be produced from a variety of plant material that has a material or wood fiber. Research the manufacture of paper pulp has been done Goenadi TKKS et al., 1994; Yufnal et al., 1997. Although such research has been done using low-level white mushrooms pelapuk and delignification processes performed by different stages. The experiments were conducted to test the use of enzymes in crude lignolitik biopulping and biobleaching TKKS. As the control is the treatment of pulping and bleaching using chemicals ..

Materials and Methods

TKKS derived from palm oil mills Kertajaya PTPN VII. Sp Omphalina pure culture, and P. ostreatus is a collection BPBPI. At this early stage do Omphalina sp rejuvenation pure culture, for the manufacture of inoculum is then performed. Preparation of inoculum carried out by growing cultures in Petri dishes JPP. As many as a quarter petri inoculum (7 g) was then inoculated into 250 ml of PDB that has added some 0.5% yeast extract were incubated in the dark to enhance the growth of JPP (Palmieri et al., 1997). Manufacture of pulp from TKKS done by sterilizing TKKS, which has diserpih beforehand using an autoclave (1.2 atm, 1 hour). Two types of pulp made is derived from TKKS who has dibiopulping with Omphalina sp (Bp) and which are not in biopulping (control). In this phase some 6 kg of wet TKKS (Ka 70%) inoculated and not inoculated Omphalina sp. TKKS pulping is done by menginokulasi Omphalina sp of 2.5 l culture and incubated for 20 days.

Next TKKS processed for the manufacture of pulp. In the not inoculated Omphalina TKKS sp (control) pulping is carried out by standard procedures using 14% NaOH while Omphalina sp inoculated using 10% NaOH. Some stages are performed in the manufacture of pulp is cooking, washing, drying and milling as well.

The next activity is to test the effectiveness of the enzyme lignolitik JPP on TKKS pulp bleaching process. In this activity carried out in advance the provision of enzymes ligninolitik P. ostreatus grown on medium TKKS. To provide an appropriate amount of enzyme to the bleaching process is done first with the creation of inoculum of P. menginokulasi ostreatus sp 2. on a Petri dish. Next inoculum contained in Petri in inokulasikan on sorghum. Inoculum that grow well, then inoculated into sterile TKKS which

has been put in a plastic bag (bag log) capacity of 100 g dry TKKS that has been enriched with 150 um coper sulfate and 0.075% vitamin B number to optimize production of enzymes and enzyme activity ligninolitik (Palmieri, 1997). Inoculum are given a number of 250 ml per bag log. Harvesting is done after 20 days incubation. Extraction of enzymes performed using phosphate buffer in accordance with optimum conditions. The enzyme obtained selanjutya applied to the pulp. Prior to the application of enzymes in pulp enzyme activity analysis. Furthermore, an enzyme produced from TKKS add as many as 900 ml in 360 grams of pulp into dry sterile TKKS. Incubation is carried out in a plastic bag. Moreover, it also tested the ability of culture and P. sp Omphalina ostreatus in the pulp bleaching process TKKS directly. Bleaching performed using enzymes and cultures for 4 weeks. Both the pulp is treated with enzymes or directly inoculated made sheets of paper and paper quality analysis is then performed.

At a later stage also tested the ability of the enzyme ligninolitik pulping (100%) which is extracted from TKKS. At this stage the enzyme preparation is done by preparing inoculum of P. ostreatus first. In the next phase is already available inoculum was inoculated in sterile TKKS already diserpih and cut. After incubated for 2 weeks then performed ligninolitik enzyme extraction. Ligninolitik enzyme obtained is used for pulping TKKS who was ready. Two ways a TKKS as pulp material is carried out by (L1) or without giving lipase (L0). After incubation for 2 weeks then made a sheet of paper.

Results and Discussion

In this study JPP biobleaching done either by directly or indirectly through an enzyme that is produced by JPP ligninolitik grown in TKKS. The results showed that the opacity of paper sheets obtained from the use of enzymes and cultures biobleaching qualify as newsprint. Based on ISO 14.0091.1998 opacity newsprint is above 90%. Treatment with cultured P. ostreatus produces the highest opacity (Table 1). Tensile properties of the index increased compared with controls. Treatment achieved the highest increase in the use of culture Omphalina sp. Nevertheless tensile index is still not qualify as newsprint (SNI 22.45 - 50nm / g). Absorption is very high on the use of enzymes but with the use of culture water absorption lower than in controls. Achieved the lowest water absorption on the use of culture Omphalina sp.

Table 1. Properties of paper sheets from the treatment without biopulping TKKS but with biobleaching.

Parameter	Units	Control	Bb (E JTT)	Bb (K A1)	Bb (K JTT)
Opacity	%	99,0	99,3	99,3	**99,4**
Indeks tarik	Nm/g	4,13	6,17	7,2	**11,9**
Daya serap air	g/m2	105,7	193,2	**77,9**	84,5
Formasi	NUI	58,4	43,6	16,9	13
Derajat putih		25,31	**26,85**	25,54	25,18

ote: Bb (E JTT): biobleaching using enzymes from P. ostreatus
Bb (K A1): biobleaching using culture Omphalina sp
Bb (K JTT): biobleaching using cultures of P. ostreatus

Nevertheless water absorption is still not eligible SNI (less than 20 g/m2). Other characteristics of the formation (uniformity of sheets of paper) not yet qualify as a sheet of newsprint. Similarly, the characteristics of degrees of white are not yet qualified as newsprint (SNI 55%). Nevertheless the enzyme treatment gives the highest degree of white compared with other treatments.

Properties of paper sheets on treatment with biopulping TKKS and biobleaching presented in Table 2. In the table shown that the opacity of the sheet of paper from TKKS qualify as newsprint. Tensile index increased by administering biobleching enzyme as compared with controls. Nevertheless this character is not yet qualify as a newspaper based on SNI. Absorption is still too high compared to the ISO standard. Similarly, character formation. Slightly increased the degree of white characters on enzyme treatment compared with controls.

Table 2. Properties of paper sheets on treatment with Biopulping TKKS

Parameter	Units	Bp	Bp + Bb (EJTT)	Bp + Bb (KA1)	Bp + Bb (KJTT)
Opacity	%	**99,5**	**99,4**	**99,2**	**99,0**
Indeks tarik	Nm/g	8, 06	**8,35**	6,42	7,33
Daya Serap Air	g/m2	**71,1**	85,6	144,2	80,2
Formation	NUI	17,4	15	20,5	18,9
Derajat putih		23,54	**23,86**	22,25	22,68

Note:
Bp: biopulping use
Bb (E JTT): biobleaching using enzymes from P. ostreatus
Bb (K A1): biobleaching using culture Omphalina sp
Bb (K JTT): biobleaching using cultures of P. ostreatus

Enzymes that play a role in bleaching is MNP while working on a compound substrate lac nonfenolik. In addition to MNP also play a role in bleaching can lower kappa number. MNP works in depolimerisasi lignin by oxidizing MnII be MnIII form simple phenolic compounds. The results showed that the addition of 0.2 mN MnSO4 enough to process biobleaching using MNP. MNP is an essential enzyme for biobleaching hard wood by P. chrysosporium. The ability of the enzyme from P. MNP ostreatus seems still needs to be improved so that more optimum in the bleaching process. Nevertheless the enzyme laccase can also be used for pulp bleaching process which is made from sulfite process. In this study the pulping process is not through sulfitasi but through the provision of soda. The difference this process which may lead to less effective laccase enzyme activity. In addition, the results showed that biopulping Phanerochaeta chrysosporium is at 390C while Cerisporium subvermispora Lip 27-320C. In this study the possibility needs to be re-defined the optimum temperature for enzyme activity in addition to other conditions such as pH and moisture so that the enzyme activity in a more optimum biobleaching process.

Experiments to do biopupling using enzymes alone (100% biopulping) produces sheets of paper that still needs to be upgraded (Figure 1). treatment combinations

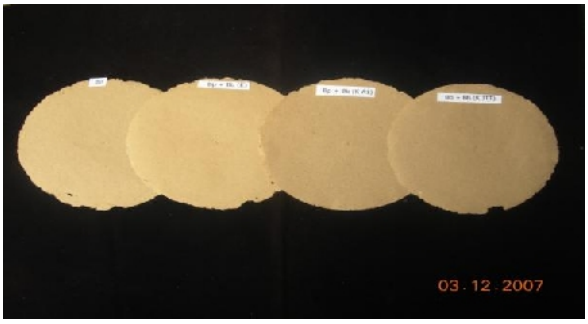

Figure 1. Sheet of paper and biobleaching biopulping TKKS through (semi-chemical, 30%). Control, and biobleacing enzyme biopulping P. ostreatus, P. biopulping and culture biobleaching ostreatus, and P. biopulping and culture biobleaching ostreatus. (from left to right)

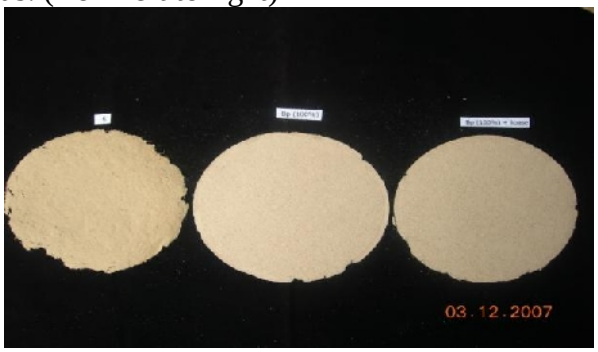

Figure 2. Sheets of paper from TKKS biopulping results using the enzyme 100%. Control (left), without lipase (middle), and with lipase (right).
chemistry and biology seem to produce sheets of paper from TKKS better.

Conclusions and Suggestions

Improved physical properties of paper sheets can be done with the treatment TKKS biobleaching and accompanied biopulping biobleaching. Characteristics of the sheet of paper only and biopulping accompanied biobleaching biobleaching using enzymes and cultures JPP produce sheets of paper that meets the requirements particular to the nature of opacity. Optimization of temperature, humidity, and pH should be determined to obtain the optimum pulping and bleaching. Combination of chemical and biological treatment seems to be considered to produce a sheet of paper from TKKS with better quality.

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Nutritional Profile of Freeze-dried Red Seaweeds From Semporna, Sabah

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Abstract
Three varieties of Kappaphycus alvarezii, which were widely cultivated in Semporna , Sabah include Aring-Aring, Crocodile Skin, and Green Flower was dried through freezed drying process and then analyzed to determine its proximate chemical composition, vitamin C, mineral element, and also fatty acid profile. The seaweeds found were high in dietary fiber (81.80 – 84.60%) and ash (6.36 – 8.97%) and low in lipid content (0.40 – 0.54%) on dry weight (DW) basis. Protein content in these seaweeds are range from 4.67 – 4.73% DW. Vitamin C content in these seaweeds is significantly high (21.10 – 32.57 mg/100g). The PUFA content in Aring-Aring was 8.02%, Crocodile Skin 8.45% and Green Flower 12.12%. This study was conducted to give a basic nutritional data for these three varieties of Kappaphycus alvarezii as guidance for further investigation especially for heat sensitive components such as antioxidant.
Keywords: Nutritional profile, red seaweed, proximate values, freeze-dried

1. Introduction

The chemical composition of seaweed around the world had been well documented, but still there is no report available on the nutritional composition of the different variety in *Kappaphycus alvarezii especially processed gone through freeze dry method*. It started to gain its popularity around Asia region and now it had spread over to other countries like South Africa and Pacific Island (Bindu et al., 2010). Looking at the increasing demand on seaweed product such as food (Phang & Wee, 1991), and industrial product (Phang, 1998), it show the importance of the study on the three variety of *Kappaphycus alvarezii* which includes *Aring-Aring*, *Crocodile Skin,* and *Green Flower.* The information obtained is important in providing data on searching for nutritious and healthy food product from the sea especially seaweed based on the potential nutrient content in it.

2. Material and methods

2.1 Collection of samples

The three seaweeds were collected from the coastal areas of Semporna, Sabah. All three seaweed was harvested at 50 days and it was washed thoroughly with distilled water to remove the holdfasts and epiphytes. It was then placed into a freezer (-20ºC) immediately after collection and freeze dried. For most of the analysis, dried sample were used except for analysis of fatty acid composition where fresh samples were used. The dried and grounded samples were stored in a plastic container and covered with aluminum foils. Inert nitrogen gas was passed into containers and samples were stored at -20ºC until further analysis. Analyses were carried out in triplicate.

2.2 Analytical Methods

2.2.1 Proximate/ biochemical analysis

Moisture content (oven method; AOAC 934.01), ash content (oven method; AOAC 942.05), crude protein (Kjedhal method; AOAC 976.05), crude lipid (Soxhlet extraction with diethyl ether; AOAC 920.39) and soluble and insoluble dietary fibers (enzymatic-gravimetric method; AOAC 991.19, 991.42) were determined (AOAC, 2000). The vitamin C of the seaweeds was

determined using spectrophotometer method (AOAC 967.22). Triplicate determinations were performed for each nutrient analysis.

2.2.2 Gas Chromatography separation of fatty acid

Fatty acids present in seaweeds were determined by using a Fison 8000 gas chromatograph (30m X 0.32mm X 0.25mm Supelco-Wax capillary column) and a Fison EL-980 flame ionization detector of their methyl esters (FAMEs). The carrier gas used was helium. It was then being identified by analyzing the retention times compared to the methyl fatty acid standard (Merck, Germany) (Sanchez-Machado et al., 2004).

2.2.3 Mineral elements

Mineral element that was present in the seaweeds was determined by atomic absorption spectrophotometer (Perkin Elmer, model 3310). The element includes Magnesium, Sodium, Potassium, Calcium, Zinc, Iron, and Copper. Sample was digested by wet ashing and dissolve in 1M HNO_3 (AOAC, 2000). Triplicate result was taken and the concentration of the elements was determined by comparing to the calibration curve of the standard elements.

2.2.4 Statistical procedure

Mean and standard deviation (SD) will be calculated and determined for every nutrient analyzed along the study. It was expressed as means ± SD (n=3) with significant differences at $p<0.05$. It was analyze by one way analysis of variance (ANOVA) using SPSS system version 19.0 for Windows.

3.0 Result and discussion

3.1 Proximate and biochemical composition

All the nutrient content of the seaweed was based on dry weight. From the result, the protein content of Aring-Aring was the lowest (4.06%) followed by Green Flower (4.50%) and Crocodile Skin (5.26%). The protein level was within range of brown seaweed and red seaweed (3 - 47% DW) (Darcy-Vrillon, 1993; Matanjun et al., 2009). The variation of the protein content in these seaweeds might due to genetic characteristic (Yeang, 2009) and the geographical differences in environment (Fleurence, 1999; Haroon et al., 2000)

While for fat content, the content is relatively low compare to other studies such as *Eucheuma cotonii* (1.1%) (Matanjun et al., 2009) and *Gracillaria changgi* (3.3%) (Norziah and Ching, 2002). The content for Aring-Aring, Crocodile Skin and Green flower were 0.47%, 0.51% and 0.44%. The differences between the seaweeds studied might due to mineral content in the environment where element such silicon, nitrogen and phosphorus play roles in determine the total content of fat in seaweeds. The low level content of fat in seaweeds makes them a good food ingredient and additive in food, health and beauty products.

Table 1. Proximate composition and vitamin C content of Aring-Aring, Crocodile Skin and Green Flower (% dry weight of samples)

Nutrient (%)	*K.alvarezii* var. Aring-Aring	*K.alvarezii* var. Crocodile Skin	*K.alvarezii* var. Green Flower
Moisture content	4.74±0.08[a]	4.67±0.03[a]	4.73±0.06[a]
Ash	6.36.±0.30[a]	7.30±0.52[a]	8.97±0.59[b]
Protein	4.06±0.06[a]	5.26±0.06[c]	4.50±0.06[b]
Crude fat	0.48±0.02[b]	0.54±0.02[c]	0.40±0.02[a]
Total dietary fiber (TDF)	84.60±1.14[b]	82.28±0.53[a]	81.80±0.72[a]
Soluble dietary fiber	71.29±1.03[b]	69.26±0.36[a]	71.27±0.43[a]
Insoluble dietary fiber (IDF)	13.31±0.15[b]	13.02±0.24[a]	10.54±0.39[a]

Values are expressed as mean ± standard deviation, n=3
Values in the same row with different superscript letters are significantly different ($p<0.05$)

The total dietary fiber in the Green Flower was the lowest (81.80%) followed by Crocodile Skin (82.28%) and Aring-Aring (84.60%). The dietary fiber content in the seaweeds studied was extremely high compare to other studies (Jiménez-Escrig and Sánchez-Muniz 2000; Mabeau and Fleurence 1993). For the soluble fiber content in Aring-Aring (71.29%) and Green Flower (71.27%), they were significantly higher than Crocodile Skin (69.26%). Soluble dietary fiber normally will be associated with having hypocholesterolemic and hypoglycemic effects, while insoluble fiber helps in decreasing the in digestive tract transit time. It seems that the seaweeds content high dietary fiber have potential in improving the health quality of human being (Burtin, 2003; Matanjun et al., 2009).

In three seaweeds that being studied, it was found that Aring-Aring has the highest level of vitamin C content (32.57 mg/100g) and was significantly higher ($p<0.05$) than Crocodile Skin (21.10 mg/100g) and Green Flower (22.55 mg/100g). It shows that these seaweeds can be a natural source of vitamin C to human.

3.2 Fatty acid composition

There was fourteen type of fatty acids were identified, most of the fatty acid identified coming from saturated fatty acid where they ranged from 62.58-69.49% for all the three seaweeds. These results were similar with other study on red seaweed (Sanchez-Machado et al., 2004). The high saturated fatty acid might occur due to hydrogenation on the double bond of unsaturated fatty acid during sample storage. The level of PUFA content in the seaweeds was ranged from 8.02-12.12%. All three seaweeds contained the essential fatty acids γ-Linolenik acid (C18:3ω6). From the result, the type of fatty acid that was found in the seaweeds is tending to be long carbon chain (C14-C24).

Table 2: Saturated fatty acid content (% of total fatty acid content) of Aring-Aring, Crocodile Skin, and Green Flower

Fatty Acid (%)	Carbon No.	*K.alvarezii* var. Aring-Aring	*K.alvarezii* var. Crocodile Skin	*K.alvarezii* var Green Flower
Myristic	C14:0	3.13±0.00[b]	3.23±0.00[c]	3.06±0.00[a]
Palmitic	C16:0	41.16±0.05[c]	34.07±0.08[a]	35.95±0.03[b]
Stearic	C18:0	1.91±0.00[b]	2.30±0.02[c]	1.50±0.01[a]
Arachidic	C20:0	-	3.18±0.01[b]	0.75±0.03[a]
Behenic	C22:0	11.20±0.01[b]	12.93±0.01[c]	10.64±0.02
Tricosanoic	C23:0	10.43±0.00[b]	9.19±0.06[a]	10.35±0.03[b]
Lignoceric	C24:0	1.64±0.00[b]	1.68±0.00[c]	0.33±0.01[a]
Total saturated fatty acid		69.49±0.04[c]	66.59±0.07[b]	62.58±0.03[a]

FA Fatty acid, MUFAs mono-unsaturated fatty acid, PUFA polyunsaturated fatty acid
Values are expressed as mean ± standard deviation, n=3
Values in the same row with different superscripts letters are significantly different ($p<0.05$)

Table 3: Unsaturated fatty acid content (% of total fatty acid content) of Aring-Aring, Crocodile Skin and Green Flower.

Fatty Acid (%)	Carbon No.	*K.alvarezii* var. Aring-Aring	*K.alvarezii* var. Crocodile Skin	*K.alvarezii* var Green Flower
Palmitoleic	C16:1	17.52±0.04[a]	17.36±0.01[a]	21.49±0.08[b]
Oleic	C18:1ω9c	4.97±0.01[b]	6.62±0.03[c]	3.72±0.02[a]
Linoleic	C18:2ω6c	-	1.89±0.00[b]	0.77±0.00[a]
γ-Linolenic	C18:3ω6	6.23±0.02[a]	6.56±0.06[b]	9.48±0.09[c]
cis-11-Eicosenoic	C20:1	-	1.04±0.00	-
cis-8,11,14-Eicosatrinoic	C20:3ω6	-	-	1.47±0.00
Arachidonic	C20:4ω6	1.79±0.01[b]	-	0.40±0.00[a]
Total		30.52±0.05[a]	33.47±0.09[b]	37.34±0.16[c]
MUFAs		22.49	25.02	25.21
PUFAs ω6		8.02	8.45	12.12
PUFAs ω3		0	0	0

FA Fatty acid, MUFAs mono-unsaturated fatty acid, PUFA polyunsaturated fatty acid
Values are expressed as mean ± standard deviation, n=3
Values in the same row with different superscripts letters are significantly different ($p<0.05$)

3.3 Mineral element

The mineral content of Aring-Aring, Crocodile Skin and Green Flower in mg/100g sample (DW) is show in table 4. The highest macro-element found in the three seaweeds was K (463.77-464.18 mg/100g), followed by Na (219.30-222.31 mg/100g), Ca (117.93-131.47 mg/100g) and Mg (30.10-30.43 mg/100g). While for micro-element, the amount is relatively low where all three elements (Zn, Fe, and Cu) had the concentration of below 3.5 mg/100g. Mineral content will differ with each other because of several factors such as genetic species, sea condition, and seasons and also the physiology and morphology of the seaweed (Krishnaiah et al., 2008).

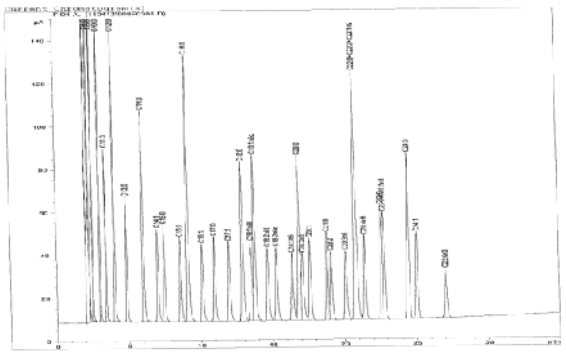

Diagram 1: Chromatogram for Methyl Ester Fatty Acid Standard

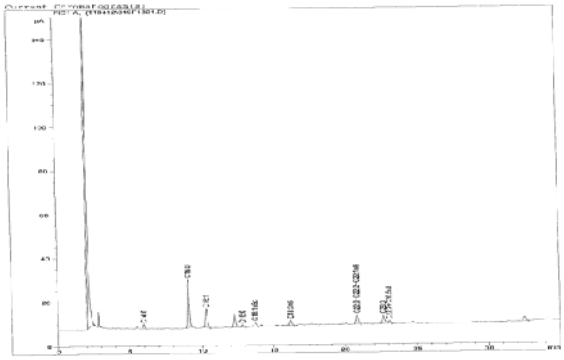

Diagram 2: Fatty acid chromatogram for Aring Aring

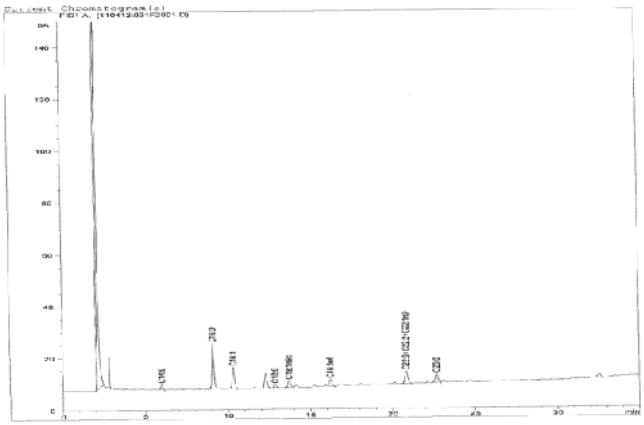

Diagram 3: Fatty acid Chromatogram for Crocodile Skin

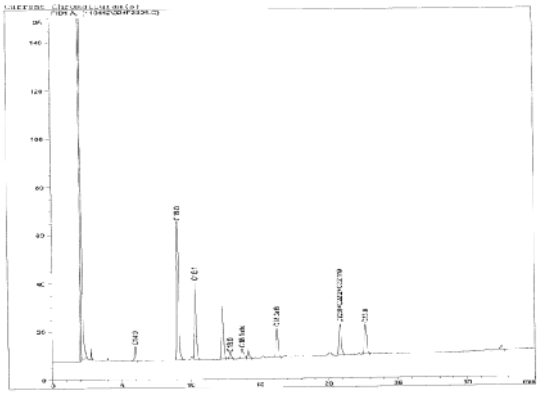

Diagram 4: Fatty acid chromatogram for Green Flower

Table 4. Mineral Contents of Three Varieties *Kappaphycus alvarezii* (mg/100g dry basic)

Variety	Mg	Na	K	Ca	Zn	Fe	Cu	Ratio Na/K
***K. alvarezii* var. Aring-Aring**	30.43± 0.04^{c}	222.31± 2.05^{a}	463.77± 0.11^{a}	131.47± 0.48^{c}	3.09± 0.09^{b}	1.33± 0.42^{a}	0.18± 0.00^{a}	0.48
***K. alvarezii* var. Crocidle skin**	30.34± 0.03^{b}	219.30± 1.25^{a}	463.85± 0.06^{a}	128.22± 0.28^{b}	2.23± 0.07^{a}	0.73± 0.00^{a}	0.88± 0.00^{b}	0.47
***K. alvarezii* var. Green flower**	30.10± 0.02^{a}	219.56± 2.20^{a}	464.18± 0.06^{b}	117.93± 0.16^{a}	2.21± 0.09^{a}	3.27± 0.00^{b}	0.06± 0.10^{a}	0.47

Values are expressed as min ± standard deviation, n=3
Values in the same row with different superscripts letters are significantly different ($p<0.05$)

4.0 Conclusion

The three variety of *Kappaphycus alvarezii* that was found widely in Malaysia was analyzed for its biochemical and mineral composition. Its nutritional composition was then being analyzed and it was found that all three seaweed contains very high amount of dietary fiber and vitamin C which is essential and beneficial to human being. It had provide a basic data toward this kind of variety for further studies on usage and application of the seaweed into food product and also nutraceutical product looking at the high nutrient value in the studied seaweeds.

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Comparison of Seed Nutmeg Oleoresin Extraction (Myrictica Houtt fragrans) Origin of North Maluku and Maceration Method Using Combined Distillation–Maceration

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Abstract

The purpose of this study was to compare the composition of oleoresin nutmeg (*Myristica fragrans* Houtt) made directly by maceration and distillation combined - maceration. nutmeg oleoresin yield amounted to 15.17 (% db) obtained by direct maceration and oleoresin extraction method combined distillation and maceration obtained yield of 20.08 (% db). While the essential oil yield itself from the water-steam distillation of the results obtained at 6.61 (% db). Ethanol extract of oleoresin analysis using GCMS method identified a total of 39 kinds of compounds with the composition of major components is methyleugenol (33.40%), myristicine (10.90%), cis-methyl isoeugenol (9.09%), elemicin (8:33%), and isocoumarin (5.61 %). For nutmeg essential oil contained 31 components of the compound, where the components are located in large numbers was sabinene (34.97%), β-phellandrene (19.9%), methyleugenol (7:55%), myristicine (5:29%) and elimicine (3:21 %). As for the essential oil is mixed with the oleoresin from the pulp remaining distillation contained 58 components that make up the compound of the oleoresin with a mixture of main components, namely: sabinene (12:38%) myristicine (10.88%), elemicin (8.93%), isocoumarin 6:26 (%), myristic acid (5.96%), and α-pinene (4.73%).

Key words : essential oil, oleoresin, maceration, maceration-distillation, GC-MS

INTRODUCTION

Nutmeg (Myristica fragrans Houtt) as a spice widely used in various fields of food, medicine and cosmetics in the form of extracts of essential oils and oleoresin. The use in these areas would require a good quality extract of nutmeg. Quality extracts influenced by extraction techniques, refinement of materials, type of solvent, extraction time, solvent concentration, the ratio material with a solvent, solvent evaporation process, purification and drying (Bombaderlli, 1991 and Vijesekera, 1991).

Nutmeg extract the chemical content in the form of volatile oil and oleoresin have been widely utilized in various fields such as food as a flavor agent in the manufacture of milk-based drinks (eggnog), meat-based foods, as well as in health and beauty such as aromatherapy, perfume, toothpaste as well as in traditional medicine. From some of the results of the study reported that the nutmeg in the form of volatile oil and oleoresin have antibacterial properties as (Stankovic, et al., 2006), natural antioxidants (Dorman, et al., 1995; Baratta et al., 1998; Lis-Balchin, 1998; Tomaino et al., 2005; Jukić et al., 2006; and Suhaj. 2006) anti-fungal / fungal (Rahman, et al., 1999) and as an ingredient in medicine.

METHOD

Seed Nutmeg (Myristica fragrance Houtt) Nutmeg seeds used in this study is nutmeg (Myristica fragrance Houtt) aged 7-9 months, the harvest month of February 2009, came from the village of Torano and Marikurubu North Maluku, entering in the quality of group A with the number of seeds 180/kg. The chemical composition of the nutmeg seed is: it contains 18.66% moisture content, ash content 1.67%, 34.63% fat content, protein content 6.96%, and 38, 07% carbohydrate content.

Nutmeg Seed Oleoresin Extraction with Direct Maceration method

A total of 40 grams of nutmeg powder at 4 ° C results digrinding pass 20 mesh sieve inserted into a 250 ml erlenmeyer containing 200 ml of solvent 96% ethanol, samples were put into a water bath shaker. at a temperature of 54oC for 4 hours with 120 rpm shake speed. Filtration using Whatman filter paper no. 1 (ekstraks1), the extract was cooled at 4 ° C for one hour to separate the fat nutmeg. Oleoresin concentration used rotary vacuum evaporator (IKA Werke RV 06 ML) at a temperature of 40oC and a pressure of 172 mbar. The extraction process was repeated once again on the same sample of nutmeg as the procedure above and obtained extract 2. The results have been weighed for the determination of the solvent evaporated oleoresin yield.

Nutmeg oleoresin extraction with a combined distillation method – Maceration

A total of 1.5 kg flour nutmeg pass 20 mesh sieve size included in the boiling kettle that has been equipped with condenser and flask clavenger, with the amount of water added as much as 6 liters. Incoming cooling water temperature is set at 4-7oC. steaming time for 4 hours starting from the first droplet. Essential oil obtained by the remaining water is removed with a filter that is passed on has been given sodium sulfate anhydrous. Nutmeg powder residue remaining in the distillation and subsequent extraction was dried by maceration to obtain oleoresin. Nutmeg essential oil yield was weighed for determination of distillation.

Characterization Component Compounds by Gas Chromatography oleoresin Composer - Mass Spectrometry (GC-MS).

Testing components constituent compounds of essential oils and oleoresin using GC-MS Shimadzu GCMS-QP2010S (Shimadzu Corporation, Kyoto, Japan) Shimadzu GCMS-QP2010S (Shimadzu Corporation, Kyoto, Japan) equipped with a capillary Column Model Number: 19091S Agilent HP-5ms-433 5% Phenyl methyl siloxane (diameter of 250 μm, length 30 m, and 0:25 μm film thickness) and FID detector is used. GC conditions: initial temperature 60 ° C raised to 250 ° C (4 ° C / minute) and then at a temperature of 250 ° C maintained for 20 minutes, with helium carrier gas flow rate 20ml/min. Compounds were identified by comparing retention index and mass spectra compared to those in the Wiley library database and the NIST library (Adams, 2004).

RESULTS

Yield

The high yield of oleoresin (20.08% bk) obtained from the combined methods of distillation and maceration due to volatile oil has been partially drawn at the time of distillation in this volatile compounds with boiling points at temperatures over 90^{o}C, while some essential oils are composed by compounds are volatile and non volatile than oloeresin the extracted during the process of maceration with the temperature 54 ^{o}C.

Profile oleoresin compounds obtained from the maceration

Oleoresin extract of ethanol by direct maceration there are 39 components of the main components are methyleugenol (percent relative area of 33.40%), myristicine (10.90%), cis-methyl isoeugenol (9:09%), elemicin (8:33%), and isocoumarin (5.61%).

Profile oleoresin compounds obtained from the combination of distillation – maceration methods

Components of Essential Oil Compounds Composer distilled. The components of the chemical constituent of essential oil of nutmeg seed based on the results of analysis using GCMS method there are 31 components, where components are located in large numbers was sabinene (34.97%), β - phellandrene (9:19%), methyleugenol (7:55%), myristicine (5:29%) and elimicine (3:21%).

CONCLUSION

Oleoresin extracted by maceration method can directly yield of 15.17% obtained with the components making up a total of 39 kinds of compounds with the five main components namely; methyleugenol (33.40%), myristicine (10.9%), cis-methyl isoeugenol (9:09%), elemicin (8:33%), and isocoumarin (5.61%). For the essential oil obtained by distillation yield of 6.61%. For nutmeg essential oil contained 31 components of the compound, where the components are located in large numbers was sabinene (34.97%), β-phellandrene (9:19%), methyleugenol (7:55%), myristicine (5:29%) and elimicine (3:21 %). As for the essential oils are mixed with the oleoresin of the remaining distillation residue contained 58 components that make up a compound of the oleoresin with a mixture of main components, namely: sabinene (12:38%) myristicine (10.88%), elemicin (8.93%), isocoumarin 6:26 (%), myristic acid (5.96%), and α-pinene (4.73%).

Acknowledgments

To the Department of Agriculture Agricultural Research Agency of the Republic of Indonesia, which has funded this research through a program KKP3T LPPM in 2010 and has facilitated so that this research can take place smoothly.

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Development Process of Frying Distillation in Capturing Flavor that Formed During Deep Frying

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Frying distillation was a new process that developed to capture the flavor that formed during deep frying. It was studied using simple water distillation apparatus. The design experiment were carried out to map the best combination between time, temperature, and ratio (between material and heating medium) that used in the frying distillation process. From the best design experiment of frying distillation will be obtained the flavor and the components will be analysed using GC-MS.

The material that used in the frying distillation process were dried shallot, dreid onion, and dried garlic. They have specific flavor when deep frying. The flavor that formed during deep frying will be captured by frying distillation and will be analysed by GC-MS. Shallot, onion, and garlic were pelled, cut, and then dried using a cabinet dryer (45-50 ^{o}C) for 22-24 hours. After that, tempering during 20-24 hours and then weighing. Dried shallot, dried onion, and dried garlic were inserted into the distillation flask and mixed with oil, until each of them was completely submerged.

There were differences in the frying distillation time, temperature, and ratio between material and heating medium for dried shallot, dried onion, and dried garlic. Each components that captured from deep frying shallot, onion, and garlic were not contain burnt attributes.

Keywords: shallot, onion, garlic, flavor, deep frying, frying distillation, distillate, flavor component

1. Introduction

Deep frying is an important method of food preparation, which immerse foods in hot oil. Deep frying is a common practice in food processing. Shallot, onion, and garlic are important seasoning spices that can be applicated in deep frying. It has specific flavor when it was crushed and cut into pieces, and then put in the deep frying oil at high temperature. Another frying method that produce typical shallot flavor is pan frying. Both of pan and deep frying method, will be produced specific flavor that can stimulate and create hunger. In Chinese cuisine such as instant noodles, fried noodles, fried rice and rice with ground pork, applicated with shallot after being deep frying then were taken out of the frying oil (Chyau and Mau, 2001).

Recently, the usage of deep fried shallot, onion, and garlic flavouring in the foods is becoming increasingly popular. Thus, the recent research did to investigate the volatile compounds of deep fried shallot flavouring. Chan, Liou, and Wu (1991) used the methods of short path distillation to separate the volatiles of deep fried shallot flavourings. Volatiles from the acidic, alkaline and neutral fractions included sulphides, pyrroles and compounds from the degradation of lipid. The lipid-degraded compounds were identified as (E)-2-hexenal, (E)-2-heptenal, (E)-2-decenal, (E,E)-2,4-heptadienal, (E,E)-2,4-decadienal, pentanoic, and hexanoic acids.

In the other hand, there is no information about the flavor that formed during frying shallot, onion, and garlic. And also there is no technology that is used to capture flavor formed during the frying shallot, onion, and garlic. To capture the delicious flavor formed during frying shallot, onion, and garlic, made a frying distillation process innovation.

Frying distillation is a new method of distillation process. Frying distillation process has the same principle with general distillation process. The difference lies in the usage of heating medium or distiller medium. Heating medium that used instead of water, steam, or organic solvent, but frying oil. The purpose of this research was to design the process of capturing flavor formed during the frying shallot, onion, and garlic and to analyze the volatile components that make up savory flavor for frying shallot, onion, and garlic.

2. Material and Methods

2.1. Materials

Shallot, onion, and garlic were purchased from local market (Yogyakarta, Indonesia). They were peeled, sliced (± 1 mm) and dried at 45-50^{o}C, 20-25 hour. During the experiment, all the samples were saved in isolated condition, using silica gel at ambient temperature. In each batch, 30 g of dried shallot mixed with frying oil, 30 g of dried onion mixed with frying oil, and 25 g of dried garlic mixed with frying oil (depend on the condition of material under the frying oil).

2.2. Experimental Design

The dried shallot, dried onion, and dried garlic samples were weighed and quantitatively transferred into a 250-ml flask, then distillated with different volume for a given time and distillation temperatures. The flavor compound of distillate was determined by GC-MS analysis.

Table 1. Experimental Design of Frying Distillation Process for Dried Shallot, Dried Onion, and Dried Garlic

Material	Given Time (minutes)	Given Temperature (^{o}C)	Ratio Between Material - Frying Oil
Dried Shallot	20, 30, 40, 50, 60, 70	140, 150, 160, 170, 180	1:5, 1:6, 1:7
Dried Onion	20, 30, 40, 50, 60, 70		
Dried Garlic	20, 30, 40, 50, 60, 70	130, 140, 150, 160, 170	1:2, 1:3, 1:4, 1:5

Determination of optimum frying distillation time carried out by looking at the large amount of distillate (distillate weight) every 10 minutes started from 20th minute and ending at 70th minute. Weighing distillate obtained performed at 20th, 30th, 40th, 50th, 60th, and 70th minute. Determination of optimum temperature was done by varied the temperature of frying distillation. The optimum frying distillation temperature was obtained from the greatest weight among the five variations of temperature. Another parameter of determination for frying distillation temperature was based on the quality of flavor that was captured. It was intended to get the flavor that escaped and avoided from burnt flavor. Determination of ratio between materials and frying oil performed after obtained the optimum time and temperature of the frying distillation.

Optimization process of frying distillation consist of time factor, temperature factor, and ratio between material and frying oil factor. Each of these factors was all factor that had undergone optimization meant that the optimum conditions to produce the best quality and quantity of the distillate. The relationship between best quality and quantity can be demonstrated by the high amount of distillate but didn't contain burnt flavor.

2.3. Frying Distillation

Each of the samples were weighed and quantitatively transfered into a 250 ml flask, then fried with different ratio between frying oil and dried shallot for a given time when the frying temperatures were respectively set.

After frying process, the distillate was collected. The best combination between time process, frying temperature, and ratio between frying oil and dried shallot will be the frying distillation condition.

2.4. Gas chromatography-mass spectrometry (GC-MS) analysis

The distillate was analyzed using AGILENT GC 6890N equipped with a 5975B MSD and HP-5ms capillary column (30m x 250 μm, film thickness 0.25 μm). Injector temperature were set at 300^{o}C, GC-MS *interface* temperature at 325^{o}C. Column temperature was initially kept at 50^{o}C for 2 min, then gradually increased to 200^{o}C with a rate of 10^{o}C/min. Helium was carrier gas, at a flow rate of 0.5 mL/min. Samples of 1 μL were injected manually and in the splitless mode. Tentative identification of the compounds was based on the comparison of their mass spectra with those of library data.

3. Result and Discussion

3.1. Determination of Frying Distillation Time

Determination of optimum conditions for the time factor was based on the weight of distillate that obtained and the quality of the flavor, which was the largest amount of distillate and flavor during frying shallot, onion, and garlic instead of burnt flavor. Longer the frying distillation taken place, heavier distillate that obtained had increased.

3.2. Determination of Frying Distillation Temperature

Frying distillation temperature was directly proportional to the amount of distillate that produced. The higher the frying distillation temperature, the bigger amount of distillate that produced previously. The heat penetration process was greater so that the process of releasing vapor components over the maximum. However, increasing frying distillation temperature was inversely proportional to the quality of the condensed flavor. The higher the frying distillation temperature, the worst quality of condensed flavor. It was associated with the accelerated process of burnt due to the higher temperature. So that the condensed flavor was the burnt flavor that was undesirable flavor in foods.

Burnt flavor was distillate quality parameter that resulted from the isolation of flavor formed during the frying shallots. According to Ojeda, et al (2001), burnt flavor formed by overcooked, and had burnt attributes, namely odor produced when food was burned. Compounds that describe burnt attribute were pyrocatechol; 3-methoxypyrocatechol; dimethoxybencebe 1.2-, 2.6-dimethoxy 4-methylphenol, 2.6-dimethoxy-4-(1-propenyl)-phenol; burnt bread. The usage of different frying temperatures caused the difference in time of appearance burnt flavor. The higher temperature, burnt flavor that was formed more quickly.

3.3. Determination of Ratio Between Dried Shallot and Frying Oil

Obtained inverse relationship between distillate weight and ratio dry shallot-cooking oil, the greater the ratio the smaller the distillate obtained. It was associated with greater volume of dried shallot-frying oil mixture that be heated so that the longer the heat transfer process. The longer time the heat taken to reach the middle part of the mixture, the smaller components that were successful to exit. So for the same time frame, the higher volume of the mixture, it will produce smaller amount of distillate.

3.4. Optimization Process of Frying Distillation and Distillate Yield

Each of the temperature, time, and ratio between material and frying oil factors were all factors that had undergone optimization, meant that the optimum conditions to produce the

best quality and quantity of the distillate. The relationship between best quality and quantity can be demonstrated by the high amount of distillate but didn't contain burnt flavor (Table 2).

Table 2. Weight of Distillate that Obtained in Several Frying Time and Temperature of Frying Distillation for Dried Shallot, Dried Onion, and Dried Garlic

Dried Shallot		Time (minutes)						
		0	20	30	40	50	60	70
Temp (^oC)	140	0	0.0851	0.1551	0.1945*	0.1945	0.1945	0.1945
	150	0	0	0.2911	0.3154*	0.3154	0.3424	0.3548
	160	0	0	0.9082	0.9792*	1.0076	1.0270	1.0270
	170	0	0	1.0390	1.1231*	1.1922	1.4637	1.7791
	180	0	0.1211	1.6586*	2.0999	2.4569	2.6364	2.6364
Dried Onion		Time (minutes)						
		0	20	30	40	50	60	70
Temp (^oC)	140	2.3511	2.8080	2.8084	2.8384*	2.8694	2.8844	2.3511
	150	2.3134	3.0320	3.0702*	3.0717	3.1014	3.1014	2.3134
	160	2.0753	4.0975*	4.1808	4.2131	4.2170	4.2221	2.0753
	170	2.5318	6.1879*	6.6392	6.6648	6.6825	7.1058	2.5318
	180	1.8143*	4.7157	5.0870	5.1549	5.7332	5.7332	1.8143*
Dried Garlic		Time (minutes)						
		0	20	30	40	50	60	70
Temp (^oC)	130	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	140	0.000	0.000	0.000	0.000	0.000	0.065	0.065
	150	0.000	0.095	0.190*	0.225	0.305	0.365	0.378
	160	0.000	0.835*	0.985*	1.065	1.175	1.220	1.230
	170	0.000	0.985*	1.210	1.305	1.370	1.415	1.460

Ratio between Dried Garlic and Frying Oil	Weight of Distillate (g) **	Weight of Distillate (g) ***	Flavor Quality (Burnt Factor)
1:2	0.80	0.47	++
Ratio between Dried Garlic and Frying Oil	Weight of Distillate (g) **	Weight of Distillate (g) ***	Flavor Quality (Burnt Factor)
1:3	0.87	0.53	+
1:4	1.00	0.55	-
1:5	0.00	00.0	-

* Sign apostrophe here indicated the frying process had entered the the burnt process
** 1^{st} condition = 160^oC, 25 minutes
*** 2^{nd} condition = 150^oC, 35 minutes

Based on the weight of distillate and quality of the flavor, the optimum conditions for capturing flavor process that formed during the frying shallot was at temperature of 170 ^oC for 30 minutes with the ratio between dry shallots and cooking oil was 1:5. The optimum conditions for capturing flavor process that formed during the frying onion was at temperature of 170 ^oC for 30 minutes with the ratio between dry shallots and cooking oil was 1:6. And the optimum conditions for capturing flavor process that formed during the frying garlic was at temperature of 160 ^oC for 25 minutes with the ratio between dry shallots and cooking oil was 1:4.

3.5. Volatile Components in the Distillate

Volatile components of frying distillation from dried shallot, dried onion, and dried garlic can be grouped into several major components. From the results of GC-MS analysis, contained pyrazine group, ketone group, aldehyde group, alcohol group, oxygen-containing heterocyclic compound group, carboxilate acid group and other components.

Pyrazine compound was the main compound in chocolate odour. According to Chambers, et al (1998), pyrazines had characteristics minty, but not the characteristic musty or sweet roasting results. Another characteristic that was like baked beans, acetone, and hydrolyzed. According Nuwiah (2008), the composition of hydrophobic acid and fructose as in Ghana cocoa roasting results in cocoa fat lindak, produced derivative compounds such as trimethyl pyrazine and dimethyl pyrazine.

The second and the third group were ketone and aldehyde group. Aldehydes and ketones were the degradation result of unsaturated fatty acid during the heating process and it was important in the Maillard reaction and flavor forming (Erikson, 1981 in Santi, 2002). The resulted ketones were also used in the Maillard reaction in addition to the usege of amino acids and reducing sugar from the shallot itself. According to Pokorny, et al (1998), acetaldehyde was the decomposition result (such as oxidation and other reactions such as retroaldolisation) of oxidized linalool storage.

Other components found in the distillate was derived from the alcohol group, oxygen-containing heterocyclic compound group, carboxilate acid group and other component. Alcoholic compound was found in the form of 2-propene-1-ol, 2-furancarboxaldehyde included in the oxygen-containing heterocyclic compounds, acetic acid included in carboxilate acid group and other components was carbon dioxide. According to Spanier, et al (1998), acetic acid had an odour description like vine strong, quick and contain some sulfur. According Pudil, et al (1998), acetic acid was one component of bergamot oil that slightly oxidized on the heating condition of 40^{o}C for 20 hours.

Table 3. The Components in the Distillate from Flavor Condensation during Frying Distillation of Dried Shallot, Dried Onion, and Dried Garlic by GC-MS Analysis

Component	**% Concentration**		
	A	B	C
Carbon dioxide	7.659	7.659	31.48
Acetaldehyde	32.442	18.266	-
Propanal	27.282	9.173	-
2-propen-1-ol	1.630	-	6.90
2-methyl propanal	8.793	-	-
2,3-butanedione	0.412	1.618	-
3-methyl butanal	8.212	8.752	-
2-methyl butanal	4.210	2.659	-
2-methyl-2-butenal	1.397	-	-
Methyl pyrazine	0.379	-	-
Component	**% Concentration**		
	A	B	C
2-furancarboxaldehyde	0.661	1.493	-
Acetic acid	1.626	21.751	-
1-hydroxy-2-propanone	2.181	2.462	-
2,5-dimethyl pyrazine	1.774	-	-
Dimethyl pyrazine	1.343	-	-
Propanal,2-methyl-iso butyraldehyde	-	7.485	-

2,3-pentanedione	-	0.355	-
2,3-dimethyl-oxirane	-	0.521	-
2,4,5-trimethyl-1,3-dioxolane	-	0.226	-
(methylthio)acetaldehyde	-	0.532	-
Furfural	-	3.139	-
Oxirane (CAS)	-	-	6.50
Oxiranemethanol	-	-	1.14
Allyl alcohol	-	-	53.98

A, Distillate from flavor condensation during frying shallot; B, Distillate from flavor condensation during frying onion; A, Distillate from flavor condensation during frying garlic

Based on those components in the distillate, there were not components that describe burnt attributes such as pyrocatechol; 3-methoxypyrocatechol; 1.2-,dimethoxybenzene 2.6-dimethoxy 4-methylphenol, 2.6-dimethoxy-4-(1-propenyl) -phenol; burnt bread. Burnt flavor was a distillate quality parameter resulted from the flavor isolation from frying shallot. According to Ojeda, et al (2001), burnt flavor formed due to overcooked process, which had burnt attributes, odor that produced when food was burned. That was to say this distillate was the result of condensation from frying process before reaching the burnt condition. This was indicated there wasn't detection of compounds that described the burnt attributes.

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Effect Roasting of Indonesian Sesame Seed (*Sesamum indicum L.*) on Odour Profil and Degree of Liking of The Oil

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Abstract

Roasting of sesame seed is regarded as important treatment for developing the odour of sesame oil, however, the effect of the roasting condition on the odour in term of sensory profile has not reported yet. The aim of this study was to evaluate the effect of roasting condition on the odour profil of sesame oil. Roasting of sesame seed was carried out at three level of roasting temperature (180, 200 and 220^{0}C) and three level of roasting time (10, 20, and 30 minutes), followed by pressing using hydraulic press at 140 kN for 5 minutes for obtaining the oil. The produced oil were evaluated for the odour profil by Descriptive Sensory Evaluation method using 8 trained panels, whereas degree of liking was determined by Hedonic Scale Scoring method using 30 untrained panels. The trained panels revealed that they perceived 13 odour stimulus in the sesame oil, namely: roasty, roasty coffee-like, roasty popcorn-like, roasty potato-like, earthy potato-like, caramel-like, mushroom-like, clove-like, burnt rubbery, burn sweet, meaty, spicy, sulforous onion-like. However their intensities were varies with the roasting condition. The odour profil was presented as spiderweb figure. The degree of liking was varies with the roasting condition and the panels. Beside the odour, it was revealed that appearence also affect the degree of liking.

Keywords: *roasting, odour profil, sesame oil, descriptive sensory evaluation*

INTRODUCTION

Sesame is reported as the most ancient oilseed known and used by human as a food source. It has been cultivated for centuries in Asia and Africa, for its high content of excellect quality of oil and protein It is used as source of oil and also widely used in bakery products and confectionary goods. Sesame oil is known highly stable against oxidative change compared to other vegetable oil although it contains olec and linoleic acid. This remarkable stability due to it contain significantly high amount of natural antioxidant, including tocopherols, sesamin, sesaminol, sesamol and sesamolin (Fukuda, *et al.,* 1986). The conventional process for producing sesame oil involves cleaning, optional dehulling, roasting, grinding and pressing/oil extraction (Fukuda and Namiki, 1988). Roasting process is the important step of in producing sesame oil, because the color, composition, flavor and stability of produced oil are influenced by the roasting condition (Yoshida *et al.,* 1995; Yoshida and Takagi, 1997). Takei *et al.* (1988) in Schieberle (1995) reported that acetylpyrazin provide strong popcorn-like aroma, and has important role in favor of sesame oil. Schieberle (1995) reported that heated white Mexican sesame seed revealed 18 aroma compounds having very high Flavour Dilution Factor, and on the basis of Odour Active Value, there were roasty, coffee-like, rubbery and caramel like which have inportant contributor to overall roasty and sulphury odour in crushed sesame seed. Tamura *et al.,* (2010) identify 29 odourant compounds and reported 9 thiols contributed to sulphurous, meaty, catty and black currant-like odor in addition of coffee-like, caramel-like, and clove like smelling. Although many people claimed the used of sesame oil due to this oil has a good flavor, and reseacher reported that roasting condition influenced the oil flavor, the odor profil

of sesame oil has not been published yet. Tashiro and co-workers reported that different strain and cultication area resulted in diferent oil content and minor components in the oil. Moreover, since no reported article on the odor/flavor profile of Indonesian sesame oil, therefore this paper reports our initial work on characterization of Indonesian sesame oil flavor.

MATERIALS AND METHODS

Materials

Sesame seeds (*Sesamum indicum* L.) were purchased from local farmer in Klaten, Central Java.

Roasting and oil extraction

Whole and cleaned sesame seed (500 g) were roasted in modified cofee roasting machine at 180^0C, 200^0C or 220^0C for 10, 20 or 30 minutes, separately. After roasting, the seed were allowed to cool until 80^0C prior to be pressed using hydraulic press at 140 kN for 5 minutes. The oils were allowed overnight at room to precipitate the impurities and the clear oil were decanted, stored at cool room until there were analyzed. The work was carried out as shown in Figure 1.

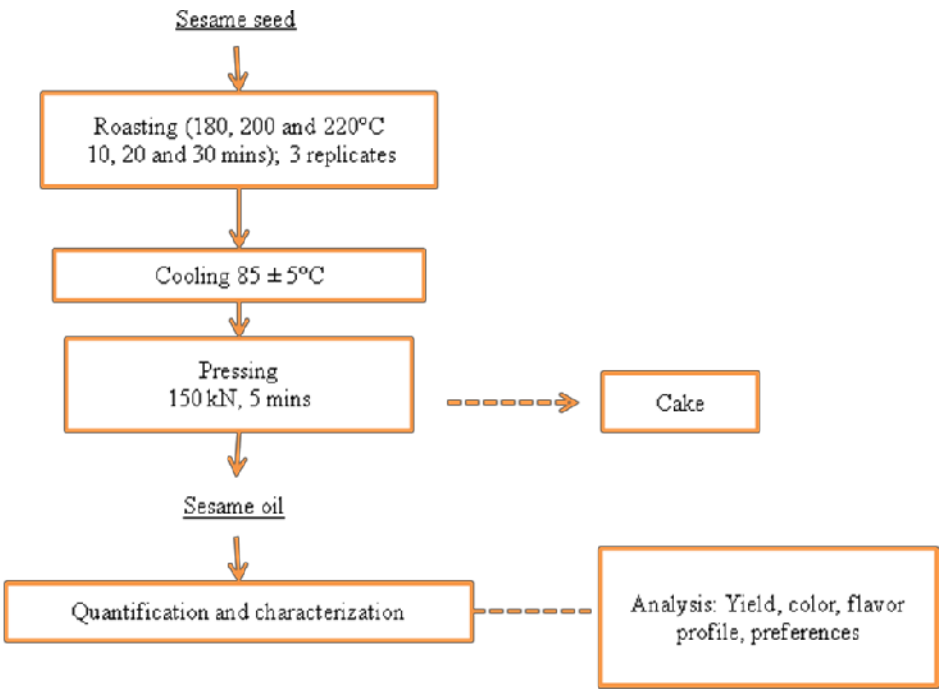

Figure 1. Research flowchart

Oil Color measurement

The measurement of oil's color was carried out based on AOCS, 1990; metode Cc 13f-92 using Lovibond Tintometer apparatus with 10 mm lovibond with cuvetes.

Sensory evaluation

Odor profile of sesame oils were determined by Quantitative Descriptive Analysis (Meilgaard, et al., (1999).

Panelis selection and training for Descriptive Analysis

Panelists were recruited on the basis of their experience on sensory evaluation, interest, time avaiability, and consumption of sesame oil. All panelists are staff and studendt in Faculty of Agricultural Technology.

Training of the panelists

During the 1st day training, the panelists were asked to describe the perceived odor of the sesame oil obtained from market (commercial sesame oil). Then, the sample standards were introduced to the panelists, and the panelists were asked to re-evaluate whether the sesame oils had these kind of odor, without rating the intensity, followed by discussion, to decide whether this oil sample has these descriptors. The standard reference intensity used for all attributes as shown in Table 1, from which the rating of the attribute intensity will be based on.

Table 1. Standard reference intensity rating used in the Quantitative Descriptive Analysis of sesame oil

Samples	Odor attributes	Intensities
Roasted sesame seed	Roasty	15
Coffee powder	Roasty, coffee-like	15
Caramel	Caramel-like	15
	Burnt, sweet	15
Popcorn	Roasty, popcorn-like	15
Washed baked potato	Roasty, potato-like	15
Unwashed baked potato	Earthy, potato-like	15
Rubber	Burnt, rubbery	15
Mixed spices	Spicy	15
Mushroom	Mushroom-like	15
Roasted meat	Meaty	15
Onion	Sulfurous, onion-like	15
Clove	Clove-like	15

On the 2nd day training, the reference samples were presented to the panelists. The panelists reviewed the reference samples and recognizing the intensity of each atributes. The panelists were then asked evaluate the odor attributes of warme up commercial sesame oil including the intensity, followed by discussion to decide which odor atributes and its intensity the sample has, until the consensus atribute and rating were assigned.

On the 3rd day training the panelists were asked to do the similar training as they have done in the 2nd day training in order to familiarized the evaluation techniques.

Sample preparation and evaluation

Nine samples sesame oil obtained from different roasting conditions were presented to the panelists in the closed bottle with three digit random number as the sample code on a tray, at room temperature, and followed monadic order. Panelists were asked to evaluate the odor of the oil in individual booth, and expressed their respons on the paper ballot

Preference test

Preference test based on aroma and color of the oil were conducted following the method as recomended by Meilgaard, *et al*., (1999), in order to measure the degree of liking to the sesame oil. This test was carried out using 30 untrained panelists.

RESULTS and DISCUSSION

Oil yield

The oil yielded from pressing of different roasting condition of the sesame seed in the range of 40.2 up to 43.3% , while from unroasted seed found to be lower than this values, Table 2.

Table 2. Oil yield (% db) obtained from different roasting conditions.

	Roasting time, minutes		
Roasting temperature, ^{0}C	10	20	30
180	42.9 ± 0.13	42.2 ± 0.14	41.1 ± 0.17
200	43.3 ± 0.14	42.5 ± 0.11	41.1 ± 0.14
220	42.6 ± 0.17	41.0 ± 0.14	40.2 ± 0.15
unroasted	39.7 ± 0.11		

All values are mean of three replicates ± standard deviation.

Roasting found to increase oil yield, it facilitate oil expression, however, if roasting is carried for prolong time may slightly decrease the oil yield.

The color of oil

The color of oil obtained from roasted sesame seed under different roasing conditions compared to unroasted seed and a sample of commercial sesame seed is shown in Table 3.

Table 3. The color of sesame oil obtained from different roasting conditions, expressed as Red and Yellow values in Lovibond Tintometer

Roasting condition (^{0}C/min)	**Red**	**Yellow**
180/10	0.80	1.45
180/20	0.95	2.00
180/30	2.35	4.60
200/10	0.90	2.45
200/20	2.40	6.35
200/30	4.35	8.80
220/10	1.55	4.00
220/20	6.60	8.50
220/30	9.20	24.10
Unroasted	0.70	1.60
Commercial sesame oil	4.90	10.00

It was found that oil of unroasted seed less red and yellow intensity, or lighter color than that from roasted seed. The longer and the roasting time and the higher the roasting temperature resulted in the darker color (brown) of the oil, as express in the higher values of redness and yellowness in lovibond tintometer scale. The increase of the color of the oil seemed to be due to the results of non-enzymatic browning reaction at elevated roasting temperature. The formation of brown substances in some thermally processed food due to non-enzymatic browning reaction Maillard-type, the reaction of reducing sugars and free amino acids or amides. As the reaction time prolong, the reaction products more accumulated, and the appearance of the oil getting darker. This results is agree with previous work (Lee *et al*., 2002) who worked on roasted safflower seed and Yoshida and Takagi (1997).

The odor profil

The odor profil obtaine by Quantitative Descriptive Analysis (QDA) using 8 trained panelists is shown in Figure 2. The panelists detected 13 odor attributes, including roasty, roasty

coffee-like, caramel-like, roasty popcorn-like, roasty potato-like, burnt, rubbery, musroom-like, burn, sweet, meaty, sulphurous onion-like, clove like, and earthy potato-like. Previous work reported by Schieberle (1995) had explained that roasting may generate more than 220 volatile compounds. These compounds of cause affect to the flavor of roasted sesame oil, especially the odor of the oil. The result of this work revealed that roasty notes were the dominant odor descriptor. The longer the roasting time and the higher the roasting temperature resulted in strongger or higher intensity of roasty notes.

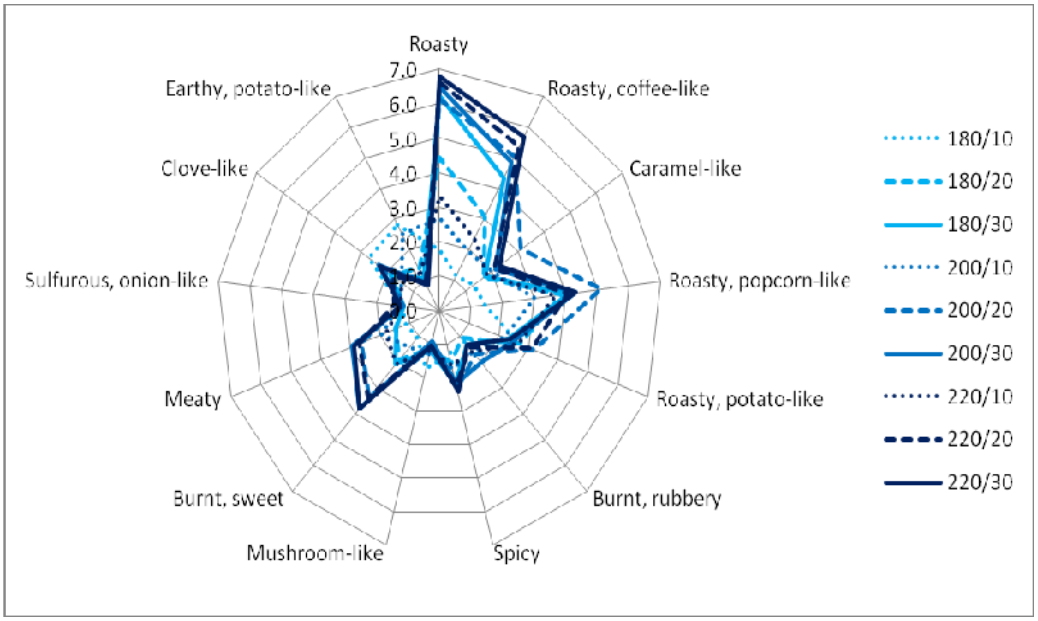

Figure 2. Odor profile of 9 sesame oils obtained from different roasting conditions (at 180, 200, and 220^{0}C for 10, 20 and 30 minutes). Intensity scale: 1 indicates very low intensity and 15 indicates equal to reference sample intensity.

Aroma and color preferences

The result of preference test on oil on the basis of oil's color and aroma using 30 untrained panel and degree of liking scale from 1(extremely dislike) until 7 (extremely like), is presented in Table 4.

Table 4. Degree of liking to the oil obtained from different roasting conditions. Value of 1 represent dislike extremely, 7 represent like extremely.

Roasting condition (^{0}C/min)	Aroma	Color
180/10	5.2	5.7
180/20	5.0	5.2
180/30	4.3	4.4
200/10	5.5	5.6
200/20	4.5	4.0
200/30	3.2	3.3
220/10	5.2	5.2
220/20	2.4	2.7
220/30	2.3	2.5
Unroasted	4.4	5.5
Commercial sesame oil	2.4	2.1

It was found that the degree of liking in the range of 2.3 until 5.7 out of 7 level of the degree og liking. It is surprising that the degree of liking to the unroasted sesame oil is high, around 5 out of 7. The oil prepared by roasting at 200^0C for 10 minutes has the highest value of the degree of liking. Roasting the sesame seed for 10 minutes prior to be pressed, found gave good oil in term of degree of liking. The longer the roasting time tend to decrease the degree of liking to the oil. Since degree of liking is varies with the consumer, it is suggested that before chosing the roasting condition, the consumer aspect should be taken into account.

CONCLUSION

Roasting of sesame seed prior to oil pressing in an important step for preparation. Roasting facilitate the oil pressing and allowing the reaction to take place within the seed, and this may resulted in enhanching the aroma of the oil. However, roasting condition should be choosen in order to meet the need of the potential consumer.

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Antioxidant Activity and Compounds of Indonesian Sesame (*Sesamum indicum* L.) Oil

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Sesame (Sesamum indicum L.*) is an important oilseed crop in the world, which can be well cultivated in Indonesia. Roasting is an important step for obtaining sesame oil from the seed. The aims of this study were to evaluate the effect of roasting, on (i) oil yield, and (ii) antioxidant activity and compounds in the oil. The roasting of the sesame seed were carried out at 180^{0}C) for 30 minutes). The roasted and unroasted seed were then pressed using hydraulic press at 140 kN for 5 minutes for obtaining the oil. The produced oil were evaluated for oil yields, the antioxidant activity, total phenolic compounds, α-tocopherol, sesamin, sesamolin, and sesamol contents of the oil. It was found that the oil yield, antioxidant activity and compound of the oil were significantly affected by roasting. The oil yields for unroasted and roasted sesame seed were 23.91 and 36.63 %, respectively. Roasting found not to affect the total phenolic compound, but increase the antioxidant activity of roasting oil almost as twice as in unroasting oil. Roasting may lowered the α-tocopherol and sesamolin contents in the oil, but increased the sesamol content. To some extent roasting of this seed also increase the degree of liking to the oil.*

***Keywords**: roasting, sesame oil, antioxidant activity, total phenolic compounds, lignans, α-tocopherol*

INTRODUCTION

Sesame (*Sesamum indicum* L.) seeds contain approximately 50% of oil and 25 % of protein, therefore it is reported as the most ancient oilseed known and used by human as a food source. It has been cultivated for centuries in Asia and Africa, for its high content of excellect quality of oil and protein. There are many kind of sesame seed in term of their colour, white, black, brown, and yellow seeds. Beside they are used as oilseeds, they also used as ingredient of some food. Ministry of Agriculture of the Republic of Indonesia, through *Balai Penelitian Tanaman Tembakau, Serat dan Kapas*, has released 4 varities of sesame plant, namely Sumberrejo1, Sumberrejo 2, Sumberrejo 3, and Sumberrejo 4.

As other oil seed, the most utilization of this seed is for vegetable oil source, and was reported as the sixth important oil seed in the world production (Lee, *et al*., 2010). Among the commonly used vegetable oils, sesame oil is known to be the most resistant to oxidative rancidity although it has high content of oleic and linoleic acid in the triglyceride. Its remarkable stability has reported due to the high content of sesamin, sesamolin, sesaminol, sesamol, and α-tocopherol (Fukuda, *et al*., 1986). Sesamin showed antioxidant activity (Yamashinta, *et al*., 2000 in Jeong, *et al*., 2004), anticarcinogenic (Hirose, *et al*., 1992 in Jeong, *et al*., 2004), blood pressure lowering (Matsumura, *et al*., 1998 in Jeong, *et al*., 2004), serum lipid lowering effects (Hirose *et al*., 1991 in Jeong, *et al*., 2004) in experimental animals and humans. Therefore, the occurence of this compound in sesame oil make sesame oil become more valueable. The process for sesame oil preparation involves cleaning, optionally dehulling, roasting, optionally grinding and pressing. Roasting is the key processing step that affect the color, composition, quality and stability of the oil produced (Yen and Shyu, 1989). Jeong *et al*. (2004) reported that roasting sesame seed at 200^{0}C for 60 minutes significantly increased the total phenolic content, radical scavenging activity,

reducing power and antioxidant activity in sesame meal extracts. Tashiro and co-workers reported that different strain and cultication area resulted in diferent oil content and minor components in the oil. Therefore, this paper reports the effect of roasting on the oil yield, antioxidant activity, total phenolic compounds, α-tocopherol, sesamin, sesamolin, and sesamol contents of the yielded oil.

MATERIALS AND METHODS

Materials

Sesame seeds (*Sesamum indicum* L.) were purchased from local farmer in Klaten, Central Java. Sesamol dan α-tokoferol standards, metanol, ethyl acetate, acetonitril, DPPH, and Folin-Ciocalteau were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). sesamin and sesamolin were isolated from sesame oil according to Reshma *et. al.,* (2009).

Roasting and oil extraction

Whole and cleaned sesame seed (500 g) were roasted in modified cofee roasting machine at 180^0C, for 30 minutes. After roasting, the seed were allowed to cool until 80^0C prior to be pressed using hydraulic press at 140kN for 5 minutes. Two other portions of whole, cleaned and unroasted sesame seed (500 g for each portion) were prepared for heated at 80^0C and unheated prior to be pressed using hydraulic press at 140kN for 5 minutes. The oils were allowed overnight at room to precipitate the impurities and the clear oil were decanted, stored at cool room until they were required for analysis.

Isolation and crystalization Sesamin dan Sesamolin

Isolation dan crystalization sesamin dan sesamolin was carried out according to reported by Reshma *et. al.,* (2009). Sesame oil (100 g) was dissolved in methanol (1:1 w/v) followed by heating at 70^oC for 10 minutes. This mixture was then, cooled to 50^oC, and allowed until 2 separate layer was formed in the separating funnel. The methanol layer was evaporated in rotary evaporator for removing the solvent. The concentrated methanol extract eas then partitioned into petroleum ether (1:2 w/v) and was keep at 4^oC for 48 hours until crystal of lignan was formed. The formed crystal was then dried in oven at 35^oC for 30 minutes fofr removing the solvent residue, followed by HPLC analysis.

Analysis of sesamol, sesamin, and sesamolin

Sesamol, sesamin and sesamolin and was analyzed by reverse phase HPLC using C18 column (150 x 4,6 mm) equiped with Rheodyne 7125 injector with 20 µl sampel loop and ultraviolet visible SPD-10A detector and was set at λ 290 nm. The mobile phase was methanol/water (70:30 v/v) at flow rate of 1 ml per minute. Sesamol, sesamin, and sesamolin in oil were quantified using calibration curve of standard sesamol and isolated sesamin and sesamolin, respectively.

Measurement of tocopherols content

Tocopherol analysis was carried out according to method repored by Aued-Pimental *et. al.,* (2006). The measurement of tocopherol was done using Shimadzu LC-10AD HPLC, equipped with a Shim-pack CLC-SIL 60 column (5µm, 150 x 4,6 mm id Shimadzu), Rheodyne injector having 20 µl sample loop, detector fluoresense (RF-10AXL) and was set at an exitation □of 300 nm and emissin □ of330 nm. The mobile phase was a mixture of hexane/ethanol (99,5 : 0,5 v/v) at a flow rate of 1.0 mL min^{-1}. Fifty mg of oil was dispersed in 5 mL of mobile phase for HPLC analysis. An aliquot sample solution was injected with fully 20 □ Ьloop. Quantification of tocopherols in oil is calculated using calibration curve of standard □ tocopherol

Measurement of total phenolic content

Measurement of total phenolic content was carried out according to Sahidi *et al*. (2004). Folin-Ciocalteau reagent (0.5 mL) was added into 0.5 mL of homogenized mixture of sesame oil and methanol (1:1 v/v), followed by addition of 1mL of saturated sodium carbonate solution. The volume of this solution was made up to 10 mL with distilled water and homogenized using a vortex mixer, and was incubated at room temperature for 45 minutes, prior to measurement of the absorbance at □ of 765 nm. The total phenolic compounds was expressed as miligram of gallic acid per gram sample using calibration curve of absorbance of methanolic gallic acid solution.

Measurement of antioxidant activity

The Antioxidant activity was expressed as free radical DPPH scavenging activity and was determined using a method reported by Suja *et. al.,* (2003). Methanolic solution of DPPH (3 mL of 0.025 g/L) was added into 1 mL of 5% methanolic sample solution and incubated at room temperature for 30 minutes, proir to be measurement of the absorbance at □ of 515nm.

Radical scavenging activity (%) = [1- {sample absorbance/control absorbance}] x 100.

RESULTS and DISCUSSION

Oil Yield

Table 1. Oil yield prepared by pressing of roasted and unroasted seed

Seed treatment	***Oil Yield* (% db)**
Roasting 180 ^{o}C 30 min	41.1^{c}
Without roasting	39.7^{b}
Without heat treatment	23.9^{a}

Each value is an average of three replicates determination.
Values with different following letters are significantly different (p<0,05)

Table 1 represent the oil yied prepared from roasted seed and unroasted seed, showing that heat treatment is needed for preparing seed for oil pressing. Roasting resulted in increasing the oil yield. Heat treatment is normally apply to oil seed prior to oil extraction, either by solvent extraction or pressing. Heating the oil seed may reduce oil viscosity, inactivate enzume, coagulate protein, rupture cell wall and membrane and make the seed soft and more pliable or plastic during pressing. However, excessive heating may has disadvantage such as produce excessive fine during pressing which affect some difficulty in the oil production. It was found in previous study that roasting 180^{0}C for 30 facilitate oil pressing. Since the oil content of the seed is 53.92%, that mean, after pressing some of the oil remains in the oil cake.

Antioxidant components

Antioxidant component analyzed in this experiment including total phenolic compounds, α-tocopherol, sesamol, sesamin dan sesamolin, Table 2.

Table 2. Antioxidant content in the pressed sesame oil prepared from roasted and unroasted seed in comparison with that in commercial sesame oil.

Seed treatment	Total Phenolic Content (milligrams gallic acid per gram oil) x 10^{-3}	Content (ppm)			
		Sesamin	Sesamolin	Sesamol	α-Tocopherol
Roasted 180 °C 30 min	2.4[a]	6855.05[b]	3996.34[b]	64.71[a]	11,836.13[b]
Unroasted	3.3[ab]	6942.01[a]	4306.47[a]	12.38[c]	16,562.95[a]
Commercial sesame oil	3.5[b]	6439.70[c]	3198.98[c]	36.12[b]	12,598.99[b]

Each value is an average of three replicates determination.
Values in the same column with different following letters are significantly different (p<0,05)

Total phenolic content.

Roasting sesame seed at 180 °C for 30 minutes found not to lower the total phenolic content in the pressed oil, Tabel 2. However, commercial oil has slightly higher total phenolic compared to the oil prepared from roasted and roasted in this study. This facts may due to the differences of the oil preparation or the variety of the oil seed.

Tocopherol content

Roasting at 180 °C for 30 minutes also found to lower the α-tocopherol content. After roasting process the tocopherol content retained about 75%. It may be due to oxidation process occurs during roasting, during which seed contact to the oxygen in the air, then oxidation potentially occurs, and the antioxidant was oxidized first. This finding is agree with previous study (Yoshida and Takagi, 1997; Lee et al., 2010), reported that □ and □ tocopherols were also derease with roasting temperature and time, but they did not find a-tocopherol. This differences may be attributed to differences of seed variety or geographical area.

Sesamol, sesamin and sesaminol content

Table 2 reveals that roasting at 180 °C for 30 minutes increase the sesamol content around five fold, concersely the sesamin and sesaminol contents slightly decrease with roasting. This finding also agree with previous study as reported by Yoshida and Takagi (1997 and Lee *et al*. (2010). Roasting may liberate sesamol from sesamolin, therefore still contribute to antioxidant activity.

Antioxidant activity

The antioxidant activity of the oil was expressed as radical scavenging capacity, as shown in Table 3, roasting at 180 °C for 30 minutes increase the antioxidant activity around 40%.

Table 3. Antioxidant activity in pressed sesame oil prepared from roasted and unroased seed in comparison with commercial sesame oil

Seed Treatment	Antioxidant activity (%)
Roasted 180 °C 30 min	42.74[b]
Unroasted	30.14[a]
Commersial sesame oil	57.66[c]
BHT	74.68[d]

Each value is an average of three replicates determination.

The increase of antioxidant activity may attributed for the increase of sesamol. Lee et al. (2010) reported that the increase of sesamol content play an important role for the increase of antioxidant activity. The other reseacher stated that there is a sinergy effect of sesamol and tocopherols that also contribute to antioxidant activity (Fukuda *et. al.*, (1986) in Yoshida and Takagi (1999). During roasting, non-enzymatic browning reaction Maillard-type also occurs, the Maillard reaction product also has antioxidant properties (Choe and Min, 2006), therefore may also contribute to the antioxidant activity.

CONCLUSION

Heat treatment , either roasting or only heat treatment up to 80ºC of sesame seed prior to oil pressing has an important role for increasing oil yield. Roasting using a modified coffee roaster at 180ºC for 30 minutes lowered the a tocopherol, sesamin and sesamolin, conversely increase the sesamol content in the pressed oil. As a result this roasting process increased the antioxidant activity. This roasting process is quick and simple technique for preparing sesame oil with good antioxidant activity.

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Application of Liquid Smoke and Smoke Powder for Process Development Instant Seasoning of Indonesian Traditional Food

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ABSTRACT

This research was initiated to determine the right formula pasta and powder curry and chicken opor seasonings. Then, determine the lowest concentration of liquid smoke as a threshold and maximum concentration of liquid smoke on each type of seasoning. Furthermore, the research results were used to determine five different concentrations on sensory testing. Based on the assessment panel's favorite, the optimum concentration can be obtained so as to produce the traditional smoke flavor.

Threshold of liquid smoke to give flavor in curry pasta is 0.025% (v/v) while in the powder 0.04% (w/v) and for chicken opor pasta is 0.015% (v/v) while in powder 0.01% (w/v). The maximum concentration in the pasta curry and chicken opor seasoning are 0.4% and 0.8% (v/v) while the powder is 1.0% and 1,25% (w/v).

The result of ANOVA test shows that pasta curry and chicken opor seasoning at concentration 0.15% and 0.25% (v/v) and powder seasoning at concentration 0.12% and 0.20% (w/v) is the optimum concentration of traditional smoke flavors.

***Keyword** : Seasonings, Liquid Smoke, Traditional flavor, engineering*

INTRODUCTION

Along with the development of the modern era, the public demands practicality. Currently, the traditional way of processing food with wood as fuel has been abandoned and replaced with the use of gas stove. In fact, the burning of wood in the traditional food processing to produce foods have the distinctive taste because the cooking process is long, the smoke flavor absorb into the food. Specific smoke flavor is quite favored by consumers. Cooking by using wood, coconut husk and coconut shell as fuel has several advantages, namely the food produced durable, has a flavor that is more comfortable and enjoyable as well as having a high flavor and distinctive (Yefrida et al, 2008). This foods such as chicken opor, curry, brongkos, beef jerky and rendang. Based on research, typical flavors found in these foods derived from organic compounds (acid, carbonyl, and phenol) that found in the smoke of burning. However, an organic compounds that contribute to the formation of the smoke flavor is phenol (Girard, 1992). The weakness of traditional cooking, among others are less consistent product quality because the optimal time and temperature can not be maintained the same, can cause environmental pollution, and allow the fire hazard, as well as the possibility of polycyclic aromatic hydrocarbon compounds are formed (benzo (a) pyrene) that are carcinogenic (Gorbatov (1971) and Maga (1987)). These compounds can be formed and can be easily attached or absorbed on the surface of food during the traditional cooking (Tigner and Leaf, 1970 b; in Leaf, 1979).

On the other hand, food processing with gas stove is more practical and cleaner can not produce the typical flavor cuisine. Flavor that generated in food processing using a gas stove have less sharp. Therefore, the flavor of foods that are processed in traditional ways

have to be raised again. The solution of the problems above by engineering a traditional flavor through the addition of smoke flavor.

Engineering traditional cooking can be done using liquid smoke or liquid smoke powder as a source of smoke flavor. However, the proportion of liquid smoke and liquid smoke powder that too much can cause a bitter taste sensation at the base of the tongue. Conversely, the proportion of the addition that too little can not produce smoke flavor. Therefore, engineering the addition of liquid smoke and liquid smoke powder is required to obtain a unique flavor that can be preferred by consumers.

Engineering can be done by determining the proportion of the lowest and the proportion of its maximum. Then, the optimum proportion of the addition of liquid smoke and liquid smoke powder is determined by sensory testing with the proportion of variation between the lowest and the proportion of maximum proportions.

RESEARCH METHODOLOGY

Materials and Research Tools

Basic materials that used in the manufacture of curry seasoning and chicken opor pasta ready to eat are spices, coconut milk and cooking oil. While the seasoning powder used spices that have been dried. For the manufacture of liquid smoke is used coconut shell, and the manufacture of liquid smoke powder is used maltodextrin as a filler. For chemical analysis, materials used for the analysis of total phenol, total acid, and proximate analysis. As a prototype of packaging used aluminum foil and plastic PE for packaging materials.

The tools used for this research is a tool for the production of pyrolysis liquid smoke there are tool consisting of pyrolysis reactor tube equipped with a thermostat and time, streamer pipe smoke and the cooling tube for condensation of liquid smoke, and liquid smoke redestilation tool consisting of a stove electricity, thermostat, penangas oil, distillate pipe thermometer, cooling pipes for condensate, and also a tool for making pasta and powder seasonings.

How To Research

Research Introduction

Determination Formula Seasonings

Formulas of ready to eat pasta seasoning is obtained by performing weighting of each fresh herb in accordance with the orientation done, then mashed and sautened.

Formulas of ready to eat powder seasoning is obtained by drying each type of fresh herbs that are used, then crushed and sifted, and weighed according to their respective orientations.

Preparation and Purification of Liquid Smoke

Liquid smoke is made from 3 kg of coconut shell through a process of pyrolysis at a temperature of 400^0C for 2.5 hours. After passing through the stages of pyrolysis, liquid smoke condensate become to redestilation at a temperature of 105^0C. Liquid smoke redestilation results then placed in dark bottles and stored in a coolroom.

Making Liquid Smoke Powder

Liquid smoke powder is made by mixing the liquid smoke and maltodextrin with a ratio of 1:1 and then dried in cabinet dryer temperature 50^0C and then stirred until evenly mixed and poured in the pan and then dried using a cabinet dryer. Once dry, the clumps are formed and then crushed with a porcelain cup to be powder.

Determination of Threshold and Maximum Concentration

Threshold testing aims to find the lowest concentration of liquid smoke to the sauce that has begun to be perceived by the panelists. It also conducted tests to determine the highest concentration of liquid smoke that can make the panelists felt sick and did not want to feel the gravy is.

Main Research

Sensory Analysis

Testing preferences and product differentiation in the sensory ready to eat seasoning is done in two batches and were evaluated by 20 panelists in each batch. The variation of the concentration used in the organoleptic test was the result obtained from the orientation of the preliminary research.

This test uses scoring method which panelists were asked to rate each of the parameters provided in the form number/ score. Scores are provided to test and test distinction of joy is in the range 1-5.

Chemical Analysis

Chemical analysis performed on this product are:

a) Analysis of water content with thermogravimetri method (Anonymous, 1990),
b) Analysis of protein content with mikrokjeldahl method (Anonymous, 1990),
c) Analysis of lipid content with soxhlet method (Anonymous, 1990),
d) Analysis of total sugar and reducing sugar with Nelson-Somogyi method (Anonymous, 1990),
e) Analysis of phenol concentration method with Senter et al., 1989; modifications to the method Plumer, 1971),
f) Analysis of total acid with titration method (Anonymous, 1990).

Making Prototype Packaging

Pasta and poeder ready to eat opor and curry seasoning that has been carried out chemical analysis then packed with plastic polyethilen use as primary packaging and aluminum foil as a secondary packaging. Furthermore, packaging is labeled to product identity.

Experimental Design

The experimental design used was Randomized Perfect Design with influential factor is the addition of various concentration of liquid smoke and liquid smoke powder and response factors to be measured is the organoleptic properties of curry sauce and chicken opor generated, which includes smoke flavor, aroma / odor of smoke, and preference as a whole. Furthermore, the data obtained statistically tested using analysis of Variance (ANOVA) to determine whether there is a difference between the panelists and assessment followed by a test DMRT (Duncan Multiple Range Test) if there is a real difference. Statistical analysis using SPSS 16. Results of sensory testing is then followed by chemical analysis.

RESULT AND DISCUSSION

Research Introduction

Determination Formula Seasonings

a. Curry Seasoning

Determination of the ready to eat pasta curry seasoning is done in two orientations to finally obtain the exact formula of spices to produce a curry sauce with a optimal taste. Spice formula is based on recipes from Ernawati, 1996. From prescription weight was obtained from each type of herbs and spices made into a paste, are solely due to the addition of chilli and turmeric recipe obtained from the color of the soup yet reddish yellow.

Table 1. Determination of formula ready to eat pasta curry seasoning

types of seasoning	recipe	weight (gram)
red onion	6 pieces	23,75
garlic	2 pieces	13,89
hazelnut	3 pieces	10,88
ginger	1 cm	1,70
turmeric	1 cm	6,28
coriander	1 tablespoon	2,59
cumin	1/4 teaspoon	0,78
klabet	1/4 teaspoon	1,08
nutmeg	1/4 teaspoon	0,70
fennel	1/4 teaspoon	0,76
tamarind	1 teaspoon	7,68
salt	1 teaspoon	7,67
chili	3 pieces	21,72
cardamom	4 pieces	0,43
galangal	1 cm	3,60
lemongrass leaves	1 sheet	9,07
bay	1 sheet	1,95
lime leaves	2 sheet	1,22
Total		115,75

In determining formulation of ready to eat curry powder seasoning is done by three times orientation until finally obtained the proper formulation of ingredients to produce curry sauce with a optimal taste. From each formula is then cooked with coconut milk as much as 1000 ml and tested to the limited panelists.

Table 2. Determination of curry powder seasoning Formula Ready to Eat

types of seasoning	I (gram)	II (gram)	III (gram)
red onion	2,61	5,22	5,23
garlic	4,18	8,36	8,36
hazelnut	10,04	20,08	20,08
ginger	0,45	0,90	0,90
turmeric	0,87	1,74	1,74
coriander	0,78	1,56	1,56
cumin	0,25	0,50	0,50
klabet	0,33	0,66	0,68
nutmeg	0,21	0,42	0,42
fennel	0,23	0,46	0,48
tamarind	1,10	2,20	3,30
salt	4,27	8,54	8,54
chili	0,13	0,26	0,28
cardamom	0,53	1,06	1,06

galangal	1,41	2,82	2,82
lemongrass leaves	0,69	1,38	1,38
bay	0,55	1,10	1,10
lime leaves	28,63	57,26	58,43

From the results of evaluation of the selected fast food seasoning curry powder on to a third orientation that could result in curry sauce with a flavor that fits.

b. Chicken Opor Seasoning

Proper formulation in the process of making smoke flavor seasoning chicken opor function to produce the most preferred flavor. Weighing in a formulation that is important to know the type and amount of ingredients needed opor. Determination of formula through several stages of orientation.

Table 3. Determination Formula Ready to Eat Pasta Seasonings opor

Types of Spices	**Orientation (grams)**			
	I	**II**	**III**	**IV**
Shallots	57.77	57.77	57.77	58
Garlic	16:59	6:59	6:59	6:59
Hazelnut	16:50	6:50	6:50	6:50
Ginger			1.84	8
Turmeric			2.77	5
Pepper	0.91	0.91	0.91	1
Coriander	0.87	0.87	0.87	0.87
Cumin	00:37	00:37	00:37	0.4
Galangal	5.60	5.60	5.60	5.60
Lemongrass leaves	1 stalk	8:16	8:16	11
Bay	2 LBR	2:01	2:01	2:01
Lime leaves			1:31	1:50
Brown sugar	17.72	20	20	26
Salt	12.63	11	11	16

The process of determining the right formula at the top through sensory testing is limited to determining flavor fast pasta seasoning opor most preferred by panelists.

In determining the formula paste opor seasoning, initially obtained orientation using a recipe from the internet. In order to taste the same formula on each test will be weighing the amount of each spice. Then, in orientation I taste sweet and salty in one liter of soup opor less appropriate. Furthermore, in orientation II, added salt and more sugar and spice leaves.

Idea appears to reverse the addition of seasoning leaf by taking the extract. The way the spices with a smooth leaf and then added water 50 ml. Then, extract the flavor leaves squeezed and filtered. The result is added at the time of extraction pemblenderan along with other spices.

At orientation III plus ginger, turmeric and lime leaves. Based on limited sensory test results showed that the flavor and aroma of spice pastes opor not fit so we need more orientation.

In the fourth orientation, the addition of some spices such as ginger, turmeric, pepper, lemon grass leaf, brown sugar and salt. Furthermore, limited sensory test carried out and obtained pasta with opor spice flavor and aroma that is appropriate. The comparison between the flavor with a sauce that is 1:8 (w / v).

Table 4. Determination Formula Powder Chicken Opor Seasoning

Types of Spices	**Recipe (Grams)**	**I (Grams)**	**II (Grams)**
Shallots	2.4	5:22	10:42
Garlic	2.4	4:13	8:26
Hazelnut	3 eggs	15:31	15:31
Ginger	-	1:35	1:35
Turmeric	-	0.62	1:24
Coriander	2.4	0.87	1.74
Cumin	0.8	0.4	0.80
Salt	10	16	16
Brown sugar	5	26	26
Galangal	1.6	1:22	2:44
Lemongrass leaves	1 stalk	1.95	1.95
Bay	2 sheets	0.80	0.80
Lime leaves	-	0.69	1:39
Pepper	-	1:00	2:00

Chicken opor seasoning flavor with formulation I are less tasty, just taste sweet and slightly salty. Orientation II done with some seasoning powder amount multiplied twice as onion, garlic, pepper, lime leaves, turmeric, coriander, cumin and galangal. Comparison of the amount of seasoning powder with a sauce that is 1:10 (w / v). Based on sensory evaluation test results obtained by flavor and aroma that is appropriate.

Liquid Smoke and Liquid Smoke Powder

3 kg of coconut shell was pyrolisated to produce liquid smoke as much as 1400 ml and 600 ml redistilled redestilat liquid smoke. While the manufacture of liquid smoke powder in this study by using liquid smoke as much as 300 ml then mixed with maltodextrin as much as 300 grams. Results of drying liquid smoke is liquid smoke powder as much as 264.8 grams so that the yield obtained from the crystallization is as much as ±44.13%. These results are similar to previous research conducted by Handayani (2004) which states that the yield resulting from the crystallization liquid smoke and maltodextrin as much as 50%.

Composition of Liquid Smoke and Liquid Smoke Powder

Table 5. The chemical composition and physical properties of liquid smoke and liquid smoke powder

Chemical composition	**Prosentase (%)**	
	Liquid Smoke	**Liquid Smoke Powder**
Fenol Acid Total Moisture Content	2,53 20,74 -	2,24 10,46 6,72
Physical Properties (odor)	Smoke specifique	Smoke specifique

The table above shows that the fraction of liquid smoke and the results redestilat liquid smoke powder, acid has a greater percentage compared with phenol. The percentage of such compounds in liquid smoke different amounts depending on the type of wood or raw material liquid smoke, component fiber base material, and pyrolysis temperature. Meanwhile, according Pszczola (1995), differences in chemical composition of liquid smoke is based on plant species, age and condition of the crop growth. Acid content of liquid smoke powder in this study were 10.46% and the liquid smoke at 20.74%. According Tranggono et.al., (1989) acid levels are several types of wood are made at 350-400^{0}C pyrolysis temperature ranged from 4.27-11.39%.

The average of phenol concentration in liquid smoke powder obtained from the analysis of 2.24% while 2.53% liquid smoke. Tranggono et.al., (2002) reported that levels of phenol in liquid smoke ranged from 2.10-5.13%. This results shows that the phenol content in liquid smoke powder and liquid smoke have very low levels.

This phenomenon can occur is possible because, although coconut shell is a hardwood that has a higher lignin content compared to other components, but the pyrolysis temperature affects the amount of decomposition of lignin compounds, namely phenol, which will be shipped in liquid smoke. Lignin will be decomposed into phenol at 350-450^{0}C, while the pyrolysis temperature in this study was 400^{0}C. Additionally redestilation temperature, ie 105^{0}C, is the temperature below the boiling point of phenol, phenol compounds which decompose during the distillation is not maximized, so not many who participated in redestilat phenol.

Moisture content in this study was conducted to liquid smoke powder using thermogravimetri. From the table shows that water content was 6.72% (wb). This shows that the moisture content is low.

Determination of Threshold Value

Threshold test used to determine the threshold value of the smoke flavor began to be perceived by the panelists. While the maximum test serves to determine the maximum proportion of liquid smoke and liquid smoke powder that can still be accepted by the panelists. According Draudt (1963), the threshold value of phenols from smoke condensate is 0.147 ppm to 0.023 ppm stimuli for taste and odor stimuli. This threshold value of phenos is used as the basis for determining the proportion of the addition of liquid smoke and liquid smoke powder in the threshold test.

a. Pasta Seasoning

Five kinds of variations in the concentration of liquid smoke added to ready to eat pasta curry and chicken opor seasoning.

Table 6. Scoring Threshold Panelists to pasta curry and chicken opor seasoning

Concentrations of Liquid Smoke (v/v)	Curry Spices		Chicken Opor	
	Smoke Smell	Smoke Flavor	Smoke Smell	Smoke Flavor
0%	2.00^{a}	2.83^{ab}	2.67^{ab}	2.67^{a}
0.015%	2.67^{ab}	3.33^{ab}	2.00^{a}	2.33^{a}
0.025%	2.33^{a}	2.50^{a}	2.33^{a}	2.67^{a}
0.035%	3.00^{ab}	3.17^{ab}	3.83^{a}	3.83^{a}
0.045%	3.67^{b}	4.17^{b}	3.33^{ab}	3.83^{a}

Note: The superscript sign with letters that are not the same in the same column indicate results significantly different (α = 5%)

Description:
(1) There is no smell / flavor of smoke
(2) Smell / flavor of smoke is very weak
(3) Smell / flavor of smoke is weak
(4) Smell / flavor of smoke is strong
(5) Smell / flavor of smoke is very strong

From the test results, indicating that the curry sauce, liquid smoke added at a concentration of 0.025% (v/v) and 0.015% (v/v) in opor sauce already can feel the flavor and aroma of the smoke by the panelists.

Furthermore, in addition to the threshold value also sought the maximum concentration values for the addition of liquid smoke in the ready to eat pasta seasoning. Initially tried 2 kinds of variations, namely the concentration of 0.5% and 1.0% (v/v). For 0.5% concentration, odor and taste of smoke from the curry sauce is thick and feels more concentrated again to 1% concentration, so that at this concentration sauce was more bitter and panelists felt sick and did not want to eat it. For the concentration chosen was 0.5% and two below the concentration, ie 0.4% and 0.3% for the panelists and get tested to the maximum concentration of liquid smoke to produce curry sauce that taste and smell of smoke was not liked by the panelists was 0.4% (v/v).

Table 7. Scoring panelists dislike of curry spice

Concentrations of Liquid Smoke (v/v)	Attributes	
	Smoke Smell	Smoke Flavor
0.3%	1.00[a]	1.33[a]
0.4%	2.33[b]	3.00[b]
0.5%	2.67[b]	3.00[b]

Description:
(1) Like
(2) Somewhat like
(3) Not Like

Note: The superscript sign with letters that are not the same in the same column indicate results significantly different (α = 5%)

Table 8. Scoring Panelist Dislike of Chicken Pasta Opor Seasoning

The proportion of Liquid Smoke	Smoke Flavor	The smell of smoke
0.5%	1.67 [a]	1.67 [a]
0.8%	**3:33 [a]**	**3:33 [a]**
0.9%	1.67 [a]	2.67 [ab]
1%	2.67 [a]	2.67 [ab]

Description:
1= Like
2 = Somewhat dislike
3 = Dislike
4 = dislike

Note: The superscript sign with letters that are not the same in one column, showed significantly different results (α = 5%).

In the table above, note that the test results of maximum smoke flavor seasoning paste opor found on the proportion of 0.8% (v / v). On the proportion of 0.8% (v / v) panelists can feel a sense of smoke, but the flavor that appears not smoke the expected sensation. Taste sensation that arises is saturated at the base of the tongue, tend to taste bitter, so this treatment is not received by panelis.Berdasarkan results can be concluded that the optimum proportion of smoke became unpopular taste panelists on the proportion of 0.8% (v / v).

The aroma is not liked by the panelists was the smell of smoke with the proportion of liquid smoke **0.8% (v / v).** The result of the assessment panel is due to the proportion of liquid smoke used is high enough so that the smell of smoke is very strong.

b. Powders Seasoning

Five kinds variations of concentration of the liquid smoke powder added to the ready to eat curry and chicken opor powder seasoning.

Tabel 9. Scoring Threshold Panelists against Curry & Chicken Opor Powder Seasoning

Concentration of Liquid Smoke Powder (b/v)	Curry Spices		Chicken Opor	
	Smoke Smell	Smoke Flavor	Smoke Smell	Smoke Flavor
0%	1.67[a]	2.50[a]	2.83[ab]	2.50[ab]
0.01%	2.83[ab]	2.83[a]	1.67[a]	1.67[a]
0.02%	2.67[ab]	2.67[a]	1.67[a]	1.83[ab]
0.03%	3.33[ab]	2.83[a]	3.33[b]	2.83[b]
0.04%	2.67[ab]	2.17[a]	2.50[ab]	2.50[ab]

Description:
(1) There is no smell / flavor of smoke
(2) Smell / flavor of smoke is very weak
(3) Smell / flavor of smoke is weak
(4) Smell / flavor of smoke is strong
(5) Smell / flavor of smoke is very strong

Note: The superscript sign with letters that are not the same in the same column indicate results significantly different (α = 5%)

From the test results, indicating that the curry sauce, liquid smoke powder added at a concentration of 0.03% (w/v) have the smoke smell that can be perceived by the panelists, while for the smoke flavor can begin to be felt in the concentration of smoke powder 0.04% (w/v). As for the taste of chicken opor spice powder began to feel taste of smoke on the proportion of 0.01%. It is known that the lowest threshold smell of smoke began to be felt on the proportion of 0.01% (w / v).

Furthermore, to find the maximum concentration tested 2 kinds of variations, namely the concentration of 0.5% and 1.0% (w/v). For 0.5% concentration, odor and taste of smoke from the curry sauce is still acceptable by panelists at a concentration of 1% while the panelists have felt disgusted and did not want to eat it. For that the selected concentration is 0.1% and two below of the concentration, ie 0.9% and 0.8% for the panelists and get tested to the maximum concentration of liquid smoke to produce the smoke-flavored curry sauce, preferably no panelist was 1.0% (w/v) and to the smell of smoke at a concentration of 0.9% (w/v).

Table 10. Scoring Panelists Dislike of Powder Curry Seasoning

Concentration of Liquid Smoke Powder (w/v)	Atribute	
	Smoke Smell	Smoke Flavor
0.8%	1.75[a]	2.00[a]
0.9%	2.00[a]	1.75[a]
1.0%	2.00[a]	2.75[a]

Description:
(1) Like
(2) Somewhat like
(3) Not Like

Note: The superscript sign with letters that are not the same in the same column indicate results significantly different (α = 5%)

Table 11. Scoring Dislike of Panelist for Powder Chicken Opor Seasoning

Flour Concentration Liquid Smoke (w / v)	Atribute	
	Smoke Odor	Smoke Flavor
0.5%	1.75 [a]	2:00 [a]
1%	3:25 [a]	2.75 [a]
1.25%	**4:00 [a]**	**4:25 [a]**

In the spice powder chicken opor indicates that the test results for the proportion of maximum smoke flavor **1.25% (w/ v).** On the proportion of 1.25% (w / v) showed the highest value is **4:00,** which means not liked **[a]** panelist. On the proportion of 1% (w / v)

several panelists disliked the taste sensation of smoke that emerged but some panelists still able to receive it. Therefore, limit the maximum proportion can be increased until the proportion of 1.25% (w / v).

While the chicken opor spice powder showed displeasure at the smell of smoke panelist for the proportion of **1.25% (w / v).** On the proportion of 1.25% (w / v) showed the highest value is **4:25,** which means not liked [a] panelist. At this proportion is less like some panelists taste sensation that arise because of the smell of smoke generated smoke is very strong. Therefore, the limit on the maximum proportions opor chicken seasoning powder that is the proportion of 1.25% (w / v).

Sensory Analysis

- Curry Spices

Table 12. Preferrence Scoring Panelists to Curry Flavored Traditional Smoke

Sample	Smoke Smell	Smoke Flavor	Overall
Pasta			
0.10%	3.60[b]	3.28[ab]	3.30[ab]
0.15%	3.53[b]	3.40[b]	3.40[b]
0.20%	3.03[a]	2.95[ab]	3.05[ab]
0.25%	3.00[a]	3.08[ab]	3.00[ab]
0.30%	2.73[a]	2.83[a]	2.90[a]
Powder			
0.12%	3.38[c]	3.08[c]	3.08[b]
0.32%	3.30[c]	2.95[bc]	3.13[b]
0.52%	2.73[b]	2.65[abc]	2.83[ab]
0.72%	2.25[a]	2.50[ab]	2.45[a]
0.92%	2.43[ab]	2.30[a]	2.475[a]

Description:
(1) Very Dislike
(2) Dislike
(3) Neutral
(4) Like
(5) Very Like

Note: The superscript sign with letters that are not the same in the same column indicate results significantly different (α = 5%)

Table 13. Scoring Difference Testing of Traditional Curry Flavored Smoke

Sample	Smoke Smell	Smoke Flavor
Pasta		
0.10%	2.33[a]	2.13[a]
0.15%	2.18[a]	2.35[a]
0.20%	3.20[b]	2.98[b]
0.25%	3.73[c]	3.93[c]
0.30%	4.00[c]	3.93[c]
Powder		
0.12%	2.25[a]	2.18[a]
0.32%	2.75[b]	2.80[b]
0.52%	3.38[c]	3.38[c]
0.72%	3.90[d]	3.88[cd]
0.92%	4.33[d]	4.13[d]

Note: The superscript sign with letters that are not the same in the same column indicate results significantly different (α = 5%)

Description:(
1) The smell and taste of smoke is very weak
(2) The smell and taste of smoke is weak
(3) The smell and taste of smoke is a bit stronger
(4) The smell and taste of smoke is strong
(5) The smell and taste of smoke is very strong

Smoke Smell

From this table, average the second batch can be seen that the highest value favorite panelist of smoke smell/ aroma from ready to eat curry paste seasoning that added with the liquid smoke at a concentration of 0.10% (v/v), so that from the five samples of the scent of curry sauce panelists most preferred smoke at a concentration of 0.10% (v/v). However, panelists at a concentration level of delight the addition of liquid smoke 0.10% and 0.15% (v/v) did not significantly different.

The apparent difference between curry sauce with concentration of liquid smoke 0.10%, and 0.15% (v/v) with 0.20%, 0.25%, and 0.30% (v/v). From the results of preference test indicated that panelists prefer a curry sauce with a low concentration of liquid smoke that is below 0.20% (v/v). The higher concentration of liquid smoke, the panelist's favorite level goes down, this proved to panelists gave a lower value. This is also supported by the results of the test scoring differences that the higher concentration of added liquid smoke, flavor/ odor of smoke generated stronger. The smell of smoke generated from the addition of liquid smoke 0.10% (v/v) did not significantly different with the concentration of 0.15% (v/v) but significantly different with three concentration on it. The stronger the smell of smoke is detected by the panelists, the level of preference of panelists decreases.

For the ready to eat powder seasoning, the highest score of panelists to smoke smell in a curry sauce that whit added the liquid smoke powder at a concentration of 0.12% (w/v), so that from the five samples of curry sauce is the most preferred smoke flavor panelists at a concentration of 0:12 % (w/v). However, the level of flavor panelists favorite in curry sauce at a concentration of 0.12% was not significantly different with the concentration of 0:32%, but significantly different with the concentration of 0.52%, 0.72% and 0.92% (w /).

From the results of preference test indicated that panelists prefer a curry sauce with concentration of liquid smoke powder is low, under 0.32% (w/v). The higher concentration of liquid smoke powder then progressively decreasing level of panelist's favorite, this proved to panelists gave a lower value. This is because the smell of smoke is produced will get stronger and sharper. This is also supported by the results of the test scoring differences that the higher of the concentration of added liquid smoke, flavor/ odor of smoke generated stronger. The smell of smoke generated from the addition of liquid smoke 0.12% (w/v) significantly different with other concentrations. The stronger the smell of smoke is detected by the panelists, the level of preference of panelists decreases.

Smoke Flavor

From the table it can be seen that the highest value of the panelist's favorite smoke flavor from the paste seasoning in curry sauce with a concentration of 0.15% (v/v), so that from the five samples of curry sauce is the most preferred smoke flavor panelists at a concentration of 0.15% (v/v). However, the smoke flavor panelist’s favorite level of curry sauce at this concentration did not differ significantly with the concentration of the other four.

From the results of preference test indicated that panelists prefer a curry sauce with a low concentration of liquid smoke was the concentration below 0.25% (v/v). The higher concentration of liquid smoke, the panelist's favorite level goes down, this proved to panelists gave a lower value. This is also supported by the results of the difference test’s scoring that the higher concentration of added liquid smoke, flavor/ odor of smoke generated stronger. The smell of smoke generated from the addition of liquid smoke 0.10% (v/v) did not differ significantly with the concentration of 0.15% (v/v) but significantly different with three concentration on it. The stronger smell of smoke is detected by the panelists, the level of preference of panelists decreases.

For ready to eat curry powder, the highest value preferences of panelists for smoke flavor is in a curry sauce that added liquid smoke powder at concentration 0.12% (w/v), so that from the fifth sample was panelists most preferred of the smell of smoke with concentration of 0.12% (w/v). However, the level of smoke flavor favorite panelist of curry sauce at this concentration did not differ significantly with the concentration of 0:32% (w/v), and significantly different with the three other concentrations.

From the results of preference test indicated that panelists prefer a curry sauce with concentration of liquid smoke powder is low at concentrations below the 0.32% (w/v). The higher concentration of liquid smoke powder then progressively decreasing level of panelist's favorite, this proved to panelists gave a lower value because the resulting smoke flavor will get stronger and sharper, causing a bitter aftertaste. Thus it can be seen that the panelists preferred the low smoke flavor. This is also supported by the results of the difference test's scoring that the higher of the concentration of added liquid smoke powder, the flavor/ odor of smoke generated stronger. The smell of smoke generated from the addition of liquid smoke powder 0.12% (w/v) differ significantly with other concentrations. The stronger of the smell of smoke's detected by the panelists, the level of panelist's preference decreases.

Overall Liking

From the table it can be seen that the highest score overall liking from panelist of the ready to eat curry pasta, which added to the liquid smoke at a concentration of 0.15% (v/v), so that the overall liking from panelists from the fifth sample was at the concentration of 0.15% (v/v). However, the overall level of panelist's favorite from curry sauce at this concentration did not differ significantly with the concentration of the other four.

From the results of preference test indicated that panelists prefer a curry sauce with a low concentration of liquid smoke was the concentration below 0.25% (v/v). The higher concentration of liquid smoke, the panelist's favorite level goes down, this proved to panelists gave a lower value.

For ready to eat curry powder, the highest value of overall liking from panelists was for smoke flavor in a curry sauce that added liquid smoke powder at concentration 0.32% (w/v), so that from the fifth sample, panelists liked the overall flavor of smoke at a concentration 0.32% (w/v). However, the overall level of panelist's favorite smoke curry sauce at this concentration did not differ significantly with the concentration of the other four.

- Chicken Opor Seasoning

Tabel 14. Scoring Preferent Test of Traditional Chicken Opor with Smoke Flavored

Sampel	Smoke taste	Smoke odor	Overall
Pasta			
0.1%	3.32^{c}	$\mathbf{3.72^{d}}$	$\mathbf{3.60^{c}}$
0.25%	$\mathbf{3.35^{c}}$	3.45^{cd}	3.42^{bc}
0.4%	2.77^{b}	2.90^{b}	3.02^{b}
0.55%	3.10^{bc}	3.12^{bc}	3.02^{b}
0.7%	2.05^{a}	2.20^{a}	2.30^{a}
Bubuk			
0.2%	$\mathbf{3.80^{b}}$	$\mathbf{3.67^{c}}$	$\mathbf{3.80^{b}}$
0.4%	3.62^{bc}	3.35^{bc}	3.70^{b}
0.6%	3.52^{b}	3.37^{bc}	3.62^{b}
0.8%	2.90^{a}	3.07^{b}	2.92^{a}
1 %	2.55^{a}	2.57^{a}	2.65^{a}

Note: The superscript sign with letters that are not the same in the same column indicate results significantly different (α = 5%)

Description:
Testing Interests:
(1) It's Not Like (4) Like
(2) Not Like (5) It's Like
(3) Neutral

Table 15. Scoring Different Test of Traditional Chicken Opor with Smoke Flavored

Sampel	Smoke odor	Smoke taste
Pasta		
0.1%	1.75^{a}	1.75^{a}
0.25%	**3.22^{b}**	**2.92^{b}**
0.4%	3.92^{c}	3.95^{c}
0.55%	3.70^{bc}	3.95^{c}
0.7%	4.55^{d}	4.55^{d}
Bubuk		
0.2%	**2.15^{a}**	**2.02^{a}**
0.4%	2.67^{a}	2.70^{b}
0.6%	3.32^{b}	3.52^{c}
0.8%	4.05^{c}	3.97^{cd}
1%	4.42^{c}	4.32^{d}

Note: The superscript sign with letters that are not the same in the same column indicate results significantly different ($\alpha = 5\%$)

Description:
Differentiation Test:
(1) The smell and taste of smoke is very weak
(2) The smell and taste of smoke weak
(3) The smell and taste a bit stronger smoke
(4) strong smoke smell and taste
(5) The smell and taste of smoke is very strong

Smoke Flavor

Taste panelists most preferred is a variation of chicken opor spice paste flavors with smoke and liquid smoke proportion of **0.25% (v/ v).** There is no real difference with the proportion of 0.1% (v / v), but for effectiveness we used the proportion of 0.25% (v / v) as a proportion of the optimum. Selection of a higher proportion in order to have antioxidant and antimicrobial higher as well. Compounds in liquid smoke that act as antimicrobial namely phenol. The proportion of 0.25% (v / v) have a higher durability and can cope with loss of smoke flavor to flavor chicken opor if prolonged storage.

Mechanism of phenolic compounds in killing microbes is the reaction between acid fenoleat with protein (in this case, microbes. In enzymatic conditions in the presence of enzymes that work naturally fenolase at neutral pH, acid fenoleat oxidized to quinones that can react with the lysine of the protein that causes these proteins can not be used in biological (Hurrell, 1984).

According Wastono (2006), liquid smoke *(liquid smoke)* may be used as a preservative because of the acid compounds, phenolic and carbonyl who have the ability to preserve food such as meat, fish, noodles, food seasonings and meatballs.

A higher proportion of liquid smoke contains higher as well. Liquid smoke contains components that are bakteristatis and bactericidal which can act as a preservative. This can happen if the smoke settles on the surface or seep into the material of smoked (Winarno, 1981). Compounds that very act as antimicrobials are compounds of phenol and acetic acid, and its role is increasing when there are two compounds together (Darmadji, 1996). Besides phenol, aldehyde compounds, acetone and ketones also has bacteriostatic and bakteriosidal power on smoke products. Girrard (1992) states that the smoke in the form of liquid affect the overall amount of smoke in smoke condensate, which reached 40% with 35 types of acid.

Volatile acid content in the smoke will decreased the pH, thus slowing the growth of microorganisms (Buckle *et al.*, 1985).

There are real differences in the proportion of 0.4%, 0.55% and 0.7% (v/v). Chicken opor seasoning with the proportion of 0.4% liquid smoke shows tend neutral rating, while the proportion of 0.55% preferred near neutral to the panelists so that is still acceptable by the panelists. Then, on the proportion of 0.70% (v / v) showed an unwelcome rating panelists. Therefore, concluded the optimum taste of smoke on the proportion of variation 0.25% (v/v). From the results of the most *high-scoring* flavor obtained a mean value of **3.35** ^{c} panelists liked sample chicken opor seasoning with proportion of liquid smoke 0.25% (v/v).

For powder fast food seasoning, the highest value preferences of panelists to smell smoke powder chicken opor seasoning with liquid smoke proportion of 0.2% (w/v) have a sense of smoke with the highest score of **3.80** ^{b} which means that panelists liked. It is known that panelists tend to like spice powder opor smoke flavor with a range of proportions of 0.2% -0.6% (w/v) with panelists on the product's favourite powder chicken opor seasoning not significantly different. While the proportion of 0.8% and 1%, which is significantly different panelists prefer less preferred. The proportion of 0.2% (w/v) was chosen as the optimum proportion because it is more efficient and economical.

The correlation between preference test results and different test results to the taste of smoke indicates that panelists prefer the taste of smoke with a low intensity. Based the result of different test above, it can be seen the proportion of the addition of liquid smoke to taste panelists affect the assessment of smoke. The greater proportion of the addition of liquid smoke and liquid smoke, the taste of flour produced stronger smoke too. On the table shows that chicken opor paste seasoning with the addition of liquid smoke proportion of 0.25% and 0.2% for the proportion of powder is the preferred product, but with low smoke flavor intensity.

The smell of smoke

Variations chicken opor pasta seasoning which showed the highest value of smoke odor and flavor of liquid smoke is the proportion of 0.1% with a value of **3.72**^{d}. However, the value is not significantly different with variations in the proportion of 0.25% (v / v) whose value is **3.45**$^{cd.}$ In consideration of antioxidant properties and durability of products like the taste of smoke, the chosen variation of the proportion of 0.25% (v / v) as a optimum proportion.

In the panelist's favourite scoring for powder chicken opor seasoning seen the highest score for the proportion of 0.2% (w/v) with a value of **3.67**^{c} which indicates neutral rather liked. On the proportion of 0.4% (w/v) with value 3.35^{bc} and 0.6% (w/v) with value 3.37$^{bc.}$ These results are not significantly different from the proportion of 0.2% (w/v). While the proportion of 0.8% (w/v) 3.07^{c} is not significantly different from the proportion of 0.4% and 0.6% (w/v). Then, the proportion of 1% (w/v) tend to be neutral is not favored with 2.57^{a} score.

Based on the correlation of preferrence test results and the different test results of the favourite shows that panelists prefer the smell of smoke with a low intensity of 0.25% on the spice paste and 0.2% for powder seasoning. Meanwhile, the strong scent of smoke less preferred panelists.

Overall

Overall, the panelists showed the highest rating in the sample that is **3.60** chicken opor pasta seasoning flavored with smoke and liquid smoke variation 0.1% (v/v) with neutral rather liked scoring. There is no real difference with the proportion of 0.25% (v/v), which the value is **3.42** ^{bc} or neutral rather liked. With consideration of the higher addition of liquid

smoke can produce high antioxidant, so the chosen proportion of 0.25% (v/v) as a optimum proportion. For the proportion of 0.4% and 0.55% (v/v) 3.02^{b} which means the scent is still accepted by the panelist. While the proportion of 0.7% (v/v), the panelists gave score 2.30^{a}, which means taste and smell strong smoke flavor is less preferred.

Panelists showed the highest rating in the sample that is **3.80^{b}** powder chicken opor seasoning with liquid smoke variation of 0.2% (w /v) with scoring tends to prefer. There is no real difference to the proportion of value 0.40% and 0.60% ^{b} 3.70 (w/v) 3.62 ^{b} with a neutral score rather liked. While the proportion of 0.8% with value 2.92^{a} and 1% (w/v) the value 2.65^{a} significant difference that is less favored by panelists. With the addition of liquid smoke economical considerations, the chosen proportion of 0.2% (w / v) as a optimum proportion. Making powder chicken opor seasoning used drying so that has higher operating costs.

Chemical Composition

Determination of Phenol and Total Acid

Determination of phenol content and total acid was to determine the components of liquid smoke flavor in the form of components phenolytic and acid compounds were absorbed into the product and its influence on the panelist's favorite.

- Curry Spices

Table 16. Results Analysis of Phenol and Total Acid Curry Spices

Sample	Concentration	Prosentage (%)	
		Phenol Content	Total Acid
pasta	0% (v/v)	1.65	2.63
	0.15% (v/v)	2.69	3.09
	0.30% (v/v)	3.13	4.77
powder	0% (b/v)	3.40	1.44
	0.12% (b/v)	4.20	2.04
	0.92% (b/v)	5.24	3.29

Phenol content in the paste seasoning without the addition of liquid smoke is 1.65%. While the phenol content in the spice paste with liquid smoke's concentration 0.15% (v/v) and 0.30% (v/v) respectively 2.69% and 3.13%, higher than the levels of phenol in smoke liquid itself, ie 2.53%. Similarly, the phenol content in the seasoning powder, powder seasoning without the addition of liquid smoke powder amounted to 3.40%. While the phenol content in the seasoning powder with liquid smoke powder's concentration 0.12% (w/v) and 0.92% (w/v) respectively for 4.20% and 5.24%, higher than the levels of phenol in liquid smoke powder itself, which is equal to 2.24%. This suggests that the seasoning paste and powder itself already contains phenolic compounds.

From the results table is also known that the higher of the concentration's use of liquid smoke to ready to eat paste seasoning or use of liquid smoke powder on ready to eat seasoning powder, then the phenol content and total acid will increase as well. Total phenol content of this acid effects on the flavor of spices produced, where Girard (1992) mentions that the phenol compounds known as the major constituents that play a role in the formation of flavor in the product and according Darmadji (1996) acid plays an important role in the organoleptic assessment on the product as a whole. More phenol and total acids contained in the material then the smoke flavor will be felt increasingly. When connected with the results of sensory analysis, the greater of uses the concentration of liquid smoke and liquid smoke powder, panelists favorite value decreases. It means that the higher phenol content and total

acid contained in the material, the value of the panelist favourites goes down because the smoke flavor is very pronounced and can give a bitter after taste.

- Chicken Opor Seasoning

Tabel 17. Results Analysis of Phenol and Total Acid Chicken Opor Seasoning

Sample	Concentration (%)	Percentage (%)	
		Phenol	Acid Total
Pasta Seasoning (v/v)	0	2.56	1.62
	0.25	2.63	2.00
	0.70	3.14	2.78
Powder seasoning (b/v)	0	1.86	1.99
	0.20	1.91	2.19
	1	2.77	2.59

From Table 17, we know that there is an increase of phenol content control pasta and powders seasoning without smoke into pasta and powder seasoning smoke flavor. The increase in phenol content was related to the use of liquid smoke 0.25% (v / v) on addition of flour paste and smoke 0.2% (w / v) of spice powder. This proves that the engineering process, adding smoke flavor can raise the level of phenol in the product but can still be accepted by consumers.

Phenolic compounds is very important in determining the quality of liquid smoke and material treated with liquid smoke. This is because phenol role in contributing to produce specific smoke flavor (Dugan, 1976). Based the table 4.13 seen that the more addition of liquid smoke and liquid smoke powder causing the higher of phenol content. The increase in phenol content was contribute the decrease of panelists preferrence in opor seasoning products for either in the form of powder or paste.

Acids derived from liquid smoke and powder liquid smoke products shipped to chicken opor seasoning. This was a favorite cause panelist to be reduced. Overall seen that the more total acid content in the product ingredients opor then decline favorite panelists.

Proximate Analysis

Table 18. Proximate Analysis Results of Ready to Eat Curry Spice

Sample	Component	Content (%wb)	Content (%db)
Pasta 0.15%	Moisture	58.12	129.01
	Fat	12.06	28.78
	Protein	5.65	13.49
	Total Sugar	6.36	15.19
	Reduction Sugar	2.33	5.57
Powder 0.12%	Moisture	8.75	9.61
	Fat	9.73	10.64
	Protein	25.17	27.58
	Total Sugar	7.91	8.67
	Reduction Sugar	4.94	5.42

Tabel 19. The Proximate Analysis Ready to Eat Chicken Opor Seasoning

Sample	Component	Content (%wb)	Content (%db)
Pasta Seasoning 0,15%	Water	37.11	59.00
	Fat	10.59	16.84
	Protein	5.71	9.08
	Total sugar	9.77	15.54
	Sugar Reduction	2.61	4.15
Powder Seasoning 0,12%	Water Levels	7.80	8.46
	Fat	5.20	5.63
	Protein	12.98	14.08
	Total sugar	19.57	21.23
	Sugar Reduction	2.65	2.87

Moisture Content

From the above table is known that the moisture content in curry paste amounted to 58.12% (wb) or 129.01% (db) and the curry powder s of 8.75% (wb) or 9.61% (db). While for the chicken opor pasta seasoning 37.11% (wb) or 59% (db) and powder of 7.8% (wb) or 8.46% (db). According to Hambali (2009) the water content of dried herbs is less than 8%.

In the spice paste, the frying process during processing can cause a decrease in water content and the spice powder, a decrease of water content occurs during the drying process of each type of seasoning. Drying is a process of removal of water from a dried material. This omission includes two stages, namely transfer of material to the surface and the stage of water evaporation from the surface to the atmosphere (Van Arsdel, 1963).

This low water content can extend shelf life because water can help prevent the destruction of food such as the microbiological, chemical, and enzymatic material by the presence of insect activity.

Fat Content

It is known from the calculation that the fat content in the curry spice paste is quite high ie 12.06% (wb) or 28.78% (db). The high fat content found in the curry spice paste is highly influenced by the oil absorption process during frying. Fat contained in the material, eg ginger rhizome only by 1.0%, 0.3% fresh red chilli, red onion 0.3%, 0.2% garlic, tamarind 0.6% (Anonim[1], 1981) and turmeric rhizome 2.7% (Thomas, 1995) so that the high content of fat in curry spice pastes due to the absorption of oil during frying. During the frying process occurs transfer cooking oil from the frying pan into the products and become a component of the product (Heid and Joslyn, 1967).

While the calculation of fat content in the curry spice powder which is 9.73% (wb) or 10.64% (db). Fat content of chicken opor seasoning paste of 10.59% (wb) or 16.84% (db). While the calculation of fat content in chicken opor seasoning powder that is 5.20% (wb) or 5.63% (db). The presence of fat in the powder tends to be a barrier against water absorption process and will reduce the tendency of granules to swell. When high levels of fat in the powder can affect the quality of ingredients during the storage process because of the high fat content in the material causes the material will be more easily damaged and rancid.

Protein Content

Protein content in the curry spice paste was 5.65% (wb) or 13.49% (db). Protein content in these products is the result of contributions from ginger rhizome with a protein content of 2.0%, 1.0% fresh red chilli, red onion 1.5%, 4.5% garlic, tamarind 2.8% (Anonim2 , 1972), and turmeric 2% (Thomas, 1995) contained in the product. While the protein content from curry spice powder is about 25.17% (wb) or 27.58% (db). In the chicken opor spice paste, protein content is 5.71% (wb) and the powder as much as 12.98% (wb).

The existence of heat treatment, such as frying and drying during processing make quality of protein in the product decreases. Heating resulted in a decrease of total amino acids, especially lysine, tryptophan, cysteine, and histidine. The higher temperatures are used, then the total amino acids will further go down (Badenhop and Hackler, 1971). Heating process carried out during the making of spice paste is frying, while in the manufacture of spice powder is drying with a cabinet dryer. Frying temperature is higher than the temperature of drying with the cabinet dryer, so the decrease in total amino acids greater.

The amount of protein content in the powder will have much effect on the functional properties of powders, especially the absorption of water. According to Kinsella (1976), high protein content will increase the ability of the material to absorb water because in general the protein has a hydrophilic group capable of binding water. So spice powder is easier to be clouding when stored in conditions that are not good.

Total Sugar and Reduction Sugar

Total sugar content found in curry spice paste is 6.36% (wb) or 15.19% (wb) and reducing sugar content of 2.33% (wb) or 5.57% (db). While the total sugar content in currry spice powder is about 7.91% (wb) or 8.67% (db) and reducing sugar content of 4.94% (wb) or 5.42% (db).

Total sugar content in the seasoning pasta chicken opor is 9.77% (wb) or 15.54% (wb) and reducing sugar content of 2.61% (wb) or 4.15% (db). In the spice powder chicken opor, total sugar content is about 19.57% (wb) or 21.23% (db) and sugar reduction content 2.65% (wb) or 2.87% (db).

From the results it is known that both the ready to eat paste and powder seasoning contain more sugar reduction when compared with non-reducing sugar.

Prototype Packaging

The uses of aluminum foil as a packaging material because of the aluminum foil is airtight packaging materials and light-tight so that the packaging is expected to spice powder that not easy clouding and spice pastes did not experience oxidation. Another function is to protect the existing one component of the spice that are phenol components which influence the flavor. Phenol is an important component that can provide the smoke flavor in the seasoning and phenol are sensitive to light and temperature, the presence of aluminum foil packaging was then phenol present in condiments can be protected.

Prototype packaging for spices powder and pasta measuring 13 cm x 12 cm (length x width) with the front containing the brand, net, and halal label while the back contains a list of compositions, serving suggestions, nutritional information, production code, barcode, and the names and addresses of companies that produce.

Conclusion

1. Precise formulation curry spice is obtained on the orientation I in spice pastes and in orientation III in spice powder. While the chicken opor seasoning paste is obtained on the orientation IV and powder in orientation II.

2. Threshold of smell and smoke flavor of liquid smoke obtained by the curry seasoning paste are 0.025% (v/v) and 0.04% (w/v). While the threshold chicken opor seasoning pasta on the proportion of 0015% (v/v) and chicken opor spice powder with the proportion of 0.01% (w/v). Maximum proportion of curry spice paste in 0.4% (v/v), and for powder 1.0% (w/v). While the maximum proportion of the less favored panelist on seasoning pasta chicken opor ie 0.8% (v/v) and then to spice powder chicken opor with the proportion of 1.25% (w/v).

3. Optimum addition of liquid smoke at a concentration of 0.15% (v/v) in the ready to eat curry paste seasoning and 0.25% (v/v) for opor chicken paste seasoning, while the addition of liquid smoke on ready to eat curry powder seasoning at a concentration of 0.12% (w/v) and 0.2% (w/v) forchicken opor powder seasoning.

Suggestion

1. Not knowing the influence of cooking time to the addition of liquid smoke and liquid smoke powder in spice curry powder and pasta ready meals, so we need further research.

2. Engineering addition of liquid smoke and liquid smoke powder on the ready to eat seasnoning produced by different water content, so the shelf life was different, it is necessary to conduct further research to determine the shelf life of products.

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A Novel Process to Prepare Chemoselectively Protected N-Phthaloyl-Chitosan without Drying of Solvent and Purging of Water Vapor

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ABSTRACT

A new process for preparing chemoselectively protected N-phthaloyl-chitosan macrospheres, which was simple and practicable, was reported in this paper. Chitosan was phthaloylated using phthalic anhydride in dimethylformamide as solvent with addition of 5% water (v/v) as cosolvent and without drying of solvent to remove the contained water and without flowing of nitrogen to purge water vapor during reaction process. As comparison, the same condition reaction was also performed to prepare N-phthaloyl-chitosan but without addition of 5% water as cosolvent. The FTIR spectra showed that chemoselectively protected N-phthaloylation of chitosan can be prepared without pretreatment of solvent drying and without treatment of water vapor purging during the reaction.

Keywords: selective, protection, phthaloyl, chitosan.

1. INTRODUCTION

Chitin is the second most abundant natural polysaccharide, generally found in the composition of crustacean shells, insects, molluscan organs, and fungi. Chitosan is obtained from chitin by alkaline hydrolysis with inorganic base; the process essentially hydrolyzes N-acetyl groups at random within the polymer backbone (Fig. 1). Chitin and chitosan have gained tremendous interest due to their properties as non-toxic, biocompatible, and biodegradable polymers. Chitin is insoluble in water and almost all organic solvents while chitosan is soluble in dilute organic acid solutions such as acetic, formic, succinic, and lactic acids at pH below 6.5. Therefore, the applications of chitin and chitosan are limited due to less solubility in water and organic solvent. In order to improve the solubility and physicochemical and biological properties, several chemical modifications of chitosan have been reported (Sajomsang, 2010).

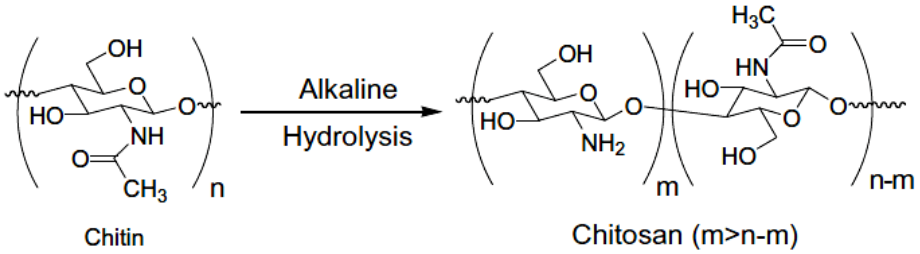


Fig. 1. Preparation of chitosan from chitin.

Phthaloylation can be a practical way for solubilization of chitosan in organic solvents, since the phthaloyl group is bulky and eliminates hydrogen from the amino group to prevent hydrogen bonding. In addition, phthaloylation can protect the amino functionalities of chitosan, and deprotection regenerates thee free of amino groups. Therefore, the N-phthaloyl group can be indispensable for both protection and solubilization. N-phthaloyl-chitosan has also proved to be most promising for conducting modification reaction with good facility and regioselectivity under mild conditions in solution (Kurita, 2001).

Phthaloylation of chitosan is usually performed in N,N-dimethylformamide (DMF) with excess phthalic anhydride at 120-130 °C for 8 h. Treatment of chitosan with phthalic anhydride, however, generally results in partial O-phthaloylation in addition to the N-substitution. With the phthaloylated product as a key intermediate, various modification reactions proceed smoothly in solution, but the *O*-phthaloyl group is an obstacle in most cases to quantitative and regioselective substitution (Nishimura *et al*, 1991). Kurita *et al* (2002) reported on a simple and reliable method to provide chemoselectively protected *N*-phthaloyl-chitosan in one step using DMF containing 5% water as a solvent. Interestingly, however, although Kurita *et al* (2002) using 5% water (v/v) as a cosolvent, he still use drying pretreatment to remove of water from the DMF as solvent and flowing nitrogen to purge of water vapor during reaction process. We report here a simple method to provide chemoselectively protected N-phthaloyl-chitosan using DMF containing 5% water as a solvent without pretreatment of drying of DMF and without flowing nitrogen to purge of water vapor during reaction process.

2. MATERIALS AND METHODS

2.1. Materials

Chitosan was obtained from deacetylated chitin that isolated from shrimp sells. N,N-dimethylformamide (DMF) and phthalic anhydride were obtained from Merck (Germany). All chemicals were used without further purification.

2.2. Synthesis of N-phthaloyl-chitosan

To a solution of 0.83 g (5.6 mmol) of phthalic anhydride in 6 mL of *N*,*N*-dimethylformamide (DMF) containing 5% (v/v) water was added 0.300 g of chitosan, and the mixture was heated at 90 °C with stirring. After 8 h of reaction, the resulting pale tan mixture was cooled to room temperature and poured into ice water. The precipitate was collected on a filter, washed with 150 mL of methanol at room temperature for 1 h, and dried to give the product as a pale tan powdery material. As comparison, the same condition reaction was also performed to prepare N-phthaloyl-chitosan but without addition of 5% water (v/v) as cosolvent. The products were characterized by recording the FTIR spectrum on a Shimadzu FTIR (IRPrestige-21).

3. RESULTS AND DISCUSSION

Protection of the functional groups of chitosan is crucial for conducting modification reactions in a well-controlled manner, and introduction of the phthaloyl group at the amine functionality is ideal not only for the protection but also for improving solubility.

Phthaloylation of chitosan is usually performed in DMF with excess phthalic anhydride at 120-130 °C for 8 h. Interestingly, however, the ds (degree of substitution) decreased to some extent on prolonged reaction. This implies the possibility of hydrolytic cleavage of the once formed ester linkages with the water produced by N-phthaloylation. Kurita *et al* (2002) reported on a simple and reliable method to provide chemoselectively protected *N*-phthaloyl-chitosan in one-step using DMF containing 5% water as a solvent. It is interesting to examine the preparation of chemoselectively protected N-phthaloyl-chitosan using DMF containing 5% water as a solvent without pretreatment of drying of DMF and without flowing of nitrogen to purge of water vapor during reaction process.

The structures of both products were confirmed by the characteristic peaks of phthalimido group (1774, 1712 cm^{-1} (C=O anhydride)), and aromatic ring (721 cm^{-1}), as shown in Fig. 2. The product prepared in DMF showed weak bands at 2600-2700 cm^{-1} (free carboxyl) and sharp ones at 1288 and 1265 cm^{-1} (ester) in the FTIR spectrum that suggests incomplete cyclization at the amino group and/or partial O-phthaloylation (Scheme 1). The product obtained in the mixed solvent (DMF and 5% water) showed very weak bands at 1288

and 1265 cm^{-1} and no appreciable bands due to ester carbonyl at about 2600-2700 cm^{-1}. This result is in accordance with the previous result (Kurita *et al*, 2002) that used DMF and 5% water as a mixed solvent and with pretreatment of drying water on DMF and by flowing nitrogen to purge of water vapor during the reaction.

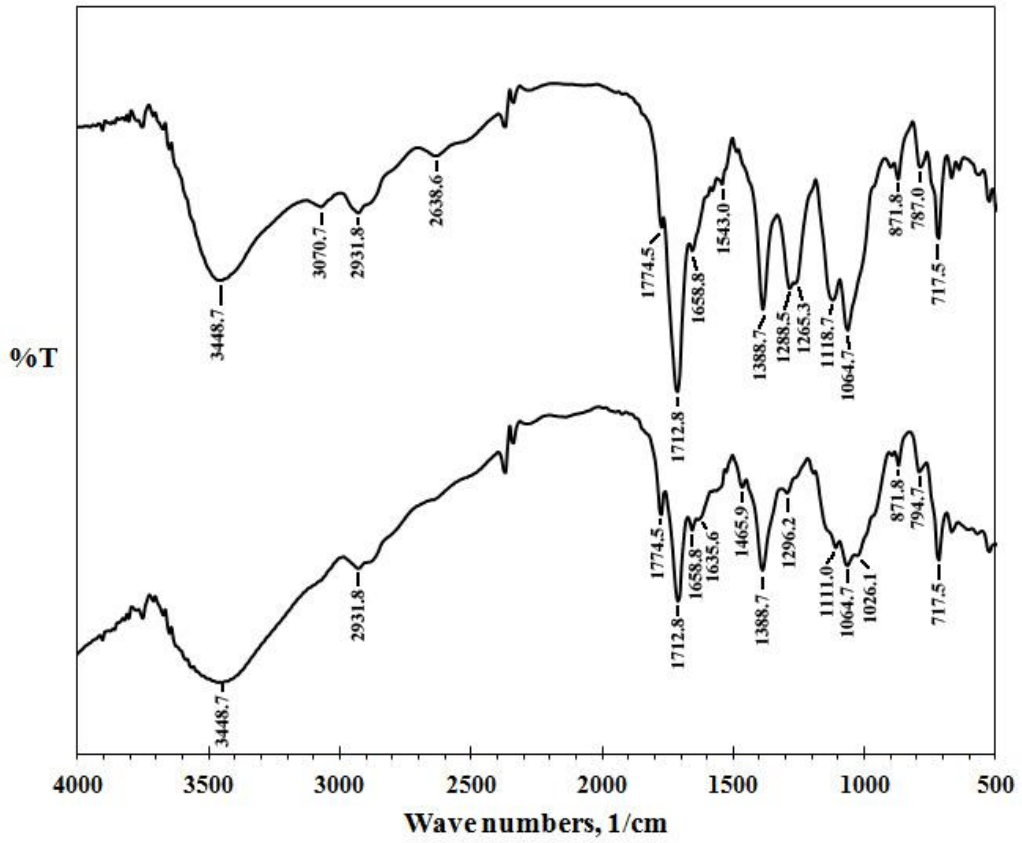
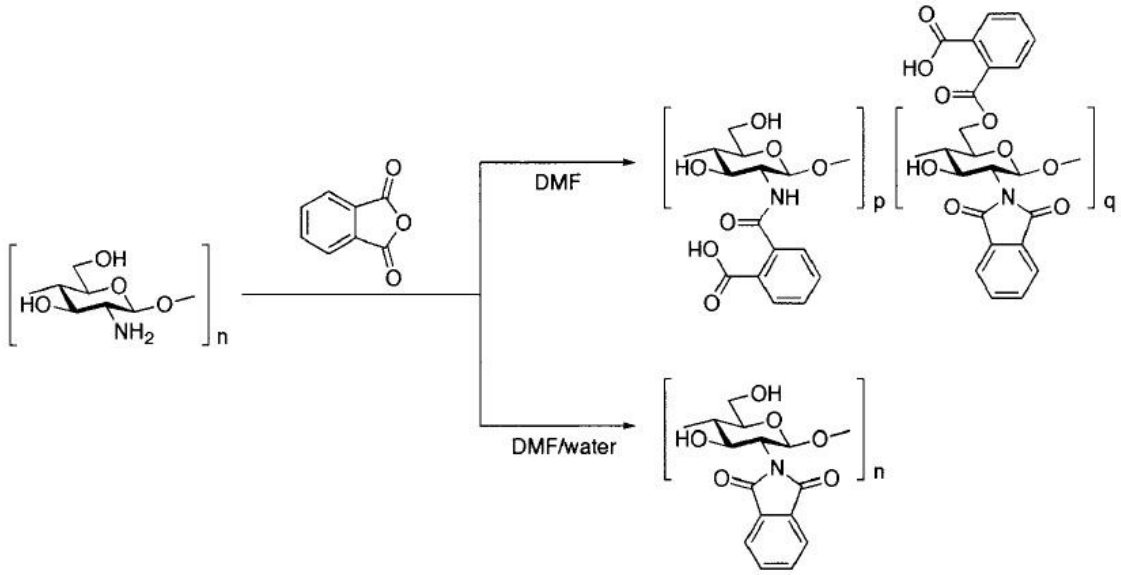


Fig. 2. FTIR spectra of phthaloylated chitosan prepared by the reaction at 90 °C for 8 h in DMF without addition of water (top) and at 90 °C for for 8 h in DMF/water (95:5) (bottom). Both products were obtained by reaction without pretreatment of drying of solvent and without purging of water vapor during reaction process.



Scheme 1

A qualitative solubility test indicated that the resulting *N*-phthaloyl-chitosan was soluble in organic solvent such as *N*,*N*-dimethylacetamide/8% LiCl. It swelled in more

common solvents such as DMF. Kurita *et al* (2002) have shown that the relatively low solubility of *N*-phthaloyl-chitosan may be partly attributable to some crystallinity as suggested by the X-ray diffractiometry**.**

CONCLUSIONS

Chemoselective N-phthaloylation of chitosan could be accomplished successfully in one-step using DMF containing 5% water as a solvent without pretreatment of drying of DMF and without flowing nitrogen to purge of water vapor during reaction process. The *N*-phthaloyl-chitosan exhibited high affinity for organic solvents, although somewhat lower than that of the product having additional *O*-phthaloyl groups. The simple procedure established here enables facile preparation of *N*-phthaloyl-chitosan, a convenient precursor for the construction of sophisticated molecular architectures based on the specialty biopolymer chitosan.

ACKNOWLEDGEMENT

This work was supported financially by a Grant of Penelitian Hibah Bersaing (No. 008/SP2H/PL/Dit.Litabmas/IV/2011) from the Directorate General of Higher Education (DGHE), Ministry of National Education, Indonesia.

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Encapsulation of Phenolic Compound from Star Fruit with Chitosan Nanoparticle

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Abstract

The use of chitosan for the encapsulation of active components has gained interest in the last years due to its mucous adhesiveness, non-toxicity, biocompatibility and biodegradability. The benefits of encapsulating active agents in a polymer matrix include their protection from the surrounding medium or processing conditions and their controlled release.In this study chitosan nanoparticles were obtained for the encapsulation of phenolic compound. Chitosan anoparticles were prepared by ionic gelation of chitosan and sodium tripolyphosphate. X-ray diffraction, FTIR, and TEM were used to structurally characterize these chitosan nanoparticle products. The penolic compound were added to the sodium tripolyphosphate solution and this was added dropwise to the chitosan solution while stirring. The effect of the encapsulating systems on the phenolic compound stability and its release properties was analyzed.The products obtained allowed to control the release of phenolic compound and therefore these encapsulating methods are a promising technique for nutraceutical and cosmetic applications.

Keyword : chitosan nanoparticle, phenolic compound,

1. Introduction

Encapsulation is a process in which thin films, generally of polymeric materials are applied to little solid particles, liquid or gases droplets. This method is used to trap active components and release them under controlled conditions. Several materials have been encapsulated in the food industry, among others, aminoacids, vitamins, minerals, antioxidants, colorants, enzymes and sweeteners[1]. Because of the many benefits offered by encapsulation,entrapped microorganisms can be used to advantage for producing dairy products such as yoghurt, cheese and frozen milk products, as well as for biomass production. The use of encapsulated microorganisms reduced the incubation time by 50% and 60% for fresh fermentedcheese production[2] and cream fermentation [3] , respectively. Sheu and Marshall[4] reported that about 40% more lactobacilli survived freezingof ice cream when they were entrapped in calcium alginate than when they were not entrapped. Moreover, encapsulation protectedthe microorganisms in batch-frozen and continuously frozen ice milk mixes[4]. The benefits of encapsulating active agents in a polymer matrix include their protection from the surrounding medium or processing conditions and their controlled release [5]

Chitosan is receiving a lot of interest in the encapsulation of active compounds due to its biocompatibility and low toxicity [6] In order to improve the use efficiency of active agent and reduce the cost of production, active agent immobilization technology is applied.

In recent years, nanotechnology has showed a significant attraction to the preparation of immobilized enzymes. Under the scale of nano, nano-materials have characteristics such as magnetism and large surface area, etc. Recently, chitosan nanoparticle have been prepared to improve its antimicrobial activity[7], and mainly used as drug carrier as reported in previous studies [8]. chitosan nanoparticles had been prepared, characterized, and used to adsorb eosin Y, acid orange from aqueous solutions [9-10]. Nanoparticles are made of natural or artificial polymers ranging min size from 10–1000 nm [11]. .Nanoparticles display unique physical

and chemical features because of effects such as the quantum size effect, mini size effect, surface effect and macro-quantum tunnel effect. Hence the aim of the present study is to investigate for the encapsulation of star fruit extract with chitosan nanoparticle

Star fruit is grown in the tropic and sub-tropic regions of the world. It is quite a popular fruit and largely planted in Southeast Asia and many other countries. It is usually consumed fresh or made into fruit juice or juice drinks. Leong & Shui [12] reported that star fruit is a good source of natural antioxidants, and the antioxidants in star fruit were found to be proanthocyanidins, -epicatechin and vitamin C [12]

Because extract studies involve several variables, the factorial experimental design was applied to extract systems. Factorial designs are widely used to investigate the effects of experimental factors and the interactions between those factors, that is, how the effect of one factor varies with the level of the other factors in a response. The advantages of factorial experiments include the relatively low cost, a reduced number of experiments, and increased possibilities to evaluate interactions among the variables. They are few papers reported in the literature on the application of factorial experiment in wastewater treatment, such as application of the central composite design and response surface methodology to the advance treatment of olive oil processing wastewater using Fenton's peroxidation [13], empirical modeling of *Eucalyptus* wood processing [14] and the optimization of ozone treatment for colour and COD removal of acid dye effluent using central composite design experiment [15]. In this study, star fruit was chosen for the purpose of investigating its extract phenol compound A 2^3 full factorial design with 20 assay at room temperature was used to evaluate the importance of solvent ratio (X1), temperature (X2),and extract time (X3).

2. Materials and methods

2.1. Chemicals and reagents

Folin–Ciocalteu reagent, sodium hidroxide, sodium tripolyphosphate and acetic acid from Merck (Darmstadt, Germany), gallic acid from Kalbe Farma.Chitosan, from laboratory analytical chemistry State Univercity of Jakarta.

2.2. Sample preparation and optimazing extraction

Several batches of star fruits were purchased from a local supermarket or wholesale center. Fresh star fruit obtained from the market was homogenized using a blender, centrifuged and filtered under vacuum. The liquid portion (juice) was used directly for antioxidant capacity and total phenolic assays.The extraction of phenolic compound from the dried powder was carried out at different ratios of solvents acetone:water (45%, 50%, and 55, respectively), to obtain suitable extraction solvent and ratio of solvent to water. Under the selected extraction solvent,different extraction temperatures (75, 85 and 90^0 C, respectively) were compared. Under the selected solvent and temperature, different extraction times (40,45,50 min, respectively), were applied for obtaining a suitable extraction time. The extraction was carried out in sealed glass bottles, which were placed into a water bath at a preset temperature.

2.3. Total phenolic compound content by Folin–Ciocalteau assay

Total phenolics were determined using Folin–Ciocalteau reagents.Gallic acid standard solution (2.0 mg/ml) was prepared by accurately weighing 0.01 g and dissolving 50 ml of distilled water. The solution was then diluted to give with concentrations working standard solutions of 1.5, 1.0, 0.5, 0.2, and 0.1 mg/ml. Forty microlitres of juice or gallic acid standard was mixed with 1.8 ml of Folin–Ciocalteu reagent (previously diluted 10-fold with distilled water and allowed to stand at room temperature for 5 min, and then 1.2 ml of sodium bicarbonate (7.5%) was added to the mixture. After standing 60 min at room temperature,

absorbance was measuredat 765 nm. Results are expressed as mg/g gallic acid equivalents (GAE).

2.4. The factorial design

The high, middle and low levels defined for the 2^3 factorial design werelisted in Table 1. The low, middle and high levels for the factors were selected according to some preliminary experiments. The factorial design matrix and total phemolic compound measured in each factorial experiment is shown in Table 2, with the low (−1),middle (0) and high (+1) levels as specified in Table 1. Total phenolic compound was determined as average of three parallel experiments. The order in which the experiments were made was randomized to avoid systematic errors. The results were analyzed with the SPSS versi 16.0 software, and the main effects and interactions between factors were determined.

Table 1 Factors and levels used in the factorial design

Factors	symbol	low	middle	high
temperature	X1	75	85	90
aceton(%)	X2	45	50	55
Contact time(min)	X3	40	45	50

2.5 Preparation of chitosan nanoparticles

Chitosan beads 500 mg was dissolved in 100 ml acetic acid 1% (v/v) to obtain chitosan solution, and raised to pH 4.6-4.8 with 10 N NaOH. STPP(1%) with pH 6.0 was slowly dropped into a chitosan solution. Under magnetic stirring at room temperature, 1 ml STPP 1% solution was added dropwise to 25 ml of chitosan solution. The mixture was stirred for a further 20 min followed by sonification. The resulting suspension was subsequently centrifuged at 12.000 rpm for 10 min. We added additional STPP into supernatant after centrifugation, and observed a milky emulsion, which implied that not at all the chitosan had been converted to nanoparticles. The mixture was stirred again for a further 20 min followed by sonification. The resulting suspension was subsequently centrifuged at 12.000 rpm for 10 min. The chitosan nanoparticles were obtained by freezing the emulsion at -4^0C. The frozen emulsion was then thawed in the atmosphere and the nanoparticles were precipitated. The weight of the collected nanoparticles mg. 20 min and then dried at 100 °C for 96 h.The Chi-tosan nanoparticle was characterized by X-Ray Diffractometry, Fourier Transform Infrared spectrometry and TEM

2.6 Encapsulation total phenol compound with chitosan nanoparticle

For doping of the total phenolic compound, the method is as follows: 0.5 g of chitosan nanoparticle was added in 25mL of total phenolic compound solution (10% w/v), and stirred vigorously at room-temperature for 24 h. The solid was then filtered, extensively washed with distilled water, and dried at 50 ◦C for1 day.

2.7. Stability of totalphenolic compounds and encapsulation efficiency

The total phenolic compound encapsulated in microspheres were quantified by Folin–Ciocalteu method after dissolving 30 mg of CNP in 20 mL of deionized water. This procedure was done after the obtention of microspheres and 3 months later in order to evaluate the stability of the encapsulated total phenolic compound over time.

Different matrix destabilizing agents (pH, temperature and chelating agents) were tested. Ten beads of control and CNP coated samples were placed in test tubes containing 10 ml of the destabilizing agent: HCl 0.1 N, NaOH 0.1 N and sodium citrate 1 and 10% w/v as a calcium chelator were assayed at ambient temperature, and distilled water was tested at 50

and 100^0 C. To facilitate the observation of the structure disintegration of beads, a colorant(methyl violet) was used as the active component. Observations were performed for 24 h.The release of the active agent in water was quantified by total polyphenols using Folin-Ciocalteau method. The assay was carried out sinking. 10 beads in test tubes with 5 ml of distilled water under continuous agitation. Control and coated chitosan humid beads were tested by triplicate. Measurements were performed at different times between 10 min and 48 h. Percentage release and effective diffusion coefficients (D) were calculated. The amount of lyophilized extract loaded in beads was estimated by dissolving a known amount of capsules in sodium citrate (10% w/v) during 20 min for control capsules and 90 min for chitosan coated beads in an Orbit- Environ Shaker (Lab-Line Instruments, USA) at 37^0 C and 125 rpm. The concentrations of lyophilized extractloaded in the beads were determined by Folin-Ciocalteau method. A blank of sodium citrate solution was also performed.The percentage of loading efficiency was calculated with the following equation:

$$\text{Loading efficiency (\%)} = \frac{L}{L0} \times 100\%$$

where L is the amount of extract determined on the solution of sodium citrate and L0 is the initial amount of extract dissolved in CNP solution.

3.Result

3.1 . Models for extract total phenolic compounds from starfruit

So far, one has at least demonstrated that extract total phenolic compounds depends on solvent ratio . A mere investigation of the individual effects of the amount of each of them upon the adsorption efficiency is not sufficient to elucidate such an interaction. This is why the use of a factorial 3^3 experiment design, by varying extract time and the temperature, could be very useful to also investigate the interactions between the major parameters involved. The results obtained after 20 attempts are summarized in Table 2.

Table 2 Design Experimental of the dependent variables to the extract parameters.

Experiment	X1	X2	X3	Total phenol (mg/g)
1	60.00	90.00	35.00	16.42
2	50.00	80.00	61.82	17.21
3	63.18	63.18	55.00	15.84
4	50.00	63.18	45.00	14.86
5	50.00	96.82	45.00	18.04
6	50.00	80.00	45.00	18.13
7	40.00	90.00	35.00	16.34
8	60.00	70.00	35.00	12.11
9	60.00	90.00	55.00	18.32
10	40.00	70.00	35.00	9.92
11	50.00	80.00	45.00	18.06
12	50.00	80.00	45.00	18.16
13	50.00	80.00	28.18	13.27
14	40.00	70.00	55.00	14.32
15	60.00	70.00	55.00	12.75
16	50.00	80.00	45.00	18.12
17	50.00	80.00	45.00	18.08
18	33.18	80.00	45.00	12.48
19	50.00	80.00	45.00	18.07
20	66.82	80.00	45.00	12.26

The mixture design for the first approach suggested 20 experimental points, each corresponding to a mixture composition. For each point an average total phenolic compound

under compression was determined. These numerical results are represented in a triangular graph, Fig. 1, which shows the level curves of total phenolic content as a function of the composition, obtained from a quadratic regression. The final form of the polynomial model that describes the polyphenol content is the following

$$R1 = 18.10 + 0.21X1 + 1.70X2 + 0.96X3 + 0.24X1X2 - 0.17X1X3 - 0.46X2X3 - 2.02X1^2 - 0.58X2^2 - 1.01X3^2$$

The positive coefficients, corresponding to the interactions between aceton concentration and temperature X1X2 , represent the synergetic effect of the mixture of components on the expected value for total phenol content . In addition, aceton (%) and temperature had positive linear effects on total phenolic yields (Fig. 1). The optimal condition determined by RSA was at ethanol concentration of 50 %, extraction temperature of 85 °C and time of 40 min with a maximal yield of total phenolic compounds of 23.65 mg/g of dry powder. .Fig. 1 is a response surface plot showing the effect of aceton concentration and temperature on the total phenolic content at the fixed time of 45 min..

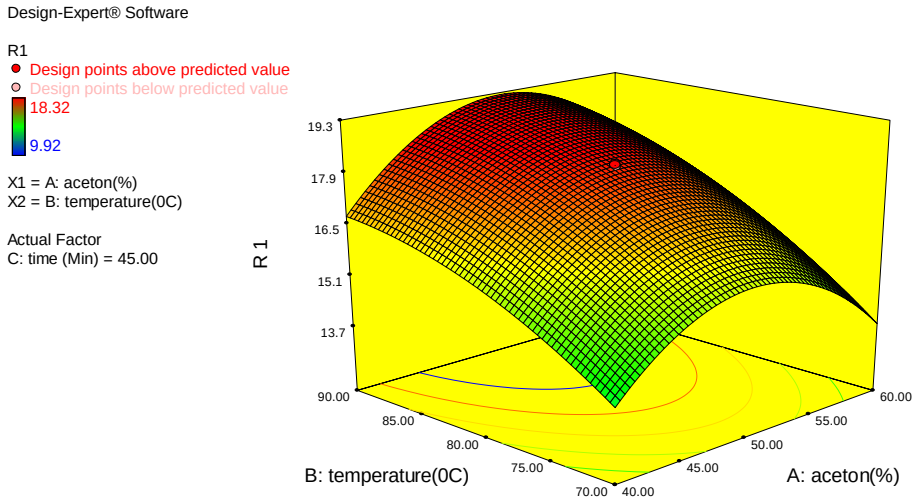

Fig. 1. Surface plot of polyphneolic yield (R1) as a function of concentration of aceton and temperature at extraction time of 45 min

Fig. 2 reports the iso-response curves for aceton and nanopowder maintaining extract time at the fixed value of 45 min. Analysis of this graph indicated that the highest total phenol content hvalue are obtained with high levels of temperature and low levels of aceton... Thus, the chosen independent variables ranged as follows: X1 = % aceton : 35–55(wvv); X2 = % temperature : 7.8-90^{0}C

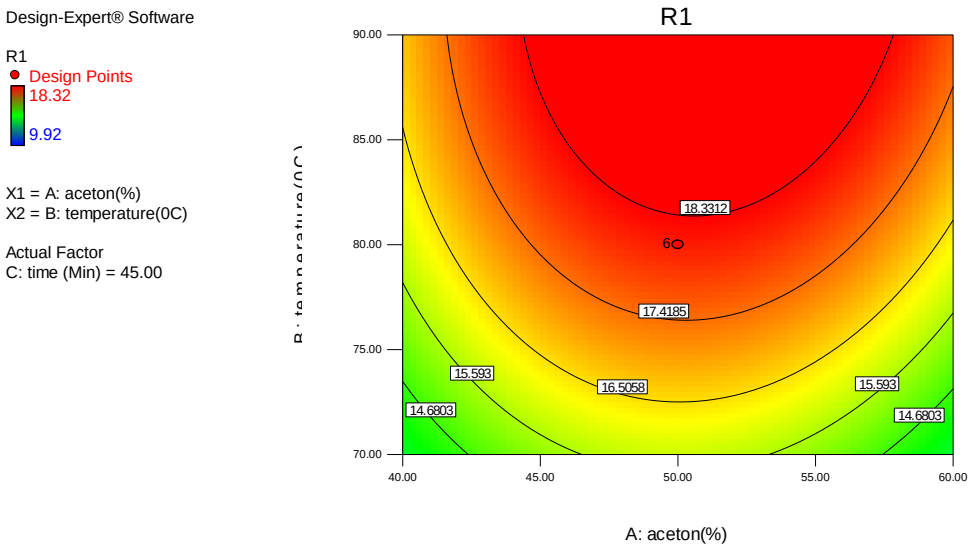

Fig. 2 Iso-response curves of formulation of nanopackaging encapsulation efficiency as a function of the factors aceton (*X*1) and temperature (*X*2), maintaining extract time (*X*3) at 45 min.

3.2 Characterization chitosan nanoparticle

3..2.1The FTIR Analysis

The FTIR spectra of nanoparticle chitosan are shown inf Fig 3. A characteristic band at 3406cm^{-1} is attributed to –NH_2 and OH groups stretching vibration and the band for amide I at 1655 cm^{-1} is seen in the infrared soectrum chitosan. Whereas in the FTIR spectra of nanoparticle chitosan the peak of 1655 cm^{-1} disapperar and 2 new peaks at 1639 cm^{-1} and 1542 cm^{-1} appears. The disappearance of the band could be attributed to the linkage between the phosphoric and ammonium ions. The nanoparticle chitosan also showed a peak for P = O at 1155 cm^{-1}. Qi et al [17] observed similar results in their study of the formation nanoparticle chitosan and chitosan film treated with phosphate.

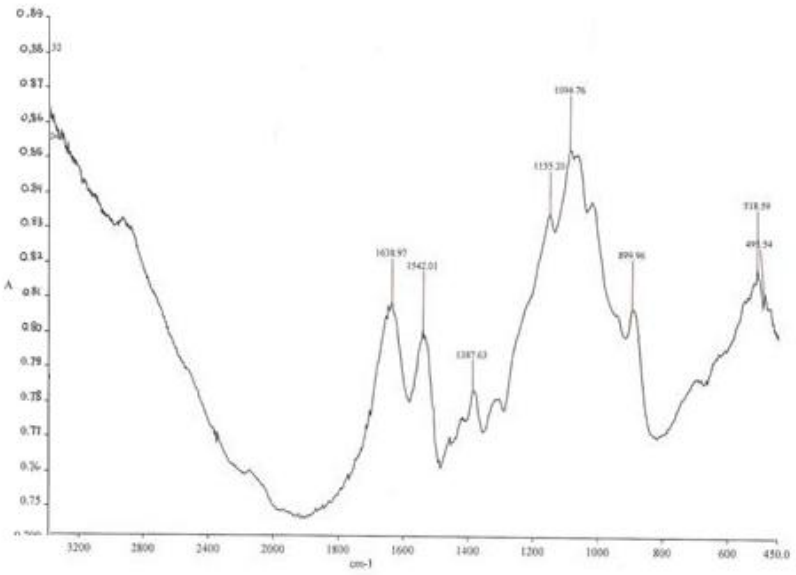

Fig 3 IR Spectra of chitosan nanoparticle

Chitosan with a pKa of 6.3 is polycationic when dissolved in acid and presents –NH_3^+ sites. STPP ($Na_5P_3O_{10}$) dissolved in water dissociates to give both hydroxyl and phosphoric ions. Since the cross linking of chitosan would be dependant on the availability of

the cationic sites and the negatively charged species, it was expected that the pH of STPP would play a significant role in same. pH would bring about a change on the extent and type cross linking. Hence in the present study, pH conditions pH 8.6 were used for reaction. At pH 8.6 both OH^- and phosphoric ions were prsent and may compete each other to interact with the $– NH_3^+$ of chitosan.

3. 2.2XRD Analysis

X-ray diffraction pattern of chitosan nanoparticle are shown in Fig 4.However, no peak is found in the difractogram of chitosan nanoparticle. The XRD pf chitosan nanoparticle is characteristic of an amoorphous polymer. The crystal structure of the amorphous chitosan nanoparticle has been destroyed after crosslinked with sodium tripolyphosphate.

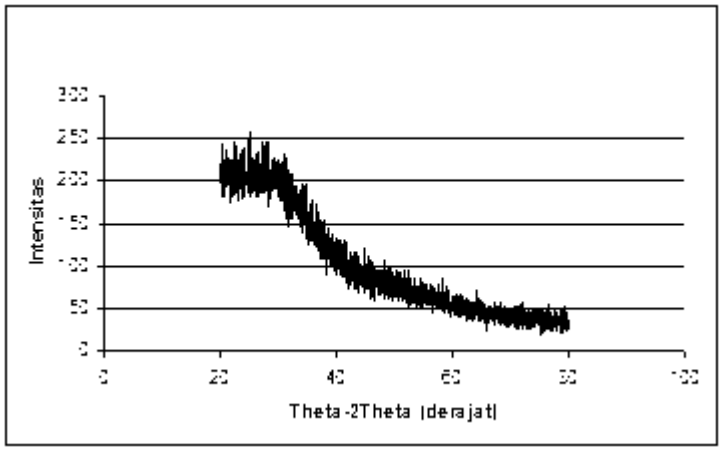

Fig 4. X-ray powder diffraction patterns of chitosan nanoparticle

Fig. 5 depicts the TEM image of chitosan nanoparticle along with SAED particles shown in inset. The average diameter of particles was about 9.1-100 nm. Zhi et al[18] was reported ,when NaOH was selected as the precipitator, the average diameter chitosan nanoparticle was 32-142 nm.

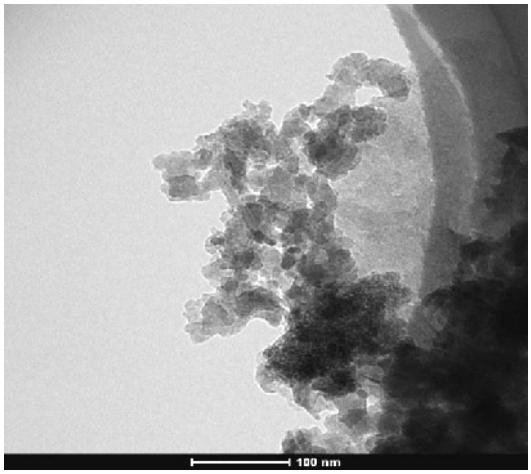

Fig.5. TEM imagesand size distribution of chitosan nanoparticle

3.3. Stability of polyphenolic compounds and encapsulation efficiency

The total polyphenol content in the obtained chitosan nanoparticle was measured to determine the encapsulation efficiency. In addition, this was also calculated after one week to study the stability of the encapsulated polyphenols over time. The encapsulation efficiency

was near 100%, after one week the polyphenol content was 100%. Therefore, chitosan nanoparticle maintainedthe stability of the polyphenols over time and are a goodvehicle for the encapsulation of these compounds. Kosaraju et al. (2006) also observed that the encapsulation of olive leaf extract by the spray-drying process did not lead to the inactivation of polyphenolic. Zhen- et al (2007) report that chitosan nanoparticle- could improve the enzyme activity by 13.17% than that of the free neutral lipase. Deladino et al. (2008) studies showed that beads without chitosan released around 50% of the polyphenol content and beads with chitosan released around 35% at 3.5 h, which is significantly lower than the released amount

3.4. Release studies

Although the pH of the skin is 5.5, the pH of cosmetic formulations can range between 5.5 and 7 (Meyer Rosen, 2005). The release studies of phenol were performed in two buffers with different pH values (pH 5.0 and 6.0). In both cases, 90% of polyphenols was delivered from phenol 3 h,while phenol-CNP slowed down the release, being at this same time 56% in pH 5.0 and 47% in pH 6.0. The higher release in pH 5.0 was due to the solubility of chitosan hydrochloride at this pH. Deladino et al. [12](prepared chitosan–alginate beads for the encapsulation of yerba mate. Their studies showed that beads without chitosan released around 50%of the polyphenol content and beads with chitosan released around 35% at 3.5 h, which is significantly lower than the released amount in the present study.

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Transition State Analysis of HMM for DNA Exon Controlling Using Bioinformatic Simulation

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Abstract

This paper describes the analysis of transition state value of HMM for DNA exon controlling using Bioinformatic simulation. Exon region in DNA is called a coding sequence (CDS) of genes in many regions at least two regions of exon. HMM model is generate using start and stop gene as a state and consist of three bases in each states. Furthermore, the region of intron in the model is able to increase the states by separating bases GT and bases AG from the length of intron. HMM properties and parameters such as Markov chain, transition state, emission state, HMM training and HMM testing is used to identify original exon region with estimated exon. The performance of estimation result shown by Correlation Coefficient (CC). Random values of transition state used for HMM train makes many differences in the CC of the model. Furthermore, the analysis of transition state values is very important to finding optimum of CC. Several models with the parameters of HMM were simulated, trained and tested for the implementation of number of states with HMM method. The simulation result predicted that the CC value is very much influenced by the value of transition state and improved the number of states on the model makes increasing of CC.

Keywords: HMM, Transition state, Exon, Correlation Coefficient (CC).

1. Introduction

To controlling exon in DNA sequences of genes *Plasmodium falciparum* has the region of exon based on coding sequence (CDS) in database from Genbank, including the region of intron has been know on CDS. In principle, start codon, exon, intron and stop codon are becomes the state to use in the structure of models. Each state of the models has many bases DNA actually start and stop codons [Nicorici, *et al.* 2003]. Transition states of the models are depends on state number and its bases on each state and transition states values minimum is 0 and maximum is 1. Trial and error in simulation using transition states values are very important to have the optimal performance of the models. Additional states in the structure of the models in regions exon and intron of DNA sequences.

Plasmodium falciparum genome belongs to eukaryotic genome and has a long DNA genome and intron or splicing process. Biological model of DNA structure from gene eukaryotic consists of some exon and intron which alternately located. CDS is a result from splicing process of intron inside the DNA and consists of some region of exon. The first region of exon in CDS starts with start codon which is ATG bases and the last region of exon, there's one of the three stop codons such as TAA, TAG and TGA bases [Samatova, 2003; Anantharaman, 2004]. In the minimum CDS, there are two region of exon which enable us to find out that there's minimum one region of intron and usually region of intron starts with G and T bases and ends with A and G bases.

HMM based finding usually gives more accurate results compared to other methods [Rabiner, 1989; Henderson, et al.1997]. In this paper, HMM's models was chosen based on HMM

method for several models with its number of states. One of the other performance parameters of HMM's models is shown by its Correlation Coefficient (CC) value for each models.

2. Materials and Methods

2.1. Materials

The materials have 152 DNA sequences of genes from genome *Plasmodium falciparum* in GenBank format with searching to: http://www.ncbi.nlm.nih.gov/entrez or www.ncbi.nlm.nih.gov/Web/Search/index.html [Alphey, 1997; Anastassiou, 2001]. Genbank format describes CDS and original DNA sequences of genes *Plasmodium falciparum*. In CDS contains at least two regions and maximum 10 regions of exons. Minimum length of sequences for this simulation is 684 base pairs (bp) and maximum length is 10095 bp. Matlab 7.0 Mathworks, Massachussets, USA was used for simulations and its hardware PC IBM Standard has specification: Processor Intel(R)Pentium(R) 4 CPU 2.8 GHz; Memory 1.99 GB of RAM; Harddisk 40 GB; Operating System Microsoft Windows XP, 2002 version.

2.2. Methods

HMM process consist of input, HMM training, HMM testing and output. Input for HMM training consists of DNA sequences, information of exon position of each sequence, transition matrix and emission distribution matrix. DNA sequence data and information of exon position of each sequence are used to set the state number for each base depend on model will be generated. Than transition matrix is defined and emission distribution matrix of each base state is calculated. Furthermore, HMM training has the both algorithms are Viterbi and Baum-Welch and needs the transition states and emission states for the process. The result of HMM training is the estimated transition states and emission states. The estimated transition and emission states are used for HMM testing process has both of the algorithms above and its result of HMM testing is the estimated states of the model. The performance of the model are the value of CC, which calculated by comparing the estimated state from HMM testing result with the original state of the input sequences [Vaisman, 1998; Samatova, 2003], the formula as equations (1).

$$CC = \frac{(TP \cdot TN) - (FP \cdot FN)}{\sqrt{(TP + FN)(TN + FP)(TP + FP)(TN + FN)}} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

where:

TP = True Positive
TN = True Negative
FP = False Positive
FN = False Negative

Several models in this simulation are using start and stop gene like as a state and consist of three bases in each states. Furthermore, the region of intron in the model is able to increase the states by separating bases GT and bases AG from the length of intron, the general structure of the model like in figure 1 below.

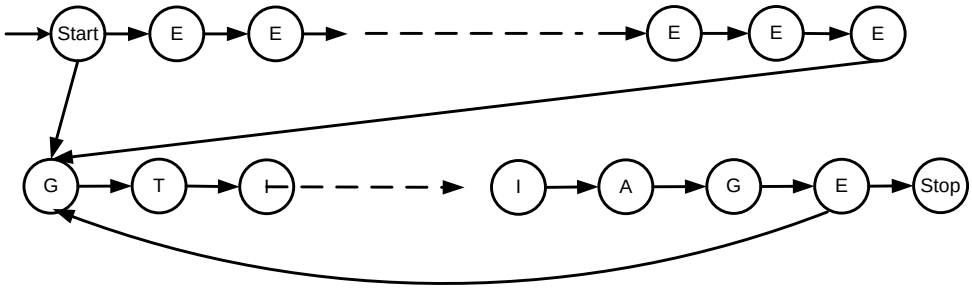

Figure1. General structures of HMM

The HMM parameters were set for the HMM training and testing method and its algorithm [Rabiner, 1989; Anastassiou, 2001].
Based on HMM method for the training uses Viterbi algorithm and testing uses both algorithms are Baum-Welch and Viterbi. HMM training and HMM testing use the same sequences. The programming is written in Matlab, there's toolbox Bio-informatics to generate DNA sequences in GenBank format and has functions of HMM training and HMM testing.

3. Result

Several models have been simulated from HMM implementation for controlling exon region in DNA sequences of genes *Plasmodium falciparum*.
The models were formed with a number of state randomly until the model is formed into 20 states, 30 states and 50 states following the general stucture of HMM as seen in figure 1. Each model simulation performed three times by using the values of the different state transition, can even use the same exon and intron structure with a number of different states.
Random value of transition state can be the analysis to find CC values optimum but the emission of each state has the distribution constant value of bases DNA sequences depends on the models. To calculate CC is by using the equation (1) and the assumption of exon is positive and intron is negative.
The simulation results with random values of transitions states for each model like as Table 1 below.

Table1. CC values for each model performed by number of state

Transition State Values (Performed by 20 states)											CC	
1	2-9	10	11-15	16	17-18	19	20	19-11	19-20	1-11	Vit	B-W
0	0.1	0.1	0.1	0.9	0.1	0.8	1	0.1	0.1	0.1	0.7077	0.6911
0	0.1	0.1	0.1	0.9	0.1	0.85	1	0.05	0.1	0	0.7131	0.6939
0	0.1	0.9	0.1	0.9	0.1	0.85	1	0.05	0.1	0	0.7481	0.7414
Transition State Values (Performed by 30 states)											CC	
1	2-12	13	14-25	26	27-28	29	30	29-14	29-30	1-24	Vit	B-W
0	0.1	0.1	0.1	0.9	0.1	0.8	1	0.1	0.1	0.1	0.6278	0.6179
0	0.1	0.9	0.1	0.9	0.1	0.8	1	0.1	0.1	0	0.7651	0.7551
0	0.1	0.9	0.1	0.9	0.1	0.85	1	0.05	0.1	0	0.7217	0.7189
Transition State Values (Performed by 50 states)											CC	
1	2-22	23	24-45	46	47-48	49	50	49-24	49-50	1-24	Vit	B-W
0	0.1	0.9	0.1	0.9	0.1	0.8	1	0.1	0.1	0	0.7520	0.7494
0	0.1	0.9	0.1	0.9	0.1	0.8	1	0.1	0.1	0.1	0.7436	0.7016
1	2-17	18	19-45	46	47-48	49	50	49-19	49-50	1-19	Vit	B-W
0	0.1	0.9	0.1	0.9	0.1	0.8	1	0.1	0.1	0	0.7727	0.7661

The simulation result of several HMM structure above, the values of CC has been influenced by transition state value and the improved states on the model. The graphic optimum CC values in this simulations result as figure 2; where HMM testing using algorithm Viterbi is better than using Baum-Welch algorithm.

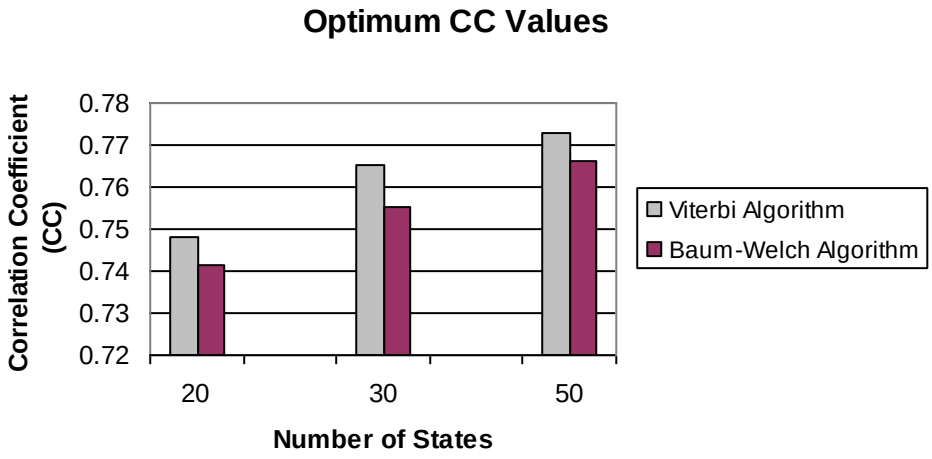

Figure 2. Optimum CC Values from simulation results.

The random value of transition state became the CC values negative, its means that the assumption of exon to be negative and intron to be positive. Meanwhile, emission state can be used to find out the distribution of emission of DNA bases in each state in accordance with HMM structure that has been used.

4. Discussion

The HMM model that has been used is developed from above model which will be tested by adding states in each exon and intron regions. The forward and backtracking directions between states in the used algorithm must be matched with one of the nature of HMM is Markov's chain and eukaryotic gene structure. Algorithm Viterbi was used at the HMM training and algorithm Viterbi and Baum-Welch was used at HMM testing.

Simulation result shows that the highest optimum CC value was for the model with 50 states with HMM testing by using Viterbi algorithm is 0.7727 and by using Baum-Welch algorithm is 0.7661.

Furthermore, improvement states of the model of this study can be used by using a number of state and sequence DNA more than that already simulated because it predicted to produce higher value of CC.

Acknowledgements

The authors would like to thanks to Directorate of Higher Education (DIKTI) for fundamental research grant 2011 and Trisakti University Research Institute for all valuable discussions and suggestions.

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Synthesis of a Series of Calix[6]arenePolymers from*p-ter*-butylphenol

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Abstract

*A research has been conducted to synthesize ofa series of novel calix[6]arene-based polymers **2a-c**using p-tert-butylphenol as a starting material. It was of interest to study calix[6]arene and its derived polymers which have cavity size larger than calix[4]arene.The synthesis was carried out in several steps i.e (1) formation of p-tert-butylcalix[6]arene from p-tert-butylphenol, (2) treatment of p-tert -butylcalix[6]arene with allyl bromide under alkaline condition to yield compound **1a**, (3) esterification of **1a** to yield **1b**, (4) hydrolysis of **1b** with chloride acid to yield **1c** and (5) polymerization of **1a-c**by treatment with concentrated sulfuric acid to yield a series of polypropyl-calix[6]arenes**2a-c**. The structures of those products were observed by means of melting point, FTIR and ^{1}H NMR spectrometers. The ^{1}H NMR spectra showed that one allyl group had been incorporated to the lower rim of the p-tert-butylcalix[6]arene.The polymers **2a-c**wereobtained as brownish crystals with the melting point of 199-201; 99-101; and 101-103 ^{o}C respectively. With a tunnel-like structure of the polymers, they can be usedas adsorbents to trap heavy metal ions.*

Keywords: p-tert-butylphenol, p-tert-buthylcalix[6]arene, esterification, hydrolysis, andpolypropyl-calix[6]arenes.

1. Introduction

Calixarenes are an important class of macrocyclic molecules in supramolecular chemistry [1-2]. These compounds are cyclic oligomer of phenols linked by methylene bridges and have active groups such as –OH groups arrange the molecules. Because of the unique molecular geometry of calixarenes, its can be used as catalyst [3], ion exchange and adsorbent forcation, anion, or neutral molecule [4,5].

The development in calixarene chemistry in mainly due to the possibilities of functional modifications at the upper or lower rim to yield new macro molecules.The reactions used to modify the lower rim of calixarenethat binding hydroxygroups, include the esterification and etherification [6, 7]. Richard et al. [8], has also succeeded in modifying the lower rim of calix[6]arena by substituting the OH groups with a carboxylic acid groups.

Calixarene compounds can be used as adsorbent for heavy metal ions by modify its functional groups polar (hydrophilic groups) such as carboxyl, sulphonate, nitro, amino, amide, halide, and phosphate.To increase its polarity, it's also possible to incorporate alkenyl groups such as an allyl groupat the lower rim ofcalixarene. Ho, *et al* [9] and Shu, *et al* [10] have shown that the two allyl group at the lower rim of calix[4]arene could be incorporated by addition of allylbromide, while Kusumawardani [11] also reported that a

resin derived calix[4]arenei.e.,tetra-*p*-propenyltetraester-calix[4]arene and tetra-*p*-propenyltetra-carboxylicacid[4]arenecould be successfully synthesized by first entering the four allyl groups at the lower rim of calix[4]arene.

In order to keep functioning as an effective adsorbent and solubility in water is reduced, it can be immobilized the calixareneon a polymer or by synthesize the polymers of calixarene.Jumina, *et al* [12] has reported that the monoallylcalix[4]-arene can be polymerized under acidic conditions to generate the corresponding polypropyl-calix[4]arene polymer. Furthermore, Utomo [13] reported that the capability of this polymerto trap heavy metal cations such as Pb (II) and Cr (III)) cationsare significallygreater thanthat of the monomer. Based on this phenomenon, it would be interesting to study calix[6]arene with cavity size larger than calix[4]arene, and its polymerization as well as the polycalix[4]arene in regards to ability to trap heavy metals. Herein, we wish to report the synthesis of a series of calix[6]arene polymers from *p-tert*-buthylphenol.

2. Experimental Section

Material

All the chemicals used in this study were the highest purity available from Merck or Aldrich chemical companies and were used without further purification.

Instruments

Melting points were obtained with anelectrothermal 9100 Model Digital Melting Point apparatus,wasobtained at theLaboratory of Chemistry, StateIslamicUniversitySunanKalijaga of Yogyakarta.Infrared (IR) spectra were recorded on a Shimadzu FTIR 8201 PC Spectrophotometer and refer to KBr disks. ^{1}H NMR spectra were obtained in the designated solvent ($CDCl_3$) on a JEOL-MY500 proton Nuclear MagneticResonanceSpectrometer.

Procedure

Synthesis of *p*-t-butylcalix[6]arene

p-tert-Butylcalix[6]arene were prepared from *p-ter*-butylphenol according to the procedures reported previously [14]. Recrystalization from chloroform-methanol afforded a white solid crystal :3.5 g, 65.47%, m.p. 370-372 ^{o}C; IR (KBr) 3425 cm^{-1}(OH stretching), ν (C=C aromatic)= 1627 cm^{-1}, (*t*-butyl)=1365 cm^{-1} and ν(methylene group)= 1481 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ 10.5 **(**s,1, ArOH), δ 7.10 (m,2, ArH), δ 3.9 (s,2, CH_2), δ 1.27 (s, 9, $C(CH_3)_3$).

Synthesis of *p-t*-butyl-37-monoallyloxy-38,39,40,41,42-penta-hydroxycalix[6]arene (1a)

A mixture of 4.86 g (5 mmol) of *p-t*-butylcalix[6]arene, 0.621 g (4,5 mmol) K_2CO_3, 0.714 mL (8.25 mmol) allylbromide, and 100 mL of dry acetone was refluxed under dry N_2 condition for 48 hours. The precipitate was filtered and acetone was evaporated. The residue was recrystallized with $CHCl_3$ and CH_3OH to yield *p-t*-butyl-37-monoallyloxy-38,39,40,41,42-penta-hydroxycalix[6]arene, afforded 4.402 g (86.99 %) of yellow crystals: mp178-180^{o}C; IR (KBr): 3387 cm^{-1}(OH stretching), ν(vinyl group,C=CH_2) 987 cm^{-1};^{1}H-NMR ($CDCl_3$), δ 7.06-7.24 (m, 12, ArH), δ 5.9 (m, 1, C=CH-C), δ 5.06-5.2 (m, 2H, C=CH_2), δ 3.2-4.3 (s and dd, 12H, ArCH_2Ar), δ 2.1 (s, 2H, OCH_2C) and δ 1.2 (s, 54, $C(CH_3)_3$).

Synthesis of *p-t*-butyl-37-monoallyloxy-38,39,40,41,42-penta-estercalix[6]arene (1b)

Into a three-necked flash equipped with a reflux condensor, it was added 0.263 g (0.25 mmol) of **1a**; 0.2875 g (2.35 mmol) ethyl-2-chloroacetic; 0.355 g (2.35 mmol) NaI; 0.425 g (3.25 mmol) K_2CO_3, and 50 mL of dry aceton.The mixture was refluxed for 24 h.

The resulting mixture was allowed to cool, K_2CO_3wa filtered off and aceton was evaporated. The residu was dissolved in chloroform, and then washed with 3 x 25 ml HCl 1 M and 1 x 25 mlsaturated NaCl. The solution was dried with Na_2SO_4 anhydrous and chloroform was evaporated. The product was characterizedby means of FTIR, ^{1}H-NMR.

Synthesis of *p-t*-butyl-37-monoallyloxy-38,39,40,41,42-penta-carboxylic-acidcalix[6]arene (1c)

A mixture of **1b**(1.2 mmol; 1.73 g); 0.5 g KOH and 50 ml of ethanolwas refluxed under dry N_2 condition for 24 hours. The resulting mixture was allowed to cooland acidified with HCl 1 M. The precipitated was filtered off. The product was characterizedby means of FTIR, ^{1}H-NMR.

Polymerization of monoallyloxycalix[6]arene

To 1 g of**1a-c** in 100 mL chloroform was added concentrated sulfuric acid (0.1 mL) in 0.25 mL portions every 30 minutes. The reaction mixture was stirred at for 8 h and the polymerization was terminated by adding 0.5 mL of methanol. The mixture was decanted and the precipitate was dissolved in diethyl ether and washed until neutral. The chloroform layer was washed until neutral and combined with the ether layer. The combined layers were dried with anhydrous Na_2SO_4 and evaporated to yield the polymers **2a-c** which was dried in a desiccator.

3. Result and Discussion

3.1 Syntheses and characterizations

Synthesis of *p-t*-buthyl-37-monoallyl-38,39,40,-41,42-pentahydroxy-calix[6]arene (**1a**) was carried out by refluxing 1.1 equivalent of allyl bromide and 0.6 equivalent of K_2CO_3 in dry aceton for 48 h under N_2atmosphere.The product of this reaction was obtained as yellow crystals with the melting point of 178-182^{o}C. The scheme ofthesyntheticroute of **1a**wasshowed in Figure 1.

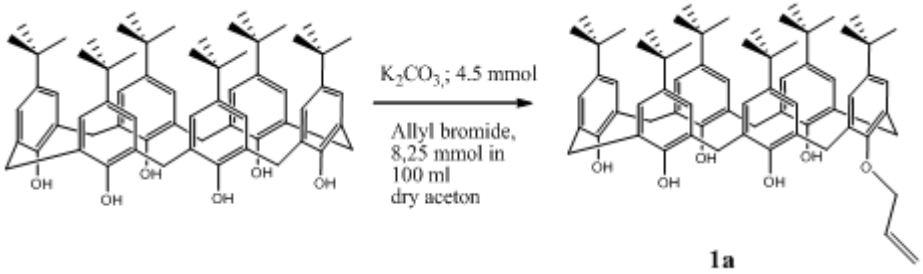


Figure 1. Scheme of the synthetic route of **1a**

Esterification of **1a** was performed using K_2CO_3, NaI and 2-ethylchloroacetate reagent. The esterification product to yield **1b**was obtained as yellow crystals with the melting point of 164-166^{o}C. Furthermore the synthesis of **1c** was done by hydrolysis reaction of **1b** and the product of this reaction was obtained as yellow white crystals with the melting point of 143-145^{o}C.

As monomers, **1a-c** were polymerized with concentrated sulfuric acid to yield polymer **2a-c**. The synthetic route of polymers were illustrated inFigure 2. The polymers **2a-c**were obtained as brown, brownish white and brown crystals respectively.

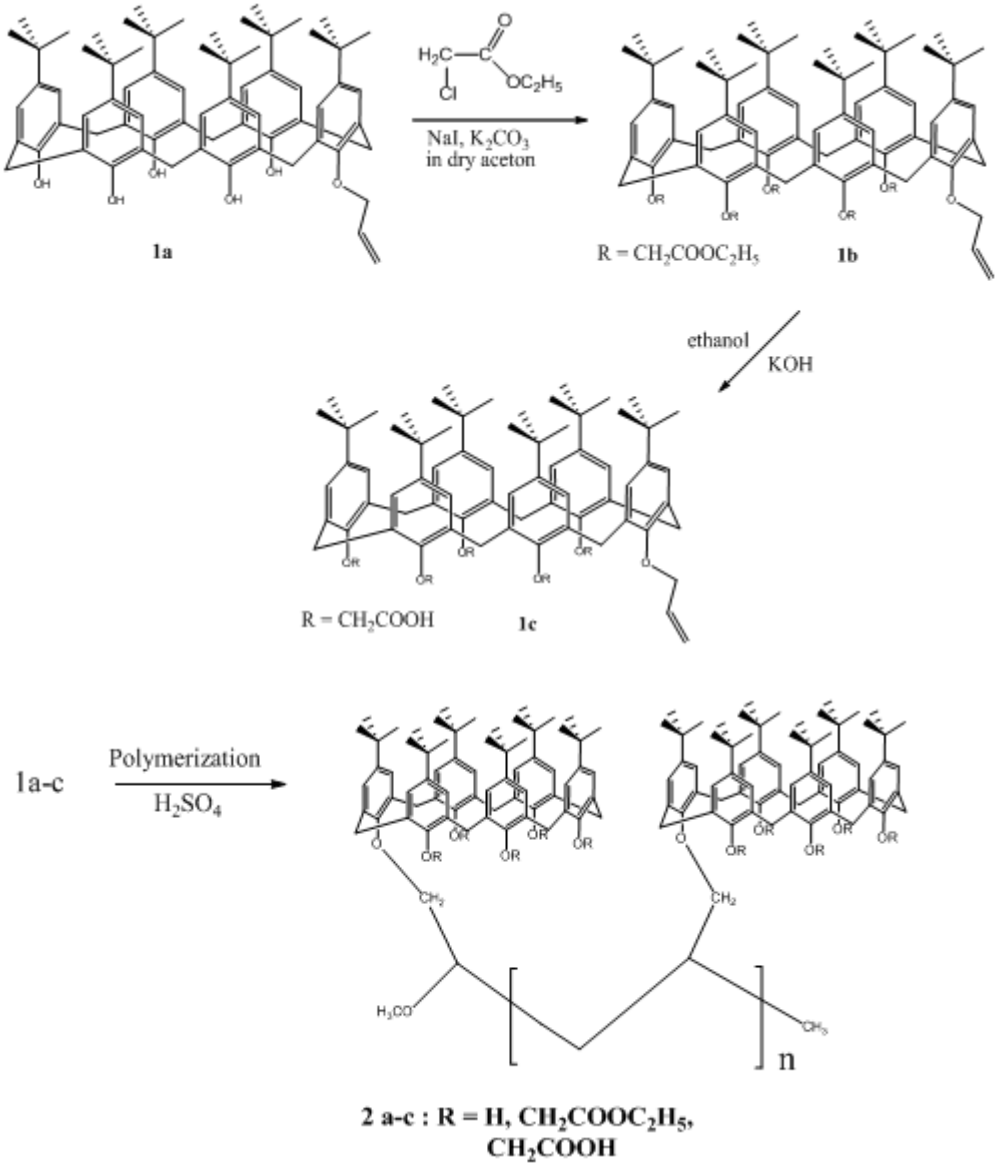


Figure 2. Scheme of thesyntheticroute of polymer 2a-2c.

The elemental analysis data and molecular weight of polymer **2a-c** were shown in Table 1. The relative molecular weight (M_n) for each polymers were obtained by UbbelohdeViscometer. The M_n of polymers **2a-c**were approximately 25,000-30,000 g/mol. The structure of all these deductions wereconfirmed by IR and ^{1}H NMR spectrum.

Table 1. Results of the polymerized products

Compounds	Results			
	m.p (°C)	Color	Rel. Mol. Weight	(n)
Poly-monoallylcalix[6]arene (**2a**)	199-201	brown	30,182	30
Poly-monoallyl-pentaestercalix[6]arene (**2b**)	99-101	brownish white	27,228	19
Poly-monoallyl-pentacarboxylic-acidcalix[6]arene(**2c**)	101-103	brown	24,612	19

3.2 IR spectrum

The structures of compound **1a-c** were confirmed by IR spectrumthat showed in Figure 3. At the IR spectrum of **1a**, it was showedthat a strong broad band of the -OH groups appeared at 3387cm^{-1} and the absorption at 1203.58 cm^{-1}, indicating the presense of C-O

derived from the bond between C benzene ring with hydroxyl oxygen atoms. This is supported by a strong absorption peak at 987.55 cm^{-1}, indicating the presence of vinyl terminal.

On the other hand, at the IR spectrum of **1b**, it can be seen that the absorption band at 3387 cm^{-1} of the hydoxyl group (-OH) dissappeared indicating that the esterification reaction has taken place. The characteristic absorption of carbonyl group (C=O) and (C-O-C) of ester appeared at 1759.08 $cm^{-1}$and 1200-1100 cm^{-1}, respectively. These data of IR spectrum certainly suggested that the hydroxyl groups were well esterifisized to yield*p-t*-buthyl-37-monoallyl-38,39,40,-41,42-pentaester-calix[6]arene (**1b**).

The successful synthesis of **1c**was showed by appearence absorption band at 3425.58 cm^{-1}, which is characteristicfor -OH groups. This indicated that the ester groups were hydrolyzed to carboxylic acid. The existence of carbonyl groups (-C=O) were showed at 1743.65 $cm^{-1}$and the absorption at 1473.62 cm^{-1} indicated the existence of methylene groups.

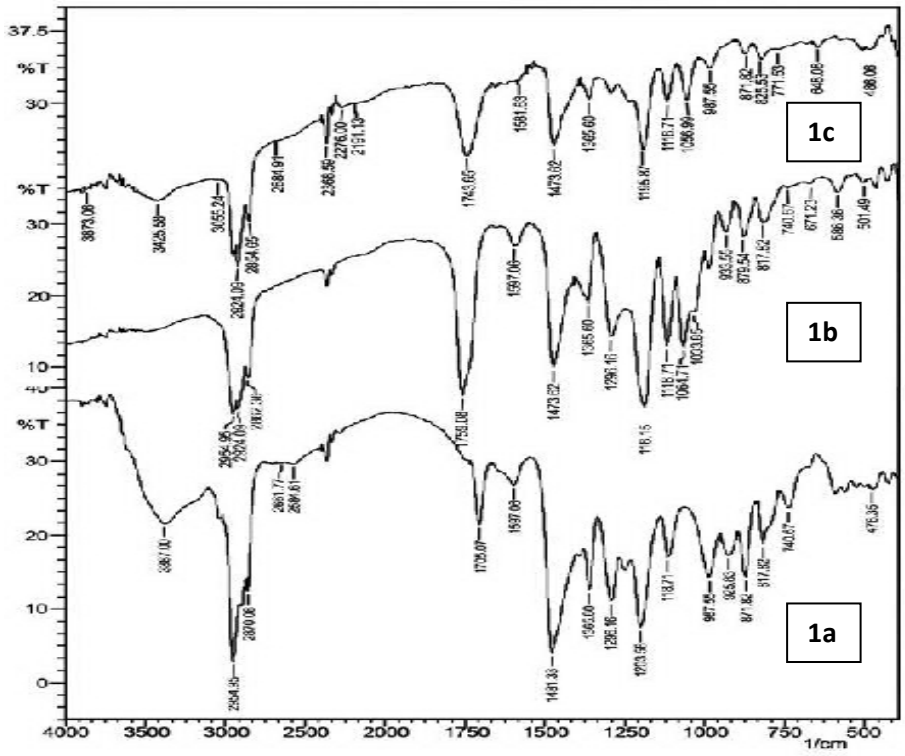


Figure 3.IR Spectrum of 1a, 1b and 1c

The structure of polymers2a-c werealso characterized by IR. In the IR spectrum, the absorption peaks of vinyl were disappeared which mean almost all of the vinyl groups were polymerized.

3.3 [1]H NMR spectrum

[1]H NMR spectrum of compound **1a** showed in Figure 4. Its showed 6 (six) signals depicting 6 differrent types of protons. Signals at δ 7.0-7.2 ppm refers to proton resonance of benzene groups. Signal at δ 5.9 ppm is predicted from one proton in the middle carbon group (-CH=) and signal at δ 5.0-5.2 ppm refers to terminal proton resonance of allyloxy (=CH_2) group. This supported by the integration of [1]H NMR spectrum shows the number of the ether linkages of the monoallyl ether was 1. This indicated that onlyone allyl group had been incorporated to the lower rim of the *p-t*-butylcalix[6]arene to form **1a**.

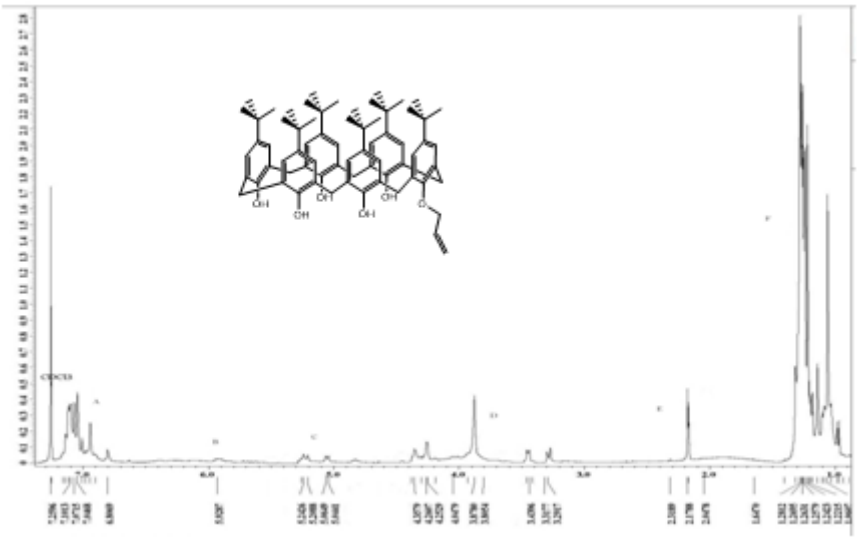
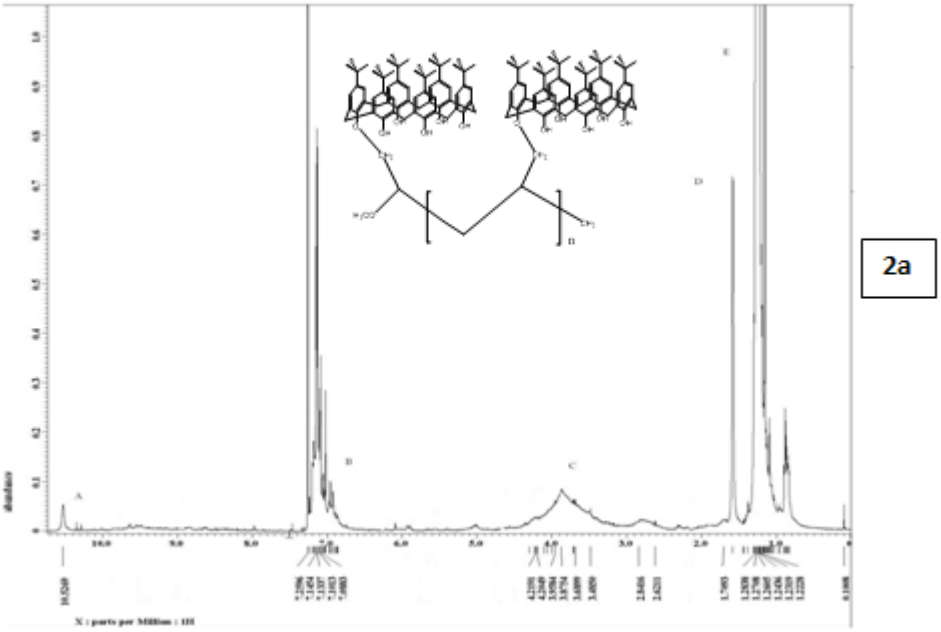
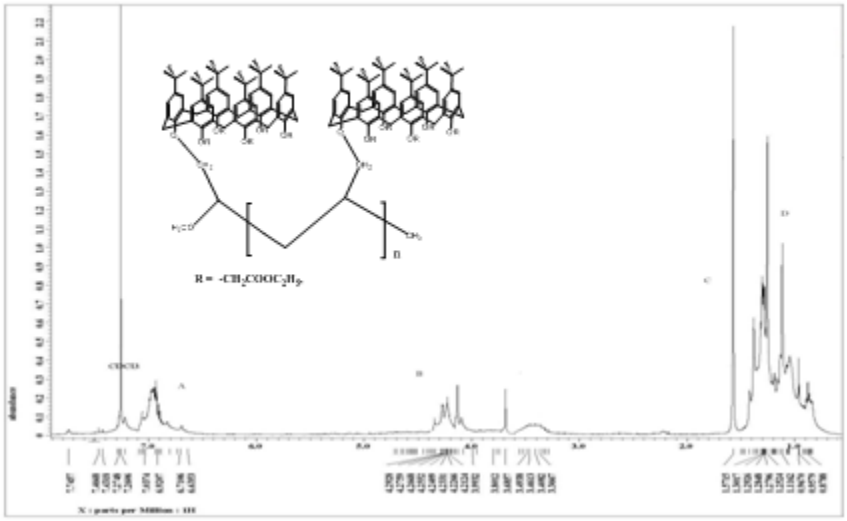


Figure 4.^{1}H NMR 500 MHz spectrum of monoallil-calix[6]arene (**1a**)

The existence of methylene brigde of calixarene (-CH_2-) protons are shown at δ 3.2-4.3 ppm. The O-CH_2- group proton and the proton tert-buthyl groups are estimated to resonate at δ 2.1 ppm and δ 1.22-1.28 ppm respectively.

On the other hand, the ^{1}H NMR spectrum of the polymers **2a-c**were showed in Fig. 5. As expected, the^{1}H NMR spectrum does not also show the existence of vinylprotons signal which resonate at δ 5.9 ppm (=CH-) and δ 5.0-5.2 (C=CH_2), indicating that the vinyl groups had been polymerized. The successful of the reaction can also be proved fromthe resonate at δ 10 ppm, which indicated the proton resonance of –OH groups. This signal disappeared in ^{1}H NMR spectrum of **2b**which mean almost all of the -OH groups were hydrolyzed.





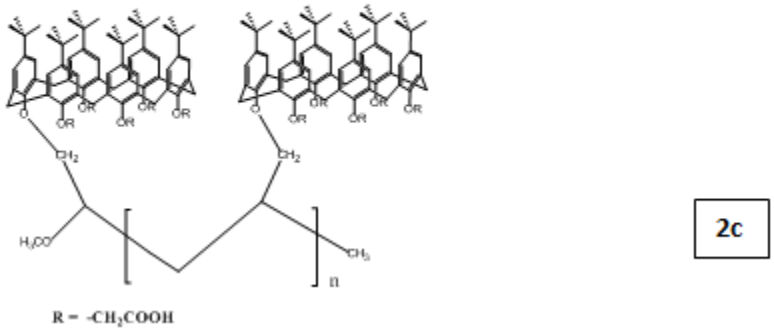


Figure 5.^{1}H NMR 500 MHz spectrum of **2a-c**

4. Conclusion

The synthesis of three new calix[6]arene-based polymers **2a-c**have been successfully produced from *p-t*ert-buthyphenol as a starting material. These polymers can be synthesized by the following steps:(1) cyclohexamerization to *p-t*ert-buthylcalix[6]arene; (2) allylation to *p-t*-buthyl-37-monoallyl-38,39,40,-41,42-pentahydroxy-calix[6]arene (**1a**); (3) esterification to *p-t*-buthyl-37-monoallyl-38,39,40,-41,42-pentaester-calix[6]arene (**1b**); (4) hydrolisis to *p-t*-buthyl-37-monoallyl-38,39,40,-41,42-pentacarboxylic-acidcalix[6]arene(**1c**); and (5) polymerization of **1a-c** to yield polymers **2a-c**. Based on IR and ^{1}H NMR analyses, it can be concluded that the synthesis of a series of calix[6]arene polymers were successfully produced. The polymers were obtained as brownish crystals with the relative molecular weight for each polymers were approximately 25,000-30,0000 g/mol.

5. Acknowledgement

Acknowledgement is directed to Indonesia Government, which supported this work via Doctorate Grant 2010 from Directorate of Higher Education, Department of National Education.

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Study of Thermal Stability of Riboflavin Synthase of *Eremothecium gossypii* Through Molecular Dynamics Approach

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ABSTRACT

Eremothecium gossypii has the enzymes that able to produce riboflavin naturally. The enzyme that responsible for the final production of riboflavin is riboflavin synthase. Riboflavin synthase catalyzes conversion of two molecules of 6,7-dimethyl-8-ribityllumazine into each one molecule riboflavin and 4-ribitylamino-5-aminouracil. In this study, we determined the interaction of riboflavin synthase isolated from Eremothecium gossypii with 6,7-dimethyl-8-ribityllumazine. We performed a computational approach to see the active sites of riboflavin synthase that play a role in the production of riboflavin. We designed riboflavin synthase isolated from Eremothecium gossypii as a model in PDB format. As a template, the structure of riboflavin synthase isolated from Schizosaccharomyces pombe with 1KZL PDB code was used. The thermal stability of enzyme had been conducted on the molecular dynamics simulation approach at 300K, 310K, 315K, 325K, 335K, and 350K. The results showed that amino acid residues which interact include Thr154, Ile169, Thr172, Val6, and Gly102 at the C-terminal domain and Thr56, Gly68, Ala70, Val109, and His108 at the N-terminal domain. Residue Thr154 was from the C-terminal domain and His108 was from the N-terminal domain, represents two-subunit of the enzyme that acts as an early stage at riboflavin catalysis reaction. These results shown that only one of active sites of the enzyme (N-terminal domain) catalyze riboflavin formation. Molecular dynamics simulation showed the calculation of RMSD values at 300 K and 315 were fluctuated in the range of 22-26 Å from the initial state. At 320 K and 335 K, fluctuation occurred in the range of 29-34 Å. At 350 K, fluctuation occurred in 38-45 Å and the domains structure had separated.

Keywords: Thermal stability, riboflavin, riboflavin synthase, 6,7-dimethyl-8-ribityllumazine, molecular dynamics, Eremothecium gossypii.

INTRODUCTION

Riboflavin is a precursor of coenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), which are required for enzymatic oxidation-reduction reaction (Sybesma, et.al., 2004). In general, the production of riboflavin can be achieved by three methods, chemical synthesis, fermentation, and biotransformation of glucose to D-ribose. Environmental and economical studies indicate that the production of riboflavin by fermentation continue to increase, due to cheaper production costs, less generated waste, and lower energy of production (Shrikant, et.al., 2006). Each year, around 3.000 tons of riboflavin is produced in the world, and 2500 tons was produced by fermentation (Choe, et.al., 2005). Some studies have shown that fermentation process using *Eremothecium ashbyii* and *Eremothecium* (*Ashbya*) *gossypii*, produce more riboflavin than other microorganisms, such as *Saccharomyces cerevisiae*, *Candida famata*, and *Bacillus subtilis* (Kato, et.al., 2006). *Eremothecium gossypii* has the enzymes that are naturally able to produce riboflavin. Amount of riboflavin produced is highly dependent on the amount of enzyme and growth conditions. The enzyme responsible for the final production of riboflavin is riboflavin synthase. Riboflavin synthase catalyzes conversion of two molecules of 6,7-dimethyl-8-ribityllumazine to one molecule each of riboflavin and 4-ribitylamino-5-aminouracil. In this study, we determined interaction of riboflavin synthase isolated from *Eremothecium gossypii* and

performed computational approach to examine the active sites of riboflavin synthase that played role in the production of riboflavin.

MATERIAL AND METHODS

The structure of riboflavin synthase isolated from *Schizosaccharomyces pombe* with 1KZL PDB code was used as a template (Gerhardt, et.al., 2002). Sequences of riboflavin synthase of *Eremothecium gossypii* from NCBI (Locus CAC07495) were used as a model and 6-carboxyethyl-7-oxo-8-ribityllumazine was used as a ligand. We designed riboflavin synthase isolated from *Eremothecium gossypii* as a model in PDB format using geno3D Protein Predict and Jigsaw modelers, and simulated using MOE2008.10. The thermal stability of enzyme were simulated using molecular dynamics approach at temperature 300K, 310K, 315K, 320K, 335K, and 350K.

RESULT

Homology Model Validation

The final result of multiple sequence alignment of riboflavin synthase from *Schizosaccharomyces pombe* and *Eremothecium gossypii* is shown in Figure 1. The result showed that homologous value was 64%. Modeling using geno3D Protein Predict obtained 3D structure in PDB format, indicates a structural similarity with RMSD deviations between models and template of 0.82 Å. RMSD deviation value is still below the maximum value of 1.70 Å, which indicates that the model and template does not have significant differences. The quality of Ramachandran plots was satisfactory for the models. Number of residues in outlier region are 2.9% for the models and 0.0% for the template. The value is still bellow the maximum value of 15%, which indicates that the model is reliable for performing further studies.

```
CLUSTAL 2.0.12 multiple sequence alignment


pdb|model|A      MFTGIVEHIGTVAEYLENDASEAGGNGVSVLIKDAAPILADCHIGDSIACNGICLTVTEF 60
pdb|1KZL|A       MFTGLVEAIG-----VVKDVQGTIDNGFAMKI-EAPQILDDCHTGDSIAVNGTCLTVTDF 54
                 ****:** **     : :*.. : .**.:: * :*. ** *** ***** ** *****:*

pdb|model|A      TADSFKVGIAPETVYRTEVSSWKAGSKINLERAISDDRRYGGHYVQGHVDSVASIVSREH 120
pdb|1KZL|A       DRYHFTVGIAPESLRLTNLGQCKAGDPVNLERAVLSSTRMGGHFVQGHVDTVAEIVEKKQ 114
                     *.******::  *::.. ***. :*****: .. * ***:******:**.**.:::

pdb|model|A      DGNSINFKFKLRDQEYEKYVVEKGFVAIDGVSLTVSKMDPDGCFYISMIAHTQTAVALPL 180
pdb|1KZL|A       DGEAIDFTFRPRDPFVLKYIVYKGYIALDGTSLTITHVD-DSTFSIMMISYTQSKVIMAK 173
                 **::*:*.*: **    **:* **::*:**.*******:* *. * * **::**: * :.

pdb|model|A      KPDGALVNIETDVNG----KLVEKQVAQYLNA--- 208
pdb|1KZL|A       KNVGDLVNVEVDQIGKYTEKLVEAHIADWIKKTQA 208
                 *  * ***:*.*  *    **** ::*::::
```

Figure 1. Sequence alignment of riboflavin synthase from *Schizosaccharomyces pombe* (1KZL)and *Eremothecium gossypii* (model); Sign (*) indicates the same amino acid (identity) and sign (*: and.) are homologous region.

Active Sites of Riboflavin Synthase of Eremothecium gossypii

Riboflavin synthase crystal from *Schizosaccharomyces pombe* is used as a template with PDB code 1KZL, determined using X-ray diffraction with a resolution of 2.10 Å. The

structure of riboflavin synthase is in the form of a complex with 6-carboxyethyl-7-oxo-8-ribityllumazine (carboxyethyllumazine). The structure of riboflavin synthase from *S. pombe* has been made with two active site pockets . Only one of the two active sites which catalyze the formation of riboflavin and the other will interact with the solvent (Fischer and Bacher, 2008).. Model of riboflavin synthase of *Eremothecium gossypii* that interact with the substrate carboxyethyllumazine, appeared to have two active sites pocket. Amino acid residues that interact include Thr154, Ile169, Thr172, Val6, and Gly102 at the C-terminal domain and Thr56, Gly68, Ala70, Val109, and His108 at the N-terminal domain (Figure 2). Residue Thr154 was from the C-terminal domain and His108 was from the N-terminal domain, represents two-subunit of the enzyme that acts as an early stage in riboflavin catalysis reaction. The result showed that only one of enzyme active sites (N-terminal domain) catalyzes riboflavin formation (Figure 3).

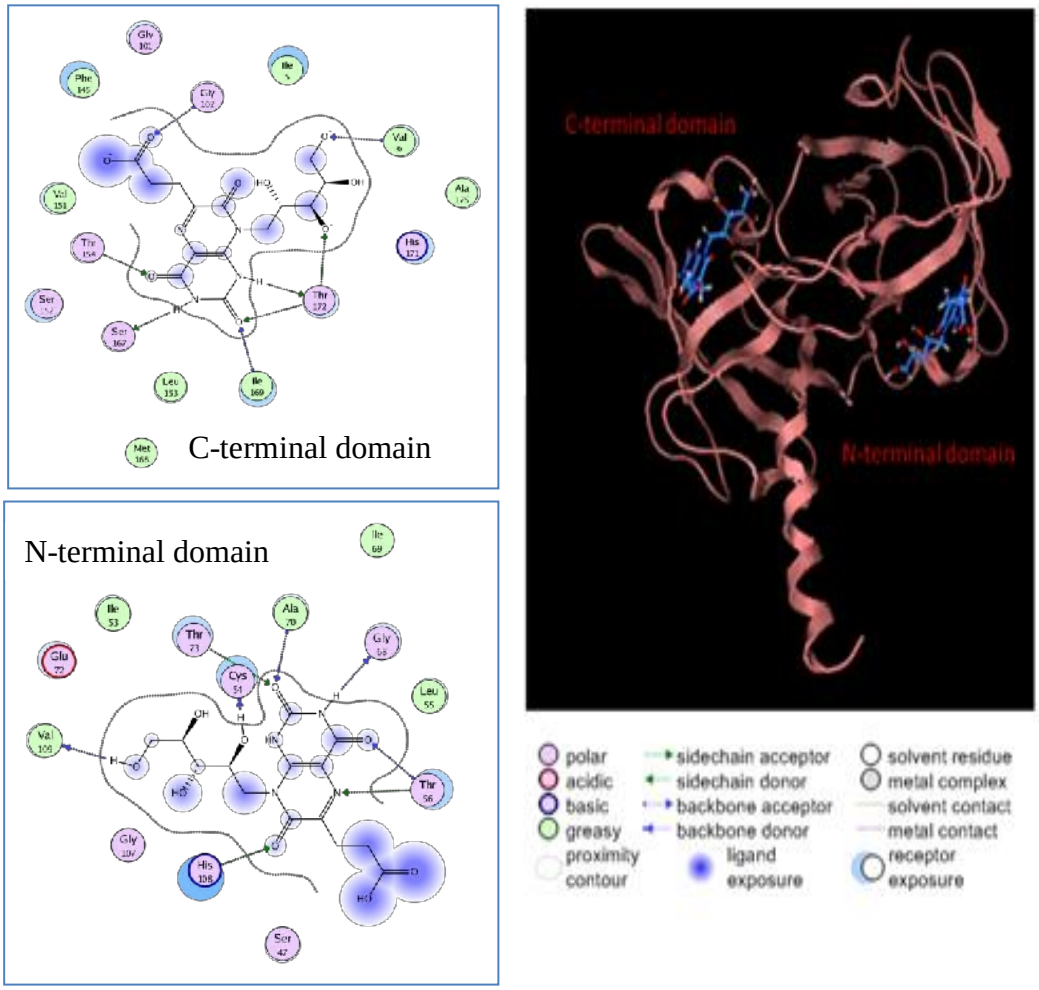

Figure 2. Active sites of riboflavin synthase and amino acid residues that interact in C-terminal domain and N-terminal domain.

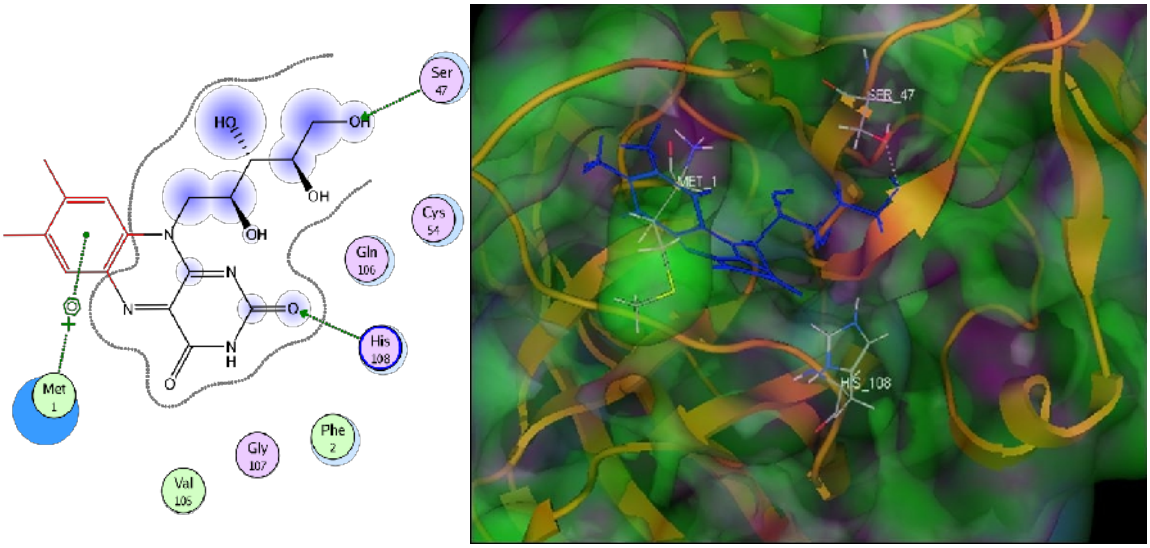

Figure 3. Active sites of the enzyme at N-terminal domain that catalyze riboflavin formation.

Thermal Stability of Riboflavin synthase of Eremothecium gossypii

The results of RMSD values versus the simulation time at 300 K showed that the value are fluctuated in the range of 22-26 Å from the initial state . RMSD value changes very

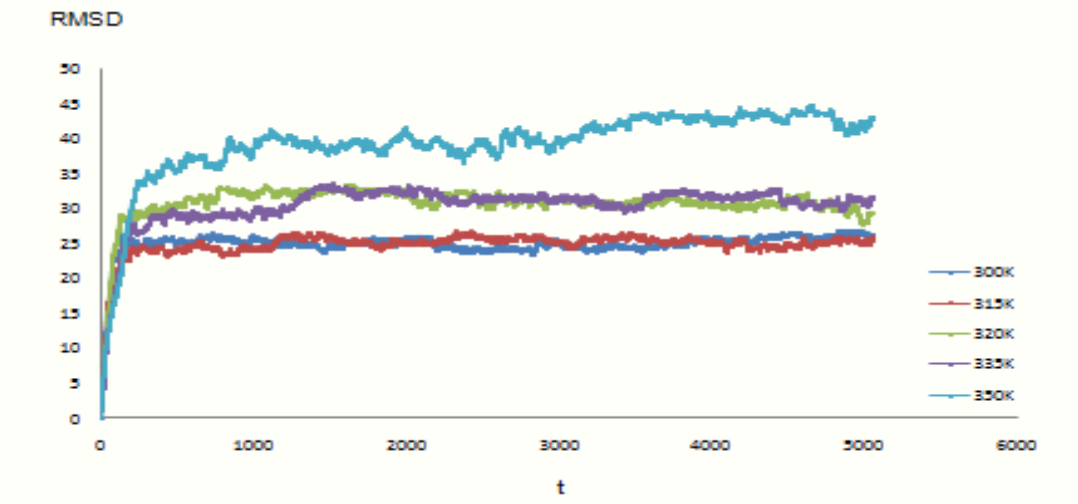

Figure 4. RMSD curves (Å) at 300 K, 315 K, 320 K, 335 K and 350 K versus the simulation time (t).

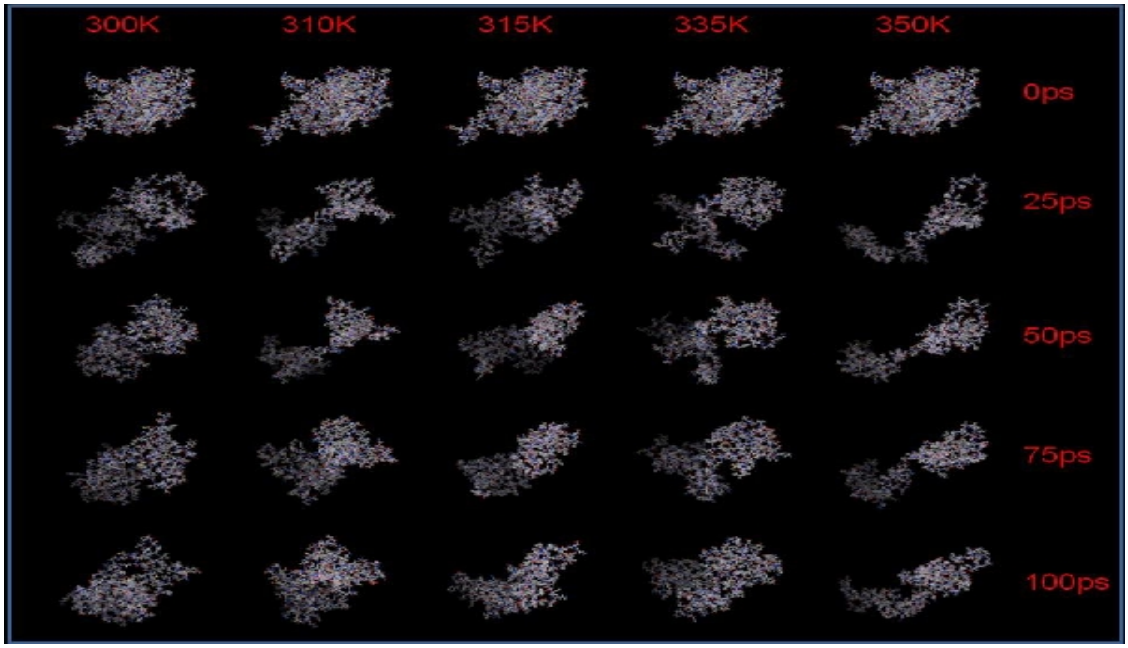

Figure 5. Conformational changes of the riboflavin synthase structure in the range of 0-100ps at temperature 300 K, 310 K, 315 K, 335 K and 350 K

sharply in the range 0-100 ps (Figure 4), indicates the occurrence of a cooperative unfolding process. Visualization results (Figure 5), indicate that the enzyme structure changes take place very rapidly, this was because the structural changes occurred followed the pattern of structural change in catalyzing formation of riboflavin from 6.7-dimethyl-8-ribityllumazine. The stability of the enzyme structure will be obtained after 100 ps with a globular shape. This form will be retained until the end of simulation. Enzyme conformational changes at 315 K does not vary much with conformational changes at 300 K, the range of 0-100ps RMSD value reaches 22 Å and fluctuating between 22-26 Å from the initial state. At 320 K and 335 K, after 100ps, the fluctuation of RMSD values increased to 29-34 Å. These results indicate the occurrence of different conformational change in unfolding process at 300 K and 315 K, which describe the instability of the structure. At 350 K, the domains structure had separated.

DISCUSSION

Thermal stability is defined as the resistance of a protein to maintain the structure in such a way as a response to high temperature so that the protein can still perform its function. RMSD values showed that the enzyme structure changes take place very rapidly for all temperature, and visualization results showed that the structural changes very sharply in the range of 0-100ps. Visualization of the results shows that the conformational changes the enzyme structure at 300 K and 315 K are most likely to catalyze the formation of riboflavin, because the N-terminal domains are not separate. From the results of dynamics simulation of the enzyme and substrate, shows that the separation of the domains will release the substrates faster thus catalyzing the process would not be possible (data not shown).

CONCLUSION

Amino acid residues of riboflavin synthase of *Eremothecium gossypii* that interact to catalyze the formation of riboflavin include: Thr154, Ile169, Thr172, Val6, and Gly102 at the C-terminal domain and the Thr56, Gly68, Ala70, Val109, and His108 in the N-terminal. Only one of active sites of the enzyme that catalyze riboflavin formation that is N-terminal domain. RMSD value at 300 K and 315 K increased to 23 Å from initial state and the simulation time in the range of 0-100ps with fluctuations around 23-27 Å and had a similar structure that

conformational changes. RMSD value at 320-350 K fluctuation occurred in 29-34Å with a conformational change towards the unstable structure.

ACKNOWLEDGEMENT

The researchers are grateful to Ridla Bakri PhD, Chairman of Department of Chemistry, Faculty of Science, University of Indonesia for his support of this research.

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Tosylation of *N*-Phthaloyl-Chitosan without Drying of Solvents and Purging of Water Vapor

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ABSTRACT

Tosylation of N-phthaloyl-chitosan, without pretreatment of solvent drying and without treatment of water vapor purging during the reaction has been studied. N-phthaloyl-chitosan was tosylated using excess tosyl chloride in dimethyl acetamide/LiCl as solvent and triethyl amine as catalyst at temperature < 10°C for 12 h. As comparison, the tosylation reaction was also performed using phthaloyl-chitosan (protection of chitosan by phthaloyl group was not chemoselective at amine groups only) as a raw material. The solvents were not dried prior to use and all subsequence reactions were not performed under nitrogen atmosphere. The result showed that by using phthaloyl-chitosan as a raw material, there is no product of tosylation can be isolated. The FTIR spectrum showed that 6-O-tosyl-N-phthaloyl-chitosan could be formed using CPNPC as raw material. It indicated that the success of tosylation can be influenced by selectivity of phthaloyl group protection on chitosan. These results showed that a tosylated-chitosan can also be prepared without pretreatment of solvent drying and treatment of water vapor purging during the reaction.

Keywords: tosyl, tosylation, phthaloyl, phthaloylation, chitosan.

INTRODUCTION

Cellulose and chitin as biopolymers are the most abundant organic compounds in Nature and estimated to be at levels approaching 1011 tons annually. Chitin has been a major structural component of animal exoskeleton since the Cambrian Period, more than 550 million years ago. The total amount of chitin harvestable without imbalancing the marine ecosystem is estimated to be 1.5.108 kg/year, mostly from the shells of crustaceans such as crab, shrimp and krill. Chitin is structurally similar to cellulose, but it is an amino polysaccharide having acetamide groups at the C-2 positions instead of hydroxyl groups. In addition to its unique polysaccharide architecture, the presence of a little amino groups (5-15%) in chitin is highly advantageous for providing distinctive biological functions and for conducting modification reactions. Chitosan is the *N*-deacetylated derivative of chitin. Actually, the names chitin and chitosan correspond to a family of polymers varying in the acetyl content. Therefore, the degree of acetylation (DA) determines whether the biopolymer is chitin or chitosan. Chitosan is the term used for the considerably deacetylated chitin that is soluble in dilute acetic acid (degree of deacetylation, DD 70%) (Zohuriaan-Mehr, 2005). Chitin and chitosan thus expected to have much higher potential than cellulose in many fields. It is a specialty biopolymer having specific properties including biodegradability, biocompatibility, and bioactivity, and it is therefore interesting not only as an abundant resource but also as a novel type of functional material (Kurita, 2001).

In spite of potential applications of chitin and chitosan, it is necessary to establish efficient appropriate modifications to explore fully the high potential of these biomacromolecules. Chemical modifications of chitin are generally difficult owing to the lack of solubility, and the reactions under heterogeneous conditions are accompanied by various problems such as the poor extent of reaction, difficulty in selective substitution, structural ambiguity of the products, and partial degradation due to severe reaction conditions.

Therefore, with regard to developing advanced functions, much attention had been paid to modification of chitosan rather than chitin (Zohuriaan-Mehr, 2005).

In order to prepare derivatives with well-defined structures and to prepare advanced functional material from chitosan, it is crucial to manipulate reactions in a well-controlled manner. Since the *p*-toluenesulfonyl (tosyl) group is bulky and the ester (tosylate) is highly reactive, it is another candidate for a substituent to prepare a soluble precursor that would enable controlled modification in solution (Kurita, 2001).

As the tosyl group is a good leaving group, many reports about tosyl-chitin and their properties have been reported (Kurita *et al*, 1992, Morita *et al*, 1994, Morita *et al*, 1999; Zou and Khor, 2005; Shimizu et al, 2007; Pourjavadi et al, 2007). There are few reports with chitosan (Nishimura et al, 1990; Jančiauskaitė and Makuška, 2008). Prior to tosylation, free amino groups of chitosan were selectively protected with phthaloyl groups to give a N-protected precursor that is soluble in organic solvent such pyridine, dimethyl acetamide (DMAc), and dimethyl sulfoxide (DMSO). Tosylation reaction was usually performed with excess *p*-toluene sulphonyl chloride (in comparison with chitin or phthaloyl-chitosan).

Although some methods of tosylation of chitin were reported using dried solvents and flowing nitrogen to purge water vapor during reaction (Morita *et al*, 1994, Morita *et al*, 1999, Zou and Khor, 2005), but the others were reported using aqueous sodium hydroxide (Kurita *et al*, 1992, Shimizu et al, 2007; Pourjavadi et al, 2007). However, tosylation of phthaloyl-chitosan were not reported clearly whether performed using dried solvent and flowing nitrogen, or without pretreatment of drying of solvents and/or without treatment of flowing nitrogen to purge of water vapor. Considering that tosylation of chitin can be performed in aqueous sodium hydroxide, it is interesting to try tosylation of protected N-phthaloyl-chitosan using without pretreatment of drying of solvents and without flowing nitrogen to purge of water vapor during reaction process and the result would be reported in this paper.

2. MATERIALS AND METHODS

2.1. Materials

Chitosan was obtained from deacetylated chitin that isolated from shrimp sells. *N*,*N*-dimethyl formamide (DMF), phthalic anhydride, *N*,*N*-dimethyl acetamide (DMAc), lithium chloride, tosyl chloride, and triethyl amine (TEA) were obtained from Merck (Germany). All reagents and solvents were of analytical grade and used without further purification. The preparation of N-phthaloyl-chitosan was performed using excess phthalic anhydride in DMF as solvent according to procedure of Nurmasari *et al* (2011).

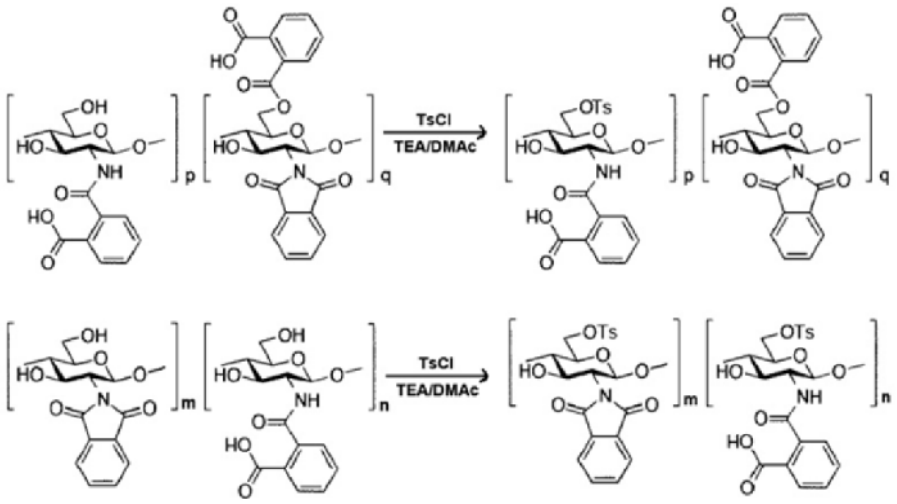
2.2. Preparation of 6-*O*-tosyl-*N*-phthaloyl chitosan

Triethylamine (9.5 ml, 68 mmol) and toluene-4-sulfonyl chloride (3.6 g, 18.8 mmol) dissolved in 20 ml DMAc were gradually added to a cooled to 4–8 °C solution of *N*-phthaloylchitosan (0.5 g, 1.88 mmol) in DMAc/LiCl (50 ml), and the mixture was stirred at 8 °C for 12 h. The precipitate obtained by pouring the solution into ice water was collected by filtration, washed with chloroform, and dried to give the product. As comparison, the tosylation reaction was also carried out in the same way but with phthaloyl-chitosan (protection of chitosan by phthaloyl group was not chemoselective) as a raw material. The product was characterized by recording the FTIR spectrum on a Shimadzu FTIR (IRPrestige-21).

3. RESULTS AND DISCUSSION

The higher reactivity of amino groups present in the main backbone of chitosan requires protection to limit their participation in reactions under tosylation of chitosan. This was done by the use of phthalic anhydride in non-dried DMF (Scheme 1) according to the procedure reported elsewhere (Nurmasari et al, 2011). Introduction of bulky phthaloyl groups prevents formation of intra- and intermolecular hydrogen bonds and consequently improves the solubility of chitosan in organic solvents such as DMF and DMAc/LiCl. Solubilization of *N*-phthaloyl chitosan in DMAc/LiCl is of the last importance for the further derivatization of chitosan in homogeneous conditions. Besides, phthaloyl groups could be removed easily regenerating free amino groups in the derivatized chitosan.

Interestingly, there is no product of tosylation can be isolated if phthaloyl-chitosan (protection of chitosan by phthaloyl group was not chemoselective) was used as a raw material. It could be occurred probably because partial O-phthaloylation of chitosan was at C-6 position. As known, a bulky tosyl groups preferentially react at the least hindered C-6 position of chitosan (Scheme 1). Although some hydroxyl groups at C-6 position were free (not phthaloylated) and could be tosylated, the degree of substitution would be relatively low. Kurita et al (1992) have shown that the resulting tosyl-chitin with a substitution degree less than 0.3 was highly hydrophilic and partially soluble in water. However, the product with a substitution degree above 0.4 become hydrophobic and was soluble in common polar organic solvents such as DMAc. These phenomenon were observed on the resulting tosyl-phthaloyl-chitosan that formed a suspended colloid at bottom part after pouring in ice water, but it could not be isolated by filtration or centrifugation. On the other hand, product of tosylation of N-phthaloyl-chitosan formed a precipitate that can be isolated easily by filtration.



Scheme 1

Formation of 6-*O*-tosyl-*N*-phthaloyl chitosan was proved by FTIR spectra. The IR spectrum of 6-*O*-tosyl-*N*-phthaloyl chitosan showed a characteristic absorption at 1188 cm^{-1} due to tosyl groups (SO_2), absorption at 818 cm^{-1} is characteristic of C-O-S, and 1643 cm^{-1} is typical of peak of aromatic C=C arising from the tosyl group (Fig. 1, bottom). Absorption at 1713 and 1774 cm^{-1} are characteristic of carbonyl in phthalimide and absorption at about 700 cm^{-1} is characteristic of aromatic arising from phthaloyl group. The bands at 1389 cm^{-1} can be attributed to $-CH_2$ bending modes.

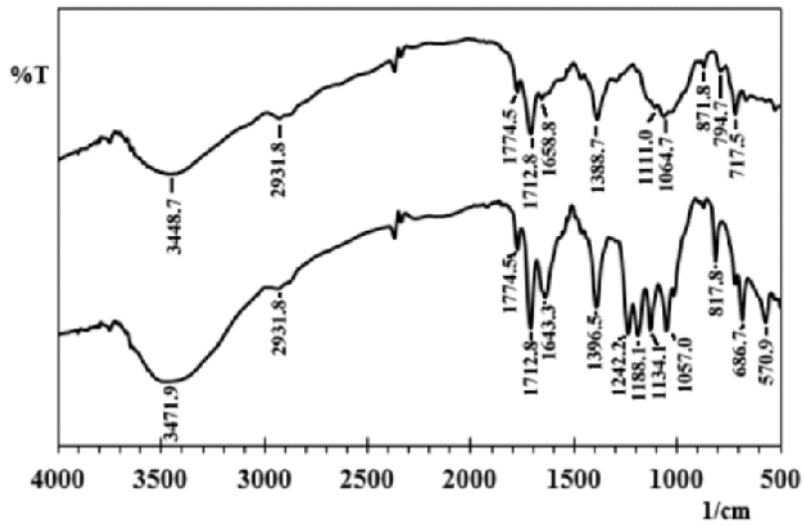


Fig. 1. FTIR spectra of N-phthaloyl-chitosan before (top) and after (bottom) were tosylated. Tosylation was carried out without pretreatment of drying of solvent and without purging of water vapor during reaction process.

The (*p*-tolylsulfonyl)oxy group is one of the most effective leaving groups widely used in carbohydrate chemistry. Since primary hydroxyl groups are more reactive than secondary ones, the regioselective reaction is expected and proved in many cases. Treatment of *N*-phthaloyl chitosan with a 10-fold excess of tosyl chloride and triethylamine in DMAc afforded 6-*O*-tosyl-*N*-phthaloyl chitosan with a very high degree of tosylation. It was proved that primary hydroxyl groups of glucosamine unit could be completely changed to tosyl groups. The use of triethylamine in the tosylation of chitosan was essential for the neutralisation of hydrogen chloride generated during tosylation (Jančiauskaitė and Makuška, 2008).

CONCLUSIONS

Activated chitosan, 6-*O*-tosyl-*N*-phthaloyl-chitosan, could be prepared successfully in one-step using N-phthaloyl-chitosan as raw material, DMAc/LiCl as a solvent, and TEA as catalyst without pretreatment of drying of solvents and without flowing nitrogen to purge of water vapor during reaction process. The simple procedure established here enables facile preparation of 6-*O*-tosyl-*N*-phthaloyl-chitosan, a convenient precursor for the construction of sophisticated molecular architectures based on the specialty biopolymer chitosan.

ACKNOWLEDGEMENT

This work was supported financially by a Grant of Penelitian Hibah Bersaing (No. 008/SP2H/PL/Dit.Litabmas/IV/2011) from the Directorate General of Higher Education (DGHE), Ministry of National Education, INDONESIA.

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International Conference on Bioscience and Biotechnology (ICBB) 2011

October 11th-12th, 2011, Yogyakarta, Indonesia

Oral Paper Cluster 2

Associations between Blood Lead Level and Blood Pressure among City Minibus Drivers in Purwokerto City, Indonesia

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Hypertension occurence is increasing in Indonesia. In 2009, the prevalence was 21%. Exposure to lead is well documented as the important risk factor related to the occurence of the disease. In indonesia, leaded gasoline is still being used, and no prohibition was made by the authorities. This lead to increase the risk of people who are exposed to lead pollution in the air to suffer from hypertension, including city minibus drivers. Minibus is used to mass transport in Purwokerto city, Indonesia. This research aimed to examine the association between blood lead level and blood pressure among city minibus drivers in Purwokerto City, Indonesia. The research was observational, cross-sectional study. The respondents were city minibus drivers in Purwokerto city that were not have a history of essensial hypertention in their family and have been work as minibus driver for at least one year. Blood pressure was measured using Hg sphygmomanometer and blood lead level was measured using AAS. Among 300 city minibus drivers, only 54 agreed to be recruited as respondents. Pearson's correlation was used to analyse the data. The result showed that all respondent have blood lead level >20 µg/dL and the mean of blood lead level was high, 49.99 µg/dL, whereas the mean of systole and dyastole blood pressure were 126,67 mmHg and 86,11 mmHg respectively. Hypertension prevalence was 338,89%. Pearson correlation analysis showed that blood lead level has no significant association with both systolic blood pressure (R=0.114; p= 0.413) and diastolic blood pressure (R=0,252; p=0.066). No significant association between blood lead level and hypertension (X^2=2,424, p=0,202, C=0,207).
Keywords: Blood lead level, blood pressure, hypertension

INTRODUCTION

Hypertension is the main cause of cardiovascular diseases. In Indonesia, cardiovascular diseases account for 26,3% dan ranked number two as the cause of death (Glenn *et al*., 2001; Departemen Kesehatan RI, 2007).

Hypertension is a multifactorial condition, these are genetic and environmental factors. Approximately 30% hypertension cases are related to genetic factor (Glenn *et al*., 2001). However, Sohaila *et al.* (2006) said that 90% hypertension cases are idiopathic, although it has been well understood that environmental factor has an important role in the occurence of hypertension. One environmental factor that is a risk factor for hypertension is toxic metal (Glenn *et al*., 2001).

Exposure to toxic metal that is considered to be related to increased blood pressure and hypertension occurence is exposure to lead or plumbum (Pb). Lead exposure remains one of the most important problems in terms of prevalence of exposure and public health impact (Hu *et al.*, 2007).

Most people are exposed to Pb because there are many Pb resourses in the environment. According to *Agency for Toxic Substances and Disease Registry* (ATSDR), the United States of America, one important Pb source in the developing countries is leaded gasoline (ATSDR, 2001). As such, transportation workers, including city minibus drivers are prone to exposed to Pb. This will increasing the risk of hypertension occurence.

WHO established that normal blood lead level for adult is 5-20 µg/dL. However, some research established that low level Pb exposure could increse blood pressure and causes hypertension. Some researches showed that lead blood level between 5 µg/dL to 10 µg/dL results in increased blood pressure and hypertension (Lee *et al*., 2001; Patrick, 2006; Nawrot *et al*., 2002; Chuang *et al*, 2004).

Considering the magnitude of Pb exposure and the high risk of hypertension occurence among city minibus drivers, this research was conducted to examine the the association between lead exposure and blood pressure among city minibus drivers in Purwokerto City, Indonesia.

MATERIAL AND METHODS

Materials needed for blood lead level examination are 7 ml venous blood, disposable syring 10 mL, vaccuettes 10 mL, torniquet, centrufuge, AAS and $PbSO_4$ solution with 2 ppm, 5 ppm, 9 ppm and 15 ppm. Materials needed to measure blood pressure are calibrated sphygmomanometer and stethoscope.

This research was an observational, cross sectional study. Reseach population was 300 city minibus drivers in Purwokerto City. Reseach sample was all city minibus drivers volunteer in Purwokerto City that is agree to be recruited as research respondent. The inclusion criteria for sample are men, has a good nutritional status based on body mass index measurement, do not have essensial hypertention history in their family, have been work as minibus driver for at least one year and agree to be recruited as research respondent with signing the informed consent.

The independent variable was blood lead level and the dependent variable was blood pressure and hypertension, consist of systolic and diastolic blood pressure. The mean of 3 systolic and diastolic blood pressure measurements with 5 minutes interval were collected, all of which were taken by a physician. Respondents were categorized as hypertensive if the systolic blood pressure is $\geq$140 mmHg and the diastolic blood pressure is $\geq$90 mmHg. Blood lead level is lead level in the whole blood in µg/dL scale, measured with AAS method (Nash *et al*., 2003; Glenn *et al*., 2001).

Blood samples were obtained by venipuncture. Sampling was done under the supervision of trained medical staff. Care was taken to avoid haemolysis of blood samples, disposable syringes were used and slow transfer to a 10 ml vacuettes Before taking the sample, all respondent were asked to sign informed consent. Minimum time was taken for transport of sample whereas storage was mostly avoided and when required the samples were refrigerated at −4 °C prior to analysis. Almost all samples were analyzed within 8 h after collection. Blood lead concentration was measured by Atomic Absorption Spectrophotometry (AAS) at the Research Laboratory, Jenderal Soedirman University. The assay detection limit was 1.0 µg/dL. Each sample analysis was performed in duplicate, and the mean of both measurements was used in these analyses. All blood lead levels less than 1.0 µg/dL were assigned a value of 0.5 µg/dL (Hu *et al*., 2001).

Detailed information on the volunteers was recorded in the designed questionnaire for this study. Questionnaires are usually used to gather data on health and other factors to ensure that respondent was meet the inclusion and exclusion criteria.

Data were analyse using a computerised statistic program. Univeariate analysis was used to analyse the frequency distribution of research data. In bivariate analysis, Pearson's correlation was used to analyse the association between blood lead level and blood pressure (systolic and diastolic blood pressure). The Chi-square test and contingency coeffisien were used to analyse the association between blood lead level and hypertension occurence. Blood lead level was classified as normal ($\leq$20 µg/dL), high ($\geq$20-40 µg/dL) and very high (>40 µg/dL).

RESULTS

At the end of research period, 54 volunteers of city minibus drivers were participated on the reseach. All respondent have abnormal or high blood lead level. The minimum blood lead level was 22,18 μg/dL, whereas the maximum blood lead level was 64,51 μg/dL. Mean blood lead level was 45,99 μg/dL, twofold of normal blood level recomended by the WHO. Detail data regarding frequency distribution of blood lead level can be seen in Table 1.

Table 1. Blood lead level among city minibus drivers in Purwokerto

NO.	BLOOD LEAD LEVEL	VALUE
1	Minimum	22,18
2	Maximum	64,51
3	Mean	45,99
4	Median	45,94
5	Standard deviation	10,43

Results of blood pressure measurement are summarized in Table 2. Most respondents have a normal systolic and diastolic blood pressure. The means of both systolic and diastolic blood pressure are still normal. Mean systolic blood pressure was 126,67, whereas mean diastolic blood pressure was 86,11. Pearson's correlation analysis showed that there were no significant association between blood lead level and both systolic and diastolic blood pressure, with R=0.114; p=0.413 and R=0.252, p=0.066, respectively.

Table 2. Blood pressure among city minibus drivers in Purwokerto

NO.	DATA DISTRIBUTION	BLOOD PRESSURE (mmHg)	
		SYSTOLIC	DIASTOLIC
1	Minimum	100	70
2	Maximum	170	120
3	Mean	126,67	86,11
4	Median	120	80
5	Standard deviation	15,54	12,80

The occurenc of hypertension among city minibus drivers in Purwokerto is presented in Table 3. The prevalence of hypertension was 38,89%, higher than that of Indonesian population. However, if compared with data regarding blood lead level where all respondent have high blood lead level, this prevalence is relatively low. Only 38,89% respondents are suffer from hypertension. Hypertension prevalence were higher among those with very high blood lead level. However, statistically there is no signoficant association between blood lead level and hypertension occurence among city minibus drivers in Purwokerto City. The association is very low (X^2=2.424, p=0.202, C=0,207).

Table 3. Hypertension prevalence among city minibus drivers in Purwokerto

NO.	BLOOD LEAD LEVEL	HYPERTENSION		TOTAL
		YES	NO	
1	High	3 (21,42%)	11 (78,58%)	14 (25,93%)
2	Very high	18 (45,00%)	22 (55,00%)	40 (74,07%)
	Total	21 (38,89%)	33(61,11%)	54 (100,00%)

DISCUSSION

This research showed that no significant association between blood lead level and blood pressure. The result also showed that no significant association was found between blood lead level and hypertension. High blood pressure is a multifactorial condition involving both genetic and environmental factors. Family and twin studies indicate that as much as 30 percent of hypertension, a disease defined by high blood pressure, is due to genetic causes. Rare forms of genetic hypertension have different molecular etiologies but a common pathophysiology mediated by abnormal sodium metabolism. Environmental risks for high blood pressure include dietary factors, cigarette smoking, and high alcohol intake, and possibly exposure to toxic metals such as lead (Glenn *et al*., 2001).

Several epidemiologic studies have suggested a relation between exposure to lead, particularly at blood concentrations less than 40 μg/dl, and small increases in blood pressure, but other studies have failed to find a relation (Chuang *et al*, 2004; Glenn *et al*., 2001; Lee *et al*., 2001; Navas-Acien *et al*., 2007; Patrick, 2006; Nawrot *et al*., 2002). The conflicting results among studies may be attributed to demographic differences between the populations studied, variation in genetic susceptibility, and limitations of the measures used to define lead exposure (Glenn *et al*., 2001).

Although statistical analysis revealed that there was no significant associations between blood lead level and blood pressure, this study showed that individu with higher blood lead level tends to have higher blood pressure and the prevalence of hypertension also higher among those with very high blood lead level. However, in this study data about genetic susceptibility and demographic differences in the study population were not measured.

Some studies concluded that genetic susceptibility is an important factor for lead exposure effect on blood pressure and hypertension (Olanaja and Claudio, 2000). According to Vupputuri *et al*. (2003), differences in genetic factor causes black people is more susceptible to the effect of lead exposure than white people. The main genetic factor affected the increased of blood pressure is gene polymorphism. Polymorphism of δ-ALAD gene resukted in differences in lead exposure susceptibility, as 80% blood lead are bind to δ-ALAD (Barbosa Jr. *et al*., 2005; Lee *et al*., 2001; Glenn *et al*. (2001). People with δ-ALAD-2 are more susceptible to lead exposure, because δ-ALAD-2 produces protein that is binding Pb more thigh than δ-ALAD-1 (Kelada *et al*., 2001).

Patient demographic, including age and chronic disease are other important factor that should be examine in the later research. People with occult kidney disfunction, for example, will experience disturbances in lead elimination from the body and usually have higher impact on blood pressure.

In conclusion, although this reseach established that there is no significant association between blood lead level and both blod pressure and hypertension, there is an evidence that people with very high blood lead level tends to have higher blood pressure and hypertension prevalence. As such, workers exposed to lead should be given more attention to monitor the effect of lead on their lead, especially their blood pressure (Oktem *et al*., 2004).

ACKNOWLEDGEMENT

Authors would like to thank to Jenderal Soedirman University for providing funding for this research. Thank also goes to all respondents and all research enumerator participated in this research.

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Rat Sperm Proteomic Analysis: Effect of the Antifertility Agent *Centellaasiatica*L.

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ABSTRACT

Oral intake of *Centellaasiatica*L. has been reported to have an antifertility effecton sperm quality. In this study the effects of *C.asiatica*L. treatment in male rat sperm quality and sperm protein expression were analyzed. Thirty-two adult male Sprague-Dawley rats twelve weeks old were divided into four groups: control group; low dose group (100 mg/kg body weight); medium dose group (200 mg/kg body weight); and high dose group (300 mg/kg body weight). All treated groups were force-fed with an ethanolic extract of *C.asiatica*L. and the control group was force-fed with distilled water, respectively for 42 days. Rats were sacrificed on day 43. Cauda epididymal rat sperm from control and high dose treatment were subjected to two-dimensional gel electrophoresis for comparison of protein expression profiles. Results showed that caudaepididymal sperm count and motilityin treated groups showed a significant decrease compared to control. Medium and high dose treatments showed the most significant reduction ($p<0.05$).Proteomic study revealed significant changes in sperm protein expression between control (282 spots) versus high dose treated group (234 spots). The matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis of the selected spots exhibited five spots differentially expressed identified as alpha enolase, aldolase A, sorbitol dehydrogenase, glutaminesynthetase and lipocalin. Sorbitol dehydrogenase was present in the control group, but not in the treated group. This enzyme plays an important role in sperm motility and is associated with the maturation of the germinal epithelial layer of seminiferous tubules.The lipocalin protein also disappeared in the treated group. This transport protein plays a significant role in epididymal function. It was concludedthat *C. asiatica*L.have antifertility activities in rats sperm quality and protein expression.

Keywords:Centella asiatica *L., sperm quality,proteomic*

Introduction

The development of antifertility or contraceptive agents from medicinal plants which are safe and effective with minimum side effects is a trend in pharmacological research today (Qureshi et al. 2006). Many studies conducted successfully on various plants identified compounds which have antifertility properties, but most of them are metabolically toxic to human (Sarkar et al. 2000). Previous reports suggested that some compounds in plants are capable of interfering with the production of androgenichormones, can modify spermatogenesis, or are abortifacients, as well as having spermicidal activity (Qureshi et al. 2006).

Centella asiatica L. (Pegagan, local name) is one of the popular herbs used in folk medicine in Indonesia for its wound healing, antibacteria and antioxidant-rich properties. Some active compoundshavingmedicinal properties have been described such as madecassoside, asiaticoside (glycoside), madecassic acid and asiatic acid (terpene acid). Studies have revealed the efficacy of *C. asiatica*in wound healing, improving memory, and

anticancer activity (Inamdar et al. 1996; Jaganath & Ng, 2000),while the antifertility effect of *C. asiatica*has been reported in animals (Dutta & Basu, 1968).

Despite many studies of *C.asiatica*, details of theeffectson the male reproductive system have not been fully elucidated. Therefore, in this study we aimed to investigate the effects of *C. asiatica*on sperm count, sperm motility, and sperm protein expression.

Materials and methods

Plant material

Centellaasiatica L. was obtained from a traditional market in Serdang, Selangor. The specimen was then identified and confirmed by botanist of Herbarium Laboratory, Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM), Bangi, Malaysia. *C. asiatica*(5000 g) was washed and dried at 45°C in an oven (Memmert) for 32 h prior to milling to apowder. Sample extraction procedure as described earlier was followed (Houghton & Raman, 1998). A Soxhlet apparatus was used with 90% ethanol as solvent. The sample was then concentrated using rotary evaporator (BüchiRotavapor® R-200/205) to yield approximately 88 g of a dark brown semi solid mass.

Animals

Thirty-two proven fertile adult (age 12 weeks old and weighing 260-300 g) male rats of the Sprague-Dawley strain were obtained from UKM's Animal House. They were divided into four groups of eight animals each. Animals were housed separately in polycarbonate cages (595 mm X 380 mm X 200 mm, Techniplast 1354, Italy) with saw dust as bedding material. The animals' room environment wasmaintained at 12:12h light and dark cycle for each 24h period within temperature of about 25^{o}C. Standard water and rat food pellets (Barastock Rat and Mouse Pelleted Feed, Australia) were administered *ad libitum.* General health conditions of the animals were monitored throughout the experiment. All experiments were conducted following the guidelines of the National Institutes of Health for the care and use of laboratory animals. The study was approved by the Animal Ethics Committee of the Faculty of Medicine, UKM.

Treatment

Animals were divided into four groups. The first three groups were administered with an ethanolic extract of *Centellaasiatica*at three different doses(Kumar & Gupta, 2002),i.e.a low dose (100 mg/kg), a medium dose (200 mg/kg), and a high dose (300 mg/kg), while distilled water was administered to the control group. All treatments were administered orally using a force feeding needle once a day from 10.00-11.00 am for 42 consecutive days.

Sperm count and motility assessment

After finishing 42 days of treatment, the following day,all rats were sacrificed under choloroform anesthesia. Sperm analysis was performed on the samples derived from the cauda epididymis. Sperm count was assessed using '*Improved Neubauer Haemocytometer'* based on previous reports (Comhaire & Mahmoud, 2006) and WHO manual (1999) with modification. Briefly, the cauda epididymis were minced using anatomical scissors, suspended in 15 ml of Biggers-Whitten-Whittingham (BWW)medium (Biggers et al. 1971; Kumar et al. 2007) prior to incubation in 5% CO_2incubator for 30 min at 37^{O}C to allow sperm swim-up. Sperm motility grade was determined usingWHO laboratory manual (1999). Statistical analysis was performed with SPSS 14.0 for windows software using one way ANOVA followed by LSD.

Proteomic Analysis of Rat Sperm

Cauda epididymal sperm obtained from high dose (300 mg/kg) and control groups were subjected to two dimensional gel electrophoresis for proteomic analysis. Protocols of the electrophoresis followed manual that has been previously described (Westermeier & Naven, 2002). Protein extraction from the cauda epididymal sperm in BWW mediumas described by Yunianto (2010).

Briefly, 10 ml of sperm were sucked from the surface of BWW medium and centrifuged (4000 rpm, 15 min, 4^oC). Sperm pellet yielded were extracted with 200 µl lysis buffer (7 M urea, 2 M thiourea, 4% 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 0.8% IPG buffer, 1mM PMSF) using ice in glass homogenizer and allowed to sit for 1h. After a final centrifugation (15,000 rpm, 20 min, 4^oC) the supernatant was removed, 60 mM dithiothreitol (DTT) was freshly added prior to frozen (-20^OC). Protein concentration was determined using the Bradford assay kit as previously reported (Bradford 1976, Rosenberg 1996).

A mini 2-dimensional electrophoresis system (*EttanIPGPhor Isoelectric Focusing,* GE Healthcare and Hoefer mini VE, Amersham Biosciences) was used. Isoelectric focusing was carried out in three steps (250 Vh, 500 Vh, and 8333 Vh, 50 µA per IPG strip, 20^OC, 8 h) (Lai 2006). After finishing the first dimensional electrophoresis, IPG strip was equilibrated in equilibration buffer (2% SDS, 50 mMTris-HCl pH 8.8, 6 M urea, 30% glycerol, 0.002% bromophenol blue, 65 mM DTT for fisrt step and 0.135 M iodoacetamide for the next step) (Görg et al. 1987).Each equilibration step was run on the 3D rotator (Lab-line) for 15 min.The second dimensional electrophoresis was *Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis* (SDS-PAGE)using 12.5% acrylamide gels running at 100 V 20 mA (Lai, 2006). Protein molecular marker (Fermentas) was used in the process. Gels were stained with Coomassie Brilliant Blue R-250 (Biorad), scanned using*Image Scanner III* (GE Healthcare) and analyzed with the Imagemaster 2D Platinum ver.6.0 (GE Healthcare).

Image analysis of the gels was performed on three replicative gels of each group. The overlapping replicative gels were used to obtain a reference gel of each group to enable comparison between them. Quantification of the gel images was based on the mean relative volume (%vol) of the protein spots in each experimental group. According to the significant difference in relative volume ($P<.05$), the spots were cut out, digested and analyzed by *Matrix Assisted Laser Desorption Ionization-Time of Flight* (MALDI-TOF). The results were then identified using peptide mass profiling matched to the database (www.matrixscience.com).

Results

Sperm count and motility

Table 1. Sperm count (x 10^6) and sperm motility grade of control and treatment groups at 100 mg/kg, 200 mg/kg and 300 mg/kg.

Dose (mg/kg)	Sperm number (x 10^6)	Motility grade (a-d)
Control	32.91 ± 11.24	b
100	25.61 ± 1.96	b
200	23.34 ± 1.48*	c
300	21.83 ± 2.24*	c

* Significantly different compared to control ($p<0.05$).

Motility grade according to WHO manual (1999).

a : rapid progressive motility; ≥ 25 µm/s at 37^OC and ≥ 20 µm/s at 20^OC

b : slow or sluggish progressive motility

c : non progressive motility; < 5 µm/s

d : immotility

Sperm count from treated and control groups are shown in Table1. The average rat sperm count was 32.91 x 106 ±11.24, 25.61 x 106 ± 1.96, 23.34 x 106 ± 1.48, 21.83 x 106± 2.24 for the control group, the low dose group, the medium dose group and the highdose group, respectively. Treatment with the high dose (300mg/kg) showed the most sperm count decrease as compared tolow dose (100 mg/kg) and medium dose (200 mg/kg). Thesefindings indicate that *Centella asiatica* treatment causednegative effect for sperm quality. Further LSD analysis also foundthat treatment in medium (200 mg/kg) and high dose (300mg/kg) gave the most significant value (p<0.05)in sperm count decrease as compared to the control.Sperm motility grade shown in Table 1 indicated that*Centella asiatica* treatment also produced an effect on the spermmotility grade, i.e., decrease from b (control and low dose)to c (medium and high dose). Analysis of both parameters,the sperm count and sperm motility grade, suggested a clearantifertility effectof the *Centella asiatica* treatment onmale rats.

Proteomic analysis

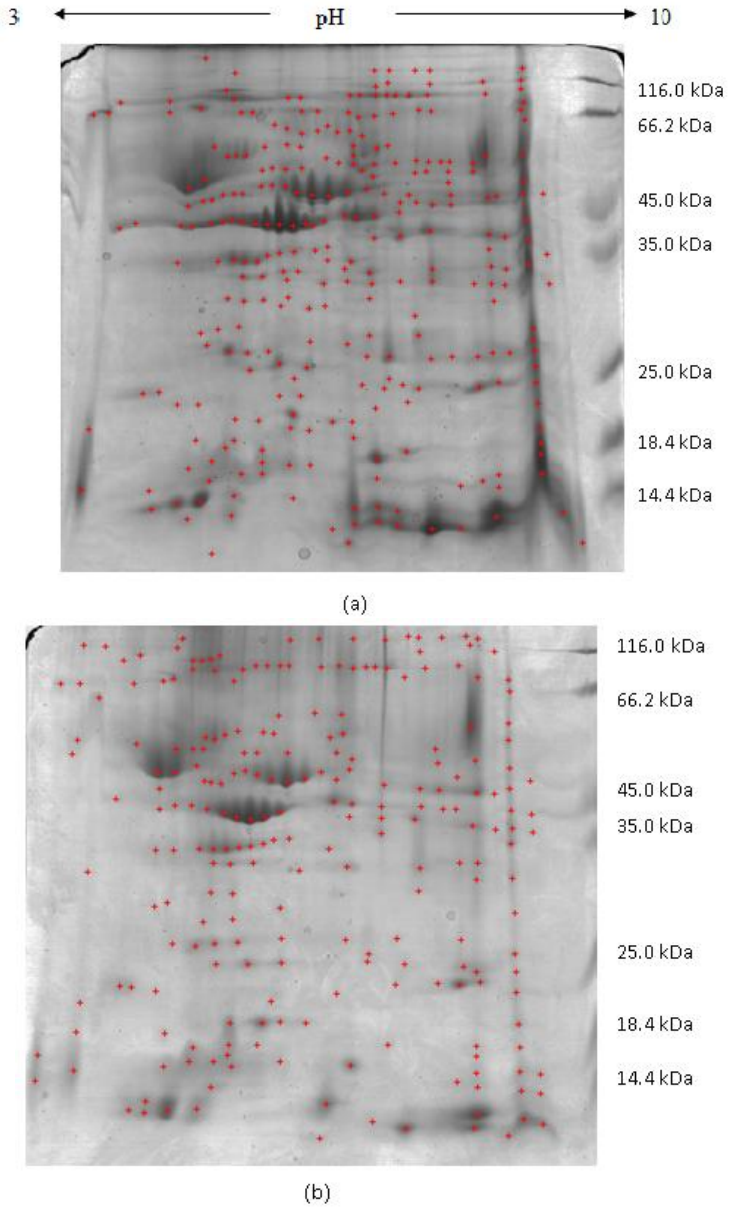

Fig 1. 2D SDS-PAGE gels (12.5%) of rat sperm using IPG strip pH 3-10, CBB stained. (a) control group (b) high dose group (300 mg/kg). Protein marker molecular weight (kDa) as shown on the right side of the gels

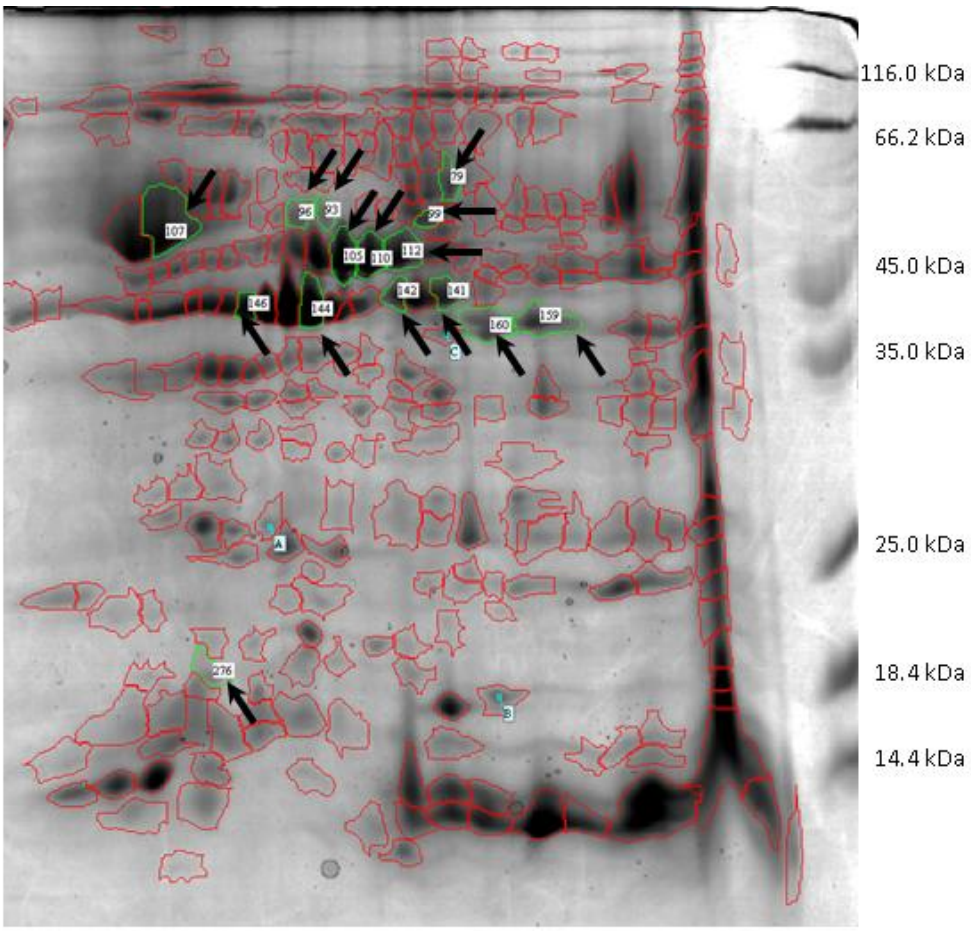

Fig. 2. 2D SDS-PAGE gel of control group showed position of reference spots. Spots appointed was the reference for quantification. Number showed reference identity for each spots identified.

Image analysis results depicted 282 versus 234 protein spots detected from control and treatment (high dose, 300 mg/kg) groups, respectively. Molecular weights (14.4 kDa-116 kDa) were as shown on the right side of the images (fig. 1). Quantification of mean relative volume (%vol) of both groups described 15 protein spots which were most significantly different ($p<.05$) in expression between the treatment as compared to the control group. Each spot was denoted by a reference identity number of protein spot in the control group (fig.2). The graphs shown in the images depicted mean relative volume's value. Nine protein spots were down-regulated (spot numbers: 1, 2, 3, 4, 6, 7, 8, 9 and 10) whereas three spots were up-regulated (spot numbers: 5, 14 and 15). Three protein spots which were present in the control groupdisappeared in the treatment group (spot numbers: 11, 12 and 13) (fig.3).

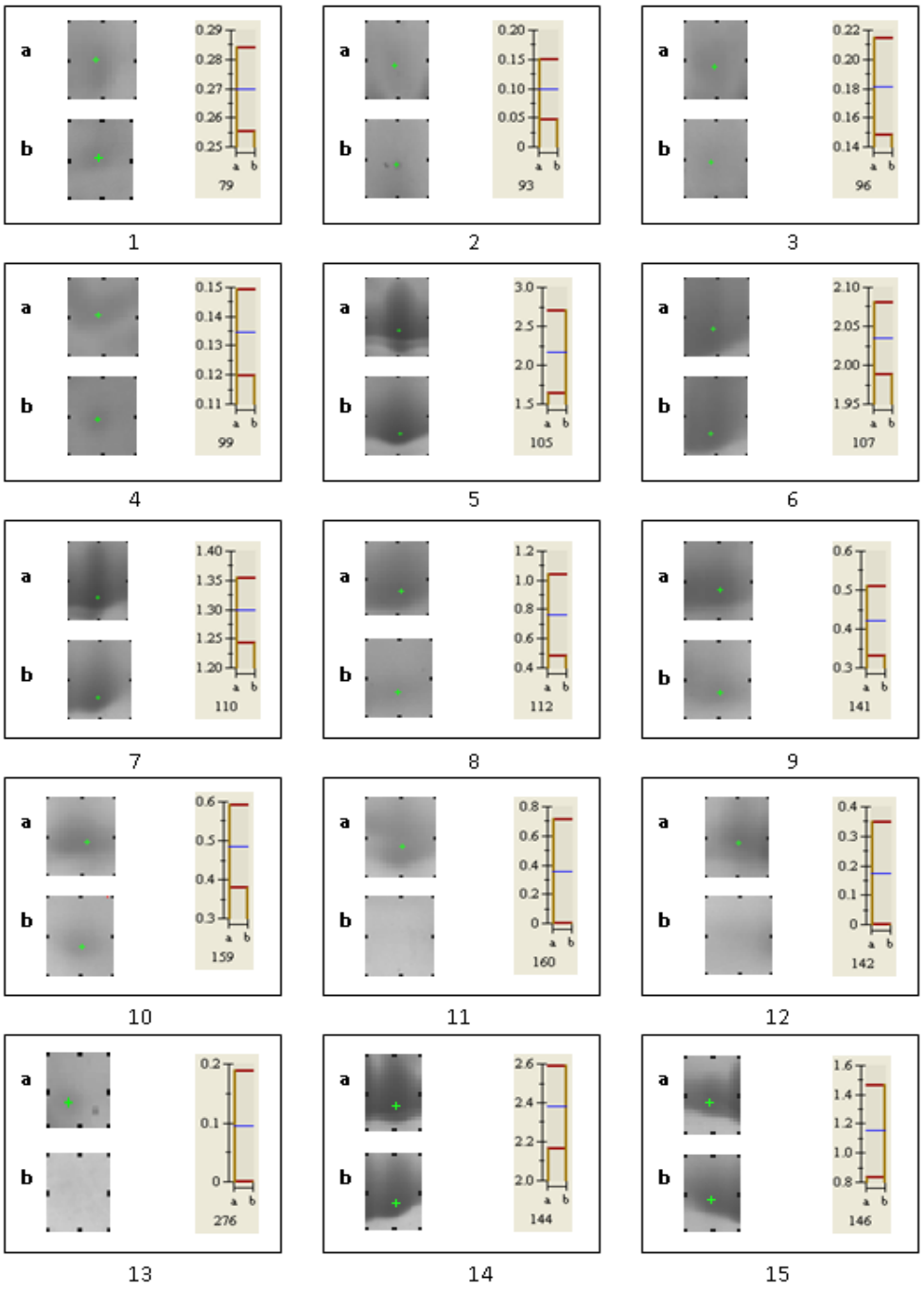

Fig. 3. Analysis result of the spots (a) control (b) treatment group showed different expression based on mean relative volume. The graph showed quantification value for each spots

Mass spectrometric analysis was performed on five protein spots (spot number 5, 10, 11, 12 and 13, correspond to reference identity spot numbers105, 159, 160, 142 and 276) selected based on the highest significance value (fig.3). Those spots met the expressional alteration criteria as reported previously (Kobayashi et al. 2009), i.e. spot number 5 was up-regulated, spot number 10 was down-regulated, whereas spot number 11, 12 and 13 disappeared. The *Probability Molecular Weight Search* (Mowse) result as described by Pappin et al. (1993) through the Mascot search engine (www.matrixscience.com) revealed spot number 5 corresponded to alfa enolase, spot number 10 corresponded to aldolase A,

whereas spot number 11, 12 and 13 corresponded to sorbitol dehydrogenase, glutamine synthetase and lipocalin respectively (table 2).

Table 2. Mascot search engine result

Spots	Score	Protein Match	Mass (Da)	pI value
5	785	Alfa enolase	54346	5.81
10	704	Aldolase A	39923	7.07
11	440	Sorbitol dehidrogenase	38780	7.14
12	321	Glutamin sintetase	41153	6.38
13	356	Lipokalin	20828	5.24

Discussion

This study was designed to investigate the antifertility effect of *Centella asiatica* on the reproductive system of male Sprague-Dawley rats. The *C. asiatica*was chosen for this study because of its medicinal properties and it's wide use by people invarious parts of the world. Results of the present study revealed the significantdecrease in sperm count of the treated groups as comparedto the control group in a dose dependent manner. There werealso a decrease in the sperm motility grade. These findings underline the antifertilityeffect of *Centella asiatica* on the male rat's reproductivesystem.

To further elucidate the antifertility effect of this plant on the sperm quality at a molecular level, proteomic analysiswas implemented. Proteomic technology is an attempt to better comprehend many biological processes in detail, including spermatogenesis. Studies based on proteomics have revealed some proteins which play a significant role in spermatogenesis (Huo et al. 2004; Zhu et al. 2006).

Mass spectrometric analysis was the key to translate the two-dimensional separation result with ahigh level of sensitivity and specificity. This method enabled a complete protein study from the proteome analysis to the structural and protein interactions (Roepstorff 1997). Analysis and identification of the proteins resulting from the Mascot search engine described four proteins belonging to the metabolic enzyme group and one transport protein. The Alfa enolase,aldolase A and sorbitol dehydrogenaseare metabolic enzymes which are involved in glycolitic processes to produce energy. Glutamine synthetase was an enzyme involved in nitrogen metabolism, whereas lipocalin was a transport protein. Two proteins that were closely associated with the spermatogenesis were lipocalin and sorbitol dehydrogenase.

One group of lipocalin that has been structurally identified is Epididymal retinoic acid-binding proteinas reported by Flower et al. (2000). Uniprot protein database (2009) described lipocalin in this study, which is also known as Epididymal-specific lipocalin-5orEpididymalretinoic acid-binding protein. Lipocalin was associated with sperm in the epididymal fluid and had the ability to bind *cis*- and *trans*-retinoic acid. It was concluded that lipocalin is involved in the retinoid carrier needed for epididymal function or sperm maturation. The image analyzed gels showedthat lipocalin expression disappeared in the treatment group. Therefore, it was suggested that epididymal function and sperm maturation did not occur optimally due to *C. asiatica*treatment.

Sorbitol dehydrogenase is a dehydrogenase/reductase enzyme whose function is to convert sorbitol into fructose(El-Kabbani et al. 2004). This process usually occurs in the liver and seminal vesicle. Previous studies (Cao et al. 2009) reported that the energy source which was metabolized into ATP was important for sperm motility. After sorbitol is converted into fructose, through the glycolytic pathway, fructose is then metabolized into ATP. The study suggested that sorbitol was used as an alternative energy source for sperm motility. Sorbitol

dehydrogenase activity was also associated with the maturation of germinal epithelial layer of seminiferous tubule(Pant et al. 1995, 2004).A previous report (Srivastava et al. 1990) showed that seminiferous tubule damage was associated with decreased activity of sorbitol dehydrogenase.

A proteomic study underlined the antifertility effect of *C. asiatica* at a molecular level. Inhibition of lipocalin enzyme due to *C. asiatica* treatment affected epididymal function and sperm maturation. The reduction activity of sorbitol dehydrogenase contributed to the inhibition of spermatogenesis and a decrease of sperm motility grade. It is suggested that lipocalin and sorbitol dehydrogenase could serve as testicular toxicity biomarkers.

Conclusion

Overall, the results of this study suggest that the ethanolic extract of *C. asiatica*isan appropriate contraceptive agent in male rats. The chemical constituents of this plant have the ability to decrease fertility and affect sperm quality. The isolation, identification and characterization of the bioactive compound(s) of *C. asiatica* that exhibited antifertility activity are under further study.

Acknowledgements

This study was supported by a grant from PostgraduatesResearch Fund, Faculty of Science and Technology,Universiti Kebangsaan Malaysia and the Ministry of Science,Technology and Innovation (MOSTI) Malaysia.

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Tapak Liman (*Elephantopus scaber L*) as Imunostimulator and Its Effect on Lymphocyte Differentiation in Mice BALB/C

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ABSTRACT

Tapak liman (*Elephantopus scaber, L*) is one of the plants that have medicinal properties and diverse roles and efforts of maintenance, improvement and restoration of health and disease treatment. The purpose of this study was to determine the effect of extracts of Tapak Liman (*Elephantopus scaber* L) as imuno stimulator to the development of lymphocytes in mice BALB / C The procedure of this study was to test extracts aquades in vivo with various treatments (control, treatment of 0.5 g / kg, 1.0 g/ kg, 2.0 g / kg) in mice BALB / C healthy for 2 weeks.

After the treatment carried out analysis of the percentage and number of cells that express $CD4^+$, $CD8^+$ and $CD4^+$ $CD8^+$ in thymus organ, using flowcytometry. Analysis of data using one-way ANOVA followed Tukey's test with SPSS. From the analysis showed that the extract of Tapak liman at various doses showed no significant effect on the percentage expression of $CD4^+$ $CD8^+$ and $CD4^+$ $CD8^+$ in thymus organs. While the analysis of the number of cells, extracts of Tapak liman show its effect on the number of cells that express $CD4^+$, $CD8^+$ and $CD4^+$ $CD8^+$ in thymus organs. Concentration of 1.0g/kg of mice showed a good effect on the increase in T helper cells ($CD4^+$), cytotoxic T cells ($CD8^+$) and Prothymosit cells ($CD4^+$ $CD8^+$).

Keyword: TapakLiman, Imunostimulator, Lymphocyte

Introduction

Elephantopus scaber Linn. is a small herb, which grows in the wild throughout the tropical regions of the world. The major phytochemical constituents of the plant are elephantopin, triterpenes, stigmasterol, epofriedelinol and lupeol (Rastogi and Mehrotra, 1990; Kritikar and Basu, 1991). The plant has been used in the Indian system of medicine as analgesic, diuretic, astringent and antiemetic. The leaves of the plant were known to be used for bronchitis, small pox and diarrhea and as a brain tonic (Sankar *et al*., 2001). Recently, it has been shown to possess anti-inflammatory and anti tumour activity in animal models (Reico, 1989) and also found to have antibacterial activity against a few standard bacterial strains (Avani and Neeta, 2005).

The genus *Elephantopus* consists of approximately 30 species distributed in the Neotropicsand the Old World, and its lectotype species, *E.scaber*, occurs in all tropical regions (Cabrera and Klein, 1980; Chen, 1985; Cao and But, 1999). InSouthern China, Hong Kong and Taiwan, the whole plant of *E. scaber*, a perennial herb (10D 50 cm in height), is well known as a folk medicine widely used in the treatment of nephritis, edema, dampness, pain in the chest, fever and cough of pneumonia, scabies, and arthralgia due to wounding (Peer and Metzger, 1980; Hsu, 1986; Tsai andLin, 1999). In Brazil, the infusion and the decoction of the whole plant are used to stimulate diuresis,reduce fever, and eliminate bladder stones (Cabrera and Klein, 1980; Poli *et al.*, 1992). It has also been popular as a medicinal herb in many countries of Southeast Asia, Latin America and Africa for a long time (Hammer and Johns, 1993; Cao *et al*., 1997).

Since the 1970's, aSince the 1970's, a number of chemical constituents and pharmacological evaluations of *E. Scaber* have been reported. For example, Kurokawa *et al.* (1970) and Govindachari *et al.* (1972) reported elephantopin, deoxyelephantopin, and isodeoxyelephantopin in this species; De Silva *et al.* (1982) found that both alcohol and chloroform extracts of *E. scaber* contain cytotoxic germacranolide-type sesquiterpene lactones; Poli *et al.* (1992) tested the aqueous and hydroalcoholic extracts of whole plants for acute toxicity, analgesic, antipyretic, anti-inflammatory, cardiovascular, diuretic, and constipating activities; Hammer and Johns (1993) reported that the plant extract of *E. scaber* was subjected to bioassays; Lin *et al.* (1995) and Tsai and Lin (1999) evaluated the hepatoprotective and anti-inflammatory effects of the Taiwanese folk medicine "Teng-Khia-U", derived from three plant species including *E. scaber*, and But *et al.* (1997) described the isolation and structure characterization of three germacranolide sesquiterpene lactones from *E. scaber.*

The aqueous extracts of roots and leaves from *E. scaber* portrayed excellent hypoglycemic effect indiabetic rats by lowering the blood glucose level and serum insulin level. A decrease in the elevated levels of glycosylated hemoglobin, liver glycogen, triglycerides and serum cholesterol in alloxan-induced hyperglycemic rats was also reported by Daisy et al. (2007).

Besides hypoglycemic activity in mice models, aqueous extract of *E. scaber* also showed significant antiinflammatory effect in both experimental acute and chronic arthritis rat models. The aqueous extract from the whole plant significantly inhibited the development of pad swelling in the acute experimental arthritis rats at a dose of 300 mg/kg while higher concentration of the extract(500 mg/kg) was required to inhibit the development of chronic joint swelling in the chronic inflammatory model (Tsai and Lin, 1999).

During thymocyte development, immature thymocytes that express both CD4 and CD8 genes must choose either a helper $CD4^+$ or cytotoxic $CD8^+$ T-cell fate. Over the past two years, there have been some important advances regarding T-cell lineage choice, including the identification of transcription factors required for CD4 gene silencing by CD8-lineage cells (RUNX3) or for $CD4^+$ T-cell differentiation (GATA3), and a better understanding of how T-cell receptor (TCR) signalling correlates CD4/CD8-lineage differentiation to MHC specificity. This review summarizes these recent advances and highlights potential links between TCR signals and nuclear effectors of lineage differentiation (Bosselut R, 2004)

The purpose of this study was to determine the effect of extracts of Tapak Liman (*Elephantopus scaber* L) as imunostimulator to the development of lymphocytes in mice BALB/C.

Materials and Methods

The type this experiment with the design of the post test only control group design. Using the 4 groups, 1 group control and 3 treatment groups, with simple randomization. Assessment is performed only when the post test, comparing the results of observations on the treatment and control groups, and among treatment groups. The samples taken at random (random) of the population reached the inclusion criteria as follows: murinestra in BALB/C female, age 6 weeks, and healthy. 12 mice BALB/C as are divided into 4 groups, each group consists of three mice. Each group of mice is given the same standard food and drink ad libitum.

Four groups of mice are given: Control(K): aquades without extract of Tapak Liman leaf Treatment1 (P1): Tapak Liman leaf extract 0.5 grams/kg body weight/day Treatment2 (P2): Tapak Liman leaf extract 1.0 g/kg body weight/day Treatment3 (P3): Tapak Liman leaf extract 2.0g/kg body weight/day.

The effect ofextractson the development of lymphocytes in thymus can be determined by measuring the percentage and number of lymphocytes expressing the express $CD4^{+,}$ $CD8^{+}$and $CD4^{+}$ $CD8^{+}$ in thymus organ using flowcytometry on the 16th day, after the treatments of Tapak Liman extract at various doses. The data obtained in the form of percentage and number of cells that express the CD will be analyzed statistically by calculating the standard deviation in each treatment and one-way ANOVA analysis to determine the effect of treatment on the development of lymphocytes. If there are real differences in each treatment will be followed by Tukey test to determined ifferences in each treatment. Analysis using SPSS. Significance with a P value<0.05.

Results and Discussion

Thymus is the primary lymphoid organs, bone marrow as a producer of T cell precursors are derivatives of progenitor cells after differentiation in the thymus to form a functional T cells, and after 4 stages of maturation involves a variety of protein expression and T cell receptor (TCR) as an end to the circulation cell T peripherals (Keer, 1998).

Transitional stages of thymocyte maturation can be characterized on phenotip cells with the TCR-CD3 complex, the presence of CD4 and CD8 coreceptor (Kuper et al., 2002).

Based on the results of flowcytometry analysis (Figure 1) Note that the percentage of CD4 + expression on the control has an average value of 13:04%, in the treatment of 0.5 g / kg had a mean value 10.67%, in the treatment of 1.0 g / kg had an average of 9.85%. While on treatment of 2.0 g / kg had an average of 10.81%. Based on the results of statistical analysis using one-way ANOVA (Appendix 8) found that the expression of CD4 +, with a significance value of 0864 (> 0.05). For the expression of CD8 + with a significance value 0676 (> 0.05), whereas for the expression of CD4 + CD8 + has a significance value 0.496 (> 0.05). It could be argued that there is no real influence liman extract of tread on all expressions of concentration versus percentage of CD4 +, CD8 +, CD4 + CD8 + in thymus organs.

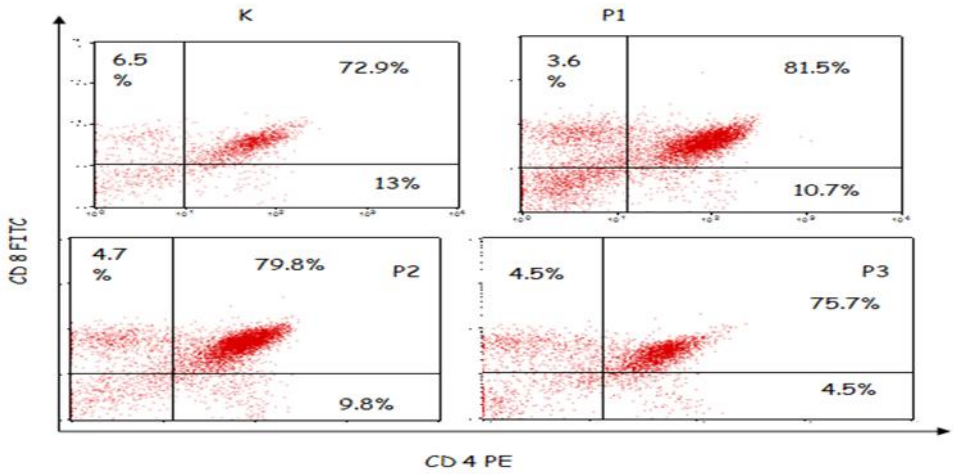

Figure 1. Cell Profile $CD4^{+}$, $CD8^{+}$ and $CD4^{+}$ $CD8^{+}$: Prosentage cell exspression $CD4^{+}$, $CD8^{+}$, $CD4^{+}$ $CD8^{+}$ on thymus. Control (K), (P1:0.5 gr/kgbw), (P2:1.0 gr/kg bw), (P3:2.0 gr/kg bw).

Total cells (Figure. 2) shows that the concentration of 1.0 g/kg increased the number of $CD4^{+}$ cells (1615946) when compared to the control and the concentration of 0.5g/kg (976 610). Concentration of 2.0 g/kg would reduce the number of cells that express $CD4^{+}$ (1438173) but still better than in the control and treatment of 0.5 g/kg. Likewise, the $CD8^{+}$

cells that express at a concentration of 1.0 g/kg caused the number of $CD8^+$cells at high (764 786) when compared to controls, 0.5 g/ kg, 2.0g/ kg. Concentration of 2.0 shows thedecline inthe number of cytotoxic cells but still higher when compared with the concentration of 0.5g/kg and control. So it can be said that the concentration of 2.0g/kg gave the best effect on the number of cytotoxic cells in mouse thymus organs. Total cells that express $CD4^+$ $CD8^+$ (prothymocyte) showed that consentration1.0g/kg showed the best effect when compared with control (7395966) and the treatment of 0.5g/kg (7,455,115), and 2.0 g treatment/kg. Showed that the concentration of 1.0g/kg increased the number of cells prothymocyte.

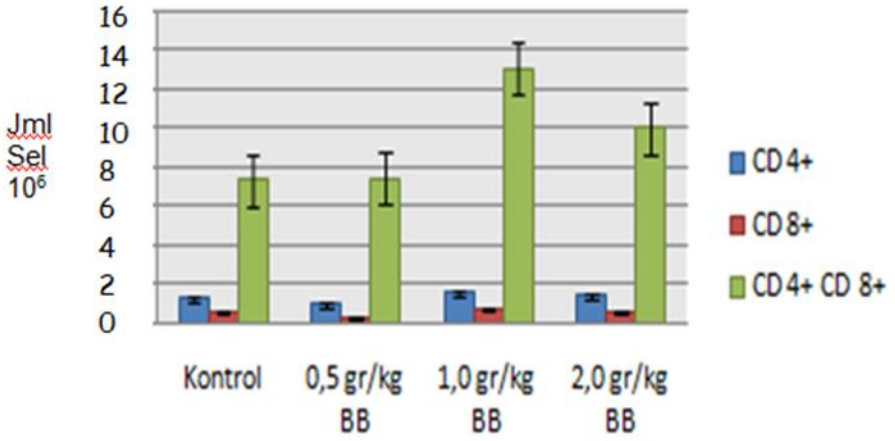

Figure 2. Total cell $CD4^+$, $CD8^+$,$CD4^+CD8^+$ on Thymus at Control, 0.5 gr/kg bw, 1.0 gr/kg bw, and 2.0 gr/kg bw.

Lymphocyte progenitor cells derived from bone marrow, some of which are relatively non-lymphocyte differentiation have migrated to the thymus and reproduce themselves, here is to obtain properties of lymphocytes T lymphocytes At first thymosit not express CD4 and CD8, cells then develop into double positive ($CD4^+$ $CD8^+$) and eventually mature into single positive ($CD4^+$ or $CD8^+$) can then log back into the blood stream, back into the bone marrow or lymphoid organs and peripheral can live several months or years (Schwarz and Bandola, 2008).

Conclusion

Extract of Tapak liman at various doses showed no significant effect on the percentage expression of $CD4^+$ $CD8^+$ and $CD4^+$ $CD8^+$ in thymus organs and $CD4^+$ $CD8^+$ in thymus organs. While the analysis of the number of cells, extracts of Tapak liman show its effect on the number of cells that express $CD4^+$, $CD8^+$ and $CD4^+$ $CD8^+$ in thymus organs. Concentration of 1.0g / kg of mice showed a good effect on the increase in T helper cells ($CD4^+$), cytotoxic T cells ($CD8^+$) and Prothymocyte cells ($CD4^+$ $CD8^+$).

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Sugar Residues and Their Variations of Distribution on Ovarian Follicles of Timor Deer (*Cervus timorensis*)

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Abstract

Timor deer is one of Indonesian tropic deer has not most which investigated in Indonesia on biology of reproduction. The data of sugar residues containing on ovarian follicle of timor deer are important to support application of reproduction technology as effort and to increase timor deer utility as livestock with maximal quality.Ovaries isolated from 2 timor deers were fixed with bouin's and embedded in paraffin wax. Sections 5 µm, were deparaffined, rehydrated and labelled with four different lectins (PHA, RCA, Con A, and LCA). Preantral follicle has founded carbohydrate with D-N-acetylgalactosamine, galactose and mannose sugar residues in cytoplasm and zona pellucida oocyte, granulosa and teca cells. The antral follicle has not founded mannose in oocyte cytoplasm. D-N-acetylgalactosamine, galactose, mannose and glucose sugar residues have founded in zona pellucida, folicular liquid, granulosa and teca cells.

Key words : sugar residues, follicle,Timor deer (Cervus timorensis), lectin

Introduction

Timor deer is one of endemic ruminant. We have four kinds of tropic deer in Indonesia, they are Sambar, Timor, Muncak and Bawean deer. The Timor deer is prospective animal to be developed as source of animal protein low cholesterol with side products that has high economic values such as velvet, skin, tail, and testis (Semiadi and Nughraha 2004).

To develop new source of animal protein by consuming animals that have high potential of meal productivity called as prospective animal, based on ministry of agriculture's decree No.362/KPTS/TN. 12/5/1990 renewed by no. 404/KPTS/OT. 210/6/2002, deer have been officially categorized as livestocks (Semiadi and Nugraha 2004).

Ex situ reproduction of deer by captivity and application of reproduction technology is very important. Artificial insemination, IVF, ICSI are technology of reproduction that have been developed for other ruminants. The data of reproduction biology are important for developing technology of reproduction. The success in captivity and application of reproduction technology must be supported by researches on the tropical deer reproduction biology either in male or female. The research of Timor deer has not been conducted in Indonesia.

The ovary is divided into the outer cortex and central medulla regions. The medulla is composed mainly of blood vessels, lymphatics, connective tissue and nerves. The cortex is in perifer arround of medulla and contains follicles (Ross *et al*. 1995). During oestrus phase, follicles will develop (folliculogenesis) with different structure components.

Sugar residues are found in all follicle development, because they are sources of energy needed for follicle ovarian development. Besides, specific sugar residues on follicle are possible as keys in specific species of gamet interaction, gamet binding, and spermatozoon penetration during fertilization (Skutelsky E, et. al, 1994).

The research on sugar residues and their variations of distribution on ovarian follicle of Timor deer must be conducted to know the component and distribution of sugar residues that play role on follicle development. Therefore, it can be data for future application of reproduction technology.

Material and Method

Materials

In this experiment, ovaries were removed from mature deers (3-4 years old). The ovaries were washed with NaCl fisiologycal solution in 30-35°C temperature. The ovaries were fixed with bouin's solution and embedded in paraffin wax.

Histochemical analysis.

The paraffin wax embedded tissue sections (5 µm thick) were incubated the slide in 60^0C temperature for 30 minutes. So its were deparaffined with xylol and rehydrated with a series alcohol. The slides were washed with PBS and than were transfered to peroxide hydrogen (H_2O_2) 0,3% in PBS for inhibiting endogen peroxidase enzym activity. The slides were washed in running wáter and PBS solution 0,01 M. So It were incubated with peroxidase labeled lectin (Table 1) for 2 hours in 37 ^{0}C temperature. And than were washed with PBS to clean the no binding lectin. Visualisation were be done with 0.05% DAB (diaminobenzidine) and 0.3% H_2O_2 in tris buffer 0.05 M, pH 7.6. Contrast staining in nucleus has be used haematoxylin. Positive reactivity of lectin in the tissue has be showed in brown colour. To know specific reactivity, on all of histochemistry staining prosedure have be used positive and negative controls.

Table 1. Kinds, source, specific sugar and dosis in this research.

Lektin	**source**	**SpecificSugar**	**Dosis (µg/ml)**
PHA	*Phaseolus vulgaris*	GalNAc	5
RCA	*Ricinus communis*	Gal, GalNAc α	5
Con A	*Canavalia ensiformis*	Man α, Glc α	10
LCA	*Lens culinaris*	Man α	5

PHA = Phaseolus Vulgaris Erythroagglutinin, RCA = Ricinus Communis Agglutinin, Con A = Concanavalin A, LCA = Lens culinaris Agglutinin. GalNAc = D-N-Acetylgalactosamine, Gal = Galactose, Man α = mannose, Glc α = glucose.

The research observed the kind of carbohydrates and their distribution on ovarian follicle. The observation on affinity and intensity of lectin positive reaction in ovarian follicle have done at 2 slide for each lectin. The result of affinity and intensity have been clastered into 5 groups. There are strength (+++), medium (++), weak (+), very weak (+/-) and negative/ have not reaction (-). Observation and documentation the result used the camera microscope (Nikon Eclips, Tokyo, Japan).

Results and Discussion

The result of lectin hystochemistry's staining show the distribution of lectin binding with PHA, LCA, RCA dan ConA reaction variated in different follicle regions(Table 2). RCA lectin is specific for carbohydrate with galactose sugar residues. RCA positive reactions occur on all follicle regions with very weak to strong intensity (Picture 1A) and on all follicle development. Those are possible as playing role of galactose on all follicle development (preantral and antral phase). RCA has weak reaction in zona pellucida of antral follicle phase (Picture 2A) but In preantral follicle has positive reaction in medium into strong intensity. In buffalo ovary, zona pellucida's antral follicle have founded galactose residues (Parillo *et al.*

1998). According to Skutelsky *et al.* (1994), β-Galactose, D-N-acetylgalactosamine dan N-acetylglucosamine are common in rodent zona pellucida. Negative reaction of RCA Lectin has recorded in dog zona pellucida (Skutelsky *et al.* 1994). In granulosa cells of antral follicle phase, RCA intensity decreases into medium as the follicle development. Therefore, RCA intencity in follicular fluid increase in last follicle development. Positive reaction in granulosa cells and follicular fluids have recorded in most mammalia such as mouse, rat, hamster, rabbit, cat, dog and pig (Skutelsky *et al.* 1994).

Table 2. Variation and distribution of sugar residues on ovarian follicles of timor deer, *C. timorensis*

Lektin	**Part of follicle**	**Tipe follicle**	
		Preantral	**Antral**
PHA	externa teca	±	+
	interna teca	+	+
	Granulosa cells	±	+++
	follicular fluid	Not formed yet	+++
	Zona pellucida	++	-
	Oocyte cytoplasm	++	++
RCA	externa teca	++	+
	interna teca	++	+
	Granulosa cells	++~+++	++
	follicular fluid	Not formed yet	+++
	Zona pellucida	++~+++	±
	Oocyte cytoplasm	+~+++	+
LCA	externa teca	+	++
	interna teca	+	++
	Granulosa cells	+++	+++
	follicular fluid	Not formed yet	+++
	Zona pellucida	±	-
	Oocyte cytoplasm	+	-
Con A	externa teca	-	±
	interna teca	-	±
	Granulosa cells	-	-
	follicular fluid	Not formed yet	±
	Zona pellucida	±	-
	Oocyte cytoplasm	-	±

- = negative, ± = very weak, + = weak, ++ = medium, +++ = strong

PHA lectin binding indicate carbohydrate present with D-N-acetylgalactosamine residue. The results show that PHA lectin react positively with very weak into weak intensity in teca cells of preantral (picture 1B) and antral follicles. Increased intensity from very weak into strong occur in granulosa cells of last antral follicle development. Strong intensity occur in follicular fluids region too. The results so far show that carbohydrate with D-N-acetylgalactosamine residue probable as play the role on granulosa cells development and follicular fluids as granulosa cells secrete, in antral follicle phase. Medium reaction have been showed in zona pellucida and oocyte cytoplasm (picture 2 B). The carbohydrate with D-N-acetylgalactosamine residue has founded on all development phases. The carbohydrate with Beta-N-acetylgalactosamine residue have founded in early antral follicle development zona pellucida of buffalo ovary. (Parillo *et al.* 1998).

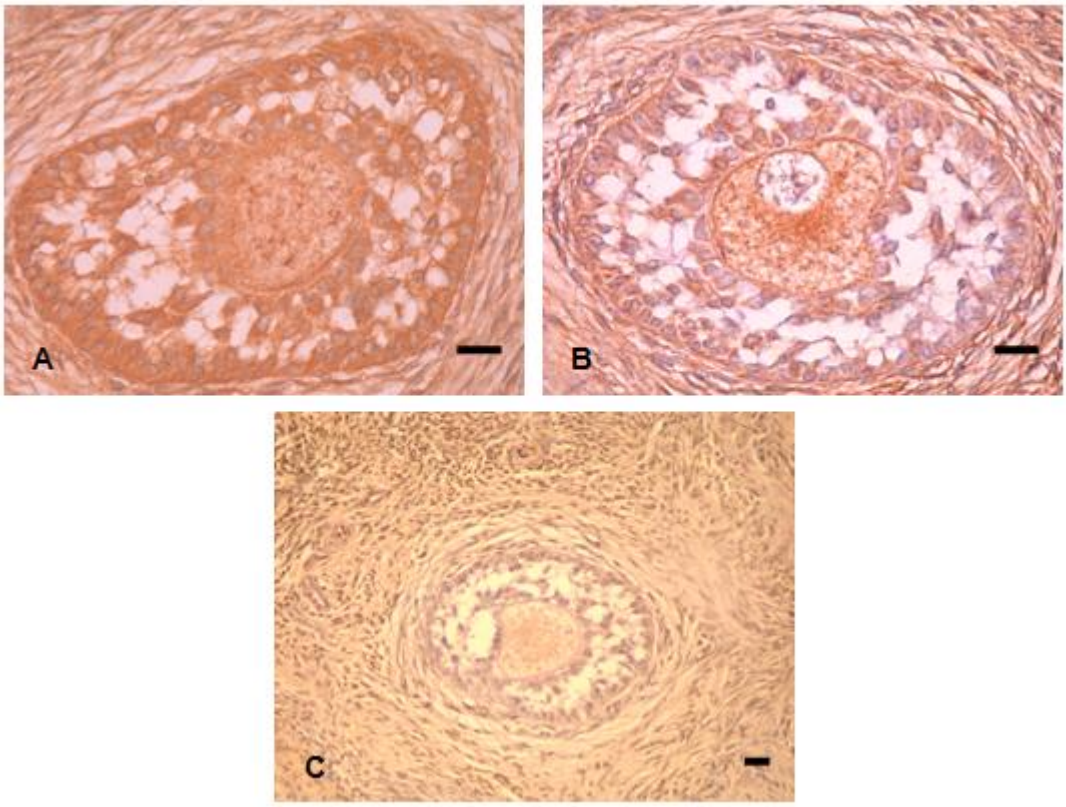
LCA lectin that indicates the carbohydrate with mannose residue show positive reaction in weak into medium intensity on interna and externa teca cells, (Picture 1C). Strong positive reaction have been seen in granulosa cells and follicular fluid. But positive reaction of LCA lectin with very weak intensity occur in zona pellucida and negative into very weak intensity in cytoplasm (Picture 2 C).

Mannose have not founded on antral follicle oocyte (zona pellucida and cytoplasm) as has recorded in cat follicle (Skutelsky *et al*. 1994) but have detected in all regions of preantral follicles (Picture 1 C). The results show that many mannose residues have founded in granulosa cells and follicular fluids. This results similar to recording in other mammal (Skutelsky *et al.*1994) and in early antral follicle of buffalo (Parillo *et al.* 1998).

Con A is the lectin detected carbohydrate with mannose and glucose residues in tissues. This results showed that have not founded mannose and glucose residues in some regions of Timor deer ovarían follicles as granulosa cells and zona pellucida of antral follicle (Picture 2 D). While this sugar residu have founded on all follicle development phase of mouse deer (Hamny *et.al.* 2008). No presented of mannose and glucose residues have also recorded in cat, dog and pig zona pellucida (Skutelsky *et al.* 1994).

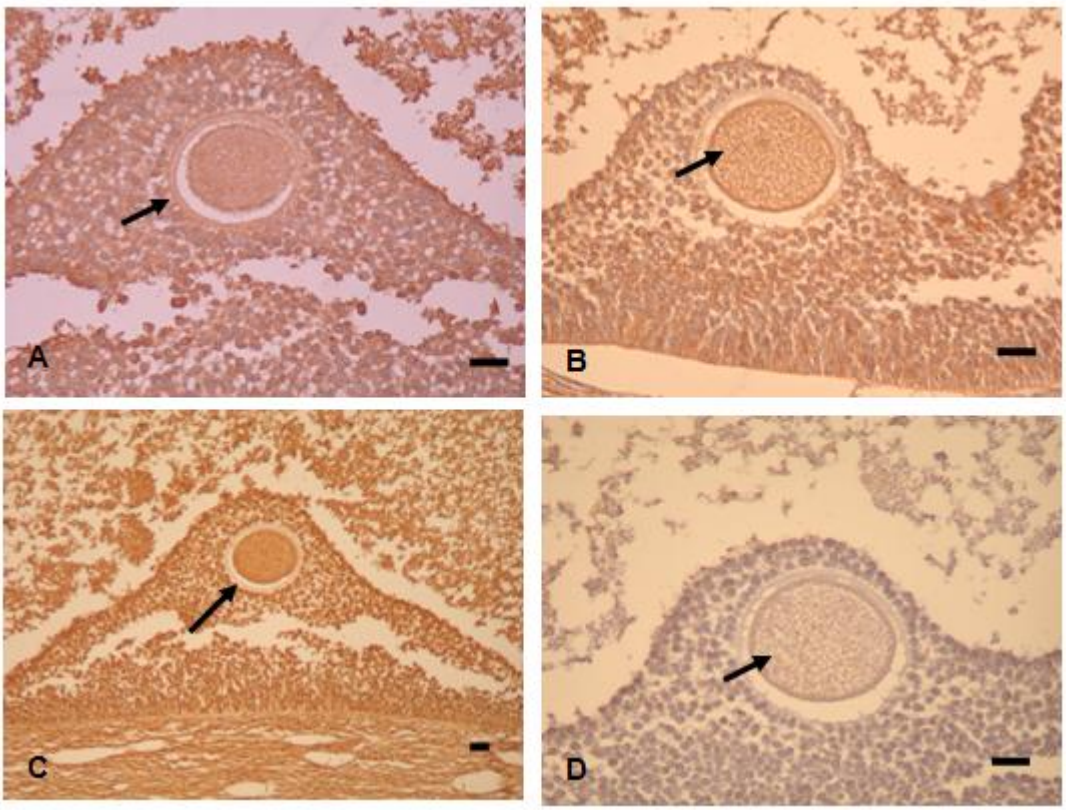
In lutein cells, PHA, LCA and RCA lectin show weak reaction, and negative reaction in Con A lectin. The positive reaction show that lutein cells contain carbohydrate with mannose, galactose dan D-N-acetylgalactosamine residues. In this research also have observed that interstitial cells arrounded follicle in ovarium contain D-N-acetylgalactosamine, galactose, mannose and any glucose residues.

Skutelsky *et al*. (1994) recorded that oocyte zona pellucida from different mammal species either show different lectin binding patterns. Sugar residues in zona pellucida are possible to play the role as spermatozoon reseptor. Carbohydrate with α-galactosyl plays the role on rat zona pellucida but in mouse, L-fucose, D-mannose and methyl mannosida have partisipated in spermatozoon binding. But not in hamster, rabbit and human; that partisipated are L-fucose and D-galactose. Antral follicle zona pellucida of Timor deer ovary have positive reaction of RCA lectin. This result demostrated that carbohydrate with galactose residues possible as play role in fertilization and in species specific of Timor deer oocyte zona pellucida.



Picture 1 Distribution lectin binding (brown) in preantral follicle. A. positive binding of RCA Lectin on all regions of follicle except on granulosa cells. B. positive binding of PHA Lectin with medium intencity on zona pellucida and oocyte cytoplasm, and weak reaction on granulosa cell matrix and teca cell. C. Positive binding of Lectin LCA on all regions of follicle. DAB, Bar 20 µm.

In follicular fluid of Timor deer have been founded any RCA lectin reaction that demostrated presented of glucose but have been founded many D-N-acetylgalactosamine, galactose dan mannose residues. Hafez and Hafez (2000) said that component and metabolite of follicular fluid were glucose, fructose, fucose, galactose, mannose, glucosamine, galactosamine, hyaluronat acid, heparin and plasminogen. There were assumted that component in follicular fluid will be different in other species. Follicular fluid of buffalo ovarian follicle contains glicoconjugate with beta-N-acetylgalactosamine, beta-galactose-(1-3)-N-acetylgalactosamine, beta-galactose-(1-4)-N-acetylglucosamine, N-acetylglucosamine, alfa-fucose, alfa-glucose, alfa-mannose and cyalic acid (Parillo *et al.* 1998).



Picture 2. Distribution of lectin binding on last phase of antral follicle. A. very weak positive reaction of RCA Lectin on zona pellucida (arrow). B. positive reaction of Lectin PHA with medium intencity on oocyte cytoplasm (arrow) and granulosa cell matrix. C. negative reaction of lectin LCA on zona pellucida (arrow). D. very weak positive reaction of lectin Con A on oocyte citoplasma (arrow). DAB, Bar 20 µm.

In oocyte cytoplasm of preantral follicle showed positive reaction of lectins that indicated present of carbohydrate with D-N-acetylgalactosamine, galactose, mannose dan glucose residues. But in antral follicle oocyte cytoplasm, mannose residues have not founded. This component dynamica indicate development proccess in oocyte cytoplasm.

CONCLUSION

1. Ovarian follicles of Timor deer contain carbohydrate with sugar residues D-N-acetylgalactosamine, galactose, mannose and glucose with intencity and variation of distribution on follicle development.
2. In early development (preantral follicle), oocyte cytoplasma and zona pellucida, granullosa cells and teca cells contain D-N-acetylgalactosamine, galactose and mannose.
3. The antral follicle has not founded mannose sugar residu on oocyte citoplasma and zona pellucida.
4. In antral follicle fase, oocyte citoplasma contain D-N-acetylgalactosamine dan galactose. zona pellucida contains low galactose. Carbohydrate with sugar residues D-N-acetylgalactosamine, galactose, mannose dan glucose have be founded on follicular fluids, granulosa and teca cells with different intencity.

ACKNOWLEDGEMENT

Thank you to Srihadi Agungpriyono, Ph.D for his lectins and DR. Marlen and the team for material in this research.

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Cholesterol Levels, High-Density Lipopolysaccharide and Triglyceride of Civet (*Paradoxurus hermaphroditus*)

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Abstract

Civet (*Paradoxurus hermaphrodites)* is wild animals included in the list of almost extinct animals. Various chemical indicators that are important in determining the health status of that animals has not been available until now. This study aims to determine the levels of cholesterol, High-Density Lipopolysaccharide (HDL) and triglyceride on the 3 health of Civet (Paradoxurus hermaphroditus). Changes in plasma concentrations of cholesterol, HDL and triglyceride underlying same physiological states were monitored. The results shown that the mean values of cholesterol levels at 181.21 ± 44.50 mg /dl, while Triglyceride levels at 55.38 ± 5.65 mg/dl and HDL levels at 47.73 ± 16.66 mg/dl. The values of these biochemical apparently healthy animal provide valuable baseline information for use in medical further studies performed with this species.

Key words: Cholesterol, Paradoxurus hermaphroditus, High-Density Lipopolysaccharide, Triglyceride

Introduction

Civet is a rare, nearly to extinct animal. For the purposes of conservation, to date there is not much literature is available related to blood biochemistry of this species to determine the animal health status. In addition to assess the metabolic condition of animals, hematological and biochemical parameters could be affected by many factors including: sex, age, reproductive status and seasonal variations (Al-Eissa et al., 2008; Çetin et al., 2009). This study specifically examines the form of metabolites in blood cholesterol, HDL and triglyceride. Other animal studies indicate the presence of variations in blood chemistry including cholesterol, triglyceride, and HDL as the goats by Widiyono and Sarmin (2011) and Mitruka and Rawnsley (1981), cattle, sheep, pig, and horse (Kraft and Duerr, 1999). This study aims to assess the preview of cholesterol, HDL, and triglycerides as beginning and basic information on blood chemistry for civet.

Materials and Method

A total of three healthy civets with 2-4 months old are used in this study. Civet adapted for two weeks in a cage and get food freely. Blood was collected through the femoral vein, immediately separated for cholesterol, HDL and triglyceride analysis. Chemical analyses were performed using spectrophotometer and standard methods described by Kraft and Duerr (1999). The data were given as mean and standard deviation.

Results

The mean and standard deviation of cholesterol, triglyceride, and HDL is shown on table 1.

ISSN: 2088-9771

Table 1. Comparison of Choleserol, HDL, and Triglyceride (Mean±SD) in Civet (our study), Adult (5 years) Caspian miniature horses, German Improved fawn goats, West African Dwarf (WAD) goats, Holstein heifer, Spanish ibex from Andalusia, Wild-dusky-footed wood rats, Wild grasscutters, *Ettawa crossbred and* Anngora rabit

Parameter	Civet (Our study)	Adult (5 years) Caspian miniature horses (Nazifi et al., 2005)	German improved fawn Goats (Žubčić, 2001)	West African Dwarf (WAD) goats (Opara et al., 2010)	Domesticated Rabbits (Al-Eissa, 2011)	Holstein heifer (Rasoli et al., 2004)	Spanish ibex from Andalusia (Pe´ rez et al., 2003)	Wild-dusky-footed wood rats (*Neotoma fuscipes) (Weber et al., 2002*	Wild grasscutters (Opara et al., 2006)	*Ettawa crossbred*	Anngora rabit (*Çetin et al., 2006)*
Cholesterol (mg/dL)	181.21±44.5	91.26±5.94	3.06±1.44	31.32±1.44	127.66±5.13 (non pregnan) 54.85±0.51 pregnan rabbits	1.82±0.06 (Summer) 2.53±0.06	53.06±21..8	127±39..90	195.65±5.49	82.47±27.26	-
HDL	47.73±16.66	25.02±2.34									
Triglyceride	55.38±5.65	5.04±1.44		47.4±4.5			37.1±37.8			35.35±16.41	158.76 ± 5.74

Discussion

This study is a preliminary study on civet related to levels of cholesterol, HDL and Triglyceride. There is little information concerning blood lipid in civet. Cholesterol concentrations in civet are 181.21(69.56-150.00) mg/dL. This concentration is higher than the concentration of cholesterol in Angora Rabbits (Çetin et al., 2009), Domestic Rabbit (AL-Eissa, 2011), West African Dwarf (WAD) goats (Opara et al., 2010), adult male and female Caspian miniature horses (Nazifi et al., 2005), mountain reedbucks (Reduncula fulvorufula) (Vahala et al., 1991), and cattle of Holstein heifer (Rasooli et al., 2004) , German improved fawn Goats (Žubčić, 2001, Spanish ibex from Andalusia (Perez et al., 2003) , and Wild-dusky-footed wood rats (*Neotoma fuscipes)* (Weber et al., 2002). However, this concentration is lower than the concentration of cholesterol in wild grasscutters (Opara et al., 2006) and similar to the value reported for adult Ottawa crossbred (Widiyono and Sarmin, 2011). It is believed that race and activities affecting the serum cholesterol levels (Guyton, 1986; Silva and Danggola, 2002).

Triglyceride concentration in the civet is 55.38+5.65 (31.58-42.85) mg/dL below the level triglyceride of female of Angora Rabbits (Çetin et al., 2009), however, higher than the values reported for adult male and female Caspian miniature horses (Nazifi et al., 2005), horses and ponies (Bauer et al., 1990), Turcoman horses (Nazifi et al., 2003), West African Dwarf (WAD) goats (Opara et al., 2010), Spanish ibex from Andalusia (Perez et al., 2003), adult Ettawa crossbred (Widiyono and Sarmin, 2011) and Domesticated and wild Asian elephant in Sri Lanka (Silva and Danggola, 2002). The difference of triglyceride and cholesterol concentration influenced by factors of species also in our opinion this is due to the circumstances of feeding conditions (Žubčić, 2001).

The concentration of lipoproteins in the HDL concentration of Civet include at level 47.73 mg / dL with a range of 15.54-48.84 mg / dL, higher than the values reported for adult male and female Caspian miniature horses (Nazifi et al., 2005). The high HDL is similar to the phenomenon known in cats that have the highest percentage of HDL of the domestic species characterized to date. Cattle have approximately equal HDL and LDL cholesterol concentrations. In sheep and horses, the majority of cholesterol circulates as HDL. In pigs, more than half of the total cholesterol circulates as LDL and VLDL combined (LATIMER et al., 2003). These species differences are due to nutritional and metabolic differences. The existence of differences in levels of cholesterol, triglycerides are due to differences of race animals, environmental temperature, sex, and hormonal changes (Al-Eissha and Alhomida, 1997), and feeding (Abrams, 1980, Opara et al., 2006). In conclusion, there is a specific preview of cholesterol, triglyceride, and HDL for Civet that can help when interpreting laboratory results in civet.

Acknowledgement

Thanks to Drh. Dwi Liliek Kusindarta, MP., Ph.D and Drh. Woro Danur Wendo for the cooperation to provide the animal for the test. My gratitude also goes to Lusi Jayatingrum, and Apriani for the co-operation in the research.

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Preview Kidney Function in Civet (Paradoxurus hermaphroditus): Especially Preview of Urea Nitrogen and Creatinin

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Abstract

Civet is a rare, nearly to extinct animal. Urea and creatinine concentrations in serum are commonly measured as indicators of renal function in clinical settings and as indices of nutritional status in animal but this reference values for the civet are not yet established. This study aimed to assess renal function seen an picture of urea nitrogen, and creatinine at 3 healthy of Civet (Paradoxurus hermaphroditus). The mean values of urea nitrogen were 8.42 ± 1.54 mg /dl while creatinine were 3.16 ±1.65 mg/dl. These results contribute to the picture of kidney function in Hermaphroditus Paradoxurus species as a preliminary determination of the health status of these animals.

Key words: Urea Nitrogen, Creatinine, Paradoxurus hermaphroditus

Introduction

Laboratory studies to evaluate function of Civet organ are more informative when results are analyzed in the context of normal values. Nutrition, age, sex, genetics, stress and transportation are all known to affect biochemical parameters observed between tropical and temperate animals (Ogunsanmi et al, 1994). Urea and creatinine concentrations in serum are commonly measured as indicators of renal function in clinical settings and as indices of nutritional status in animal. Creatinine is produced during muscle catabolism and blood levels are generally proportional to an animal's muscle mass, while urea is the end product of nitrogen metabolism and more closely reflects protein intake (Saltz and White 1991). Urinary creatinine can be used as an index of concentration when comparing urine samples because it is freely filtered at the glomerulus and excretion is relatively constant. But, even so up until this time, the operation sketch of civet's kidney as one of the wild animal seen from the urea nitrogen and creatinin projection has not been revealed much. The aim of the current study was to determine of urea nitrogen and creatinine values on these parameters in civet.

Materials and Method

Blood was collected from three healthy civet with 2-4 months old adapted for two weeks in a cage and get food freely through the femoral vein. Sample of blood immediately separated for urea nitrogen and creatinin by standard methods as described by Kraft and Duerr (1999) and Tedesco et al., (1991). The data were given as mean and standard deviation.

Results

The mean and standard deviation of urea nitrogen and creatinin is shown on table 1.

ISSN: 2088-9771

Parameters	Civet (our study)	Wild grasscutters (Opara et al., 2006)	Captive reared grasscutters (Ogunsanmi et al, 2002, n=10)	African Giant Rat (Oyewale et al, 1998)	Pangolin (Oyewale et al, 1998)	Nigerian goats (Oduye & Adedovoh, 1976) (n)	White Fulani cattle (Oduye & Fasanmi, 1971)	Human (Mcfarlene et al, 1970)	Holstein heifer (Rasooli et al., 2004)	West African Dwarf (WAD) goats (Opara et al., 2010)	Spanish ibex from Andalusia (Perez et al., 2003)	wild-dusky-footed wood rats (*Neotoma fuscipes) (Weber et al., 2002)*
Urea (mg/dl)	8.42 ± 1.54	21.87±2.84	27.00±6.86	11.71±2.3 (14)	16.40±3.89 (10)	44.07±10.81 (70)	ND	20.0±5.10	1.84±0.04 (Summer 1.65±0.04 (Winter)	37.9 ± 1.7	44.46 ±15.5	20.6±6.21
Creatinine (mg/dl)	3.16 ± 1.65	1.2±0.14	1.29±0.15	0.59±0.10 (14)	0.75±0.11(10)	ND	ND	ND		0.7 ± 0.04	1.7±0.7	0.3±0.12

Parameters	Civet (our study)	Wild grasscutters (Opara et al., 2006)	Captive reared grasscutters (Ogunsanmi et al, 2002, n=10)	African Giant Rat (Oyewale et al, 1998)	Pangolin (Oyewale et al, 1998)	Nigerian goats (Oduye & Adedovoh, 1976) (n)	White Fulani cattle (Oduye & Fasanmi, 1971)	Human (Mcfarlene et al, 1970)	Holstein heifer (Rasooli et al., 2004)	West African Dwarf (WAD) goats (Opara et al., 2010)	Spanish ibex from Andalusia (Perez et al., 2003)	wild-dusky-footed wood rats (*Neotoma fuscipes*) (*Weber et al., 2002*)
Urea (mg/dl)	8.42 ± 1.54	21.87±2.84	27.00±6.86	1.7 ± 2.3 (14)	16.40±3.89 (10)	44.07±10.81 (70)	ND	20.05 .10	1.84±0.04 (Summer 1.65±0.04 (Winter)	37.9 ± 1.7	44.46 ±15.5	20.6±6.21
Creatinine (mg/dl)	3.16 ± 1.65	1.2±0.14	1.29±0.15	0.5 9 ± 0.1 0 (14)	0.75±0.11(10)	ND	ND	ND		0.7 ± 0.04	1.7±0.7	0.3±0.12

Table 1. Comparison of urea nitrogen and creatinin (Mean±SD) in Civet (our study), Wild grasscutters, Captive reared grasscutters, African Giant Rat, Pangolin, Nigerian goats, White Fulani cattle, Human, Holstein heifer, West African Dwarf (WAD) goats, Spanish ibex from Andalusia, Wild-dusky-footed wood rats (*Neotoma fuscipes)*

ND =NOT DETERMINED

Discussion

The Urea Nitrogen's concentrate is on 8.42 ± 1.54 mg /dL lower than urea nitrogen concentrate in Wild grasscutters (Opara et al., 2006), Captive reared grasscutters (Ogunsanmi et al, 2002), Captive reared grasscutters (Ogunsanmi et al, 2002), African Giant Rat (Oyewale et al, 1998) , Pangolin (Oyewale et al, 1998), Nigerian goats (Oduye & Adedovoh, 1976), Human (Mcfarlene et al, 1970), West African Dwarf (WAD) goats (Opara et al., 2010; Ikhimioya and Imasuen, 2007), Girgentana goat (Piccione et al., 2010), mountain reedbucks (Reduncula fulvorufula) (Vahala et al., 1991), and cape hunting dogs (Vahala et al., 1990). But, the concentration is still higher than the urea nitrogen's percentage in Holstein heifer (Rasooli et al., 2004). The differences of urea nitrogen in many species of animal happen because the different of loss of extracellular fluid, the difference respond towards stress because of the heat and the food (Rasooli et al., 2004), moreover, because the different condition of excessive tissues protein catabolism associated with protein deficiency (Oduye and Adadevoh, 1976). At the same time, the concentration of creatinine, were 3.16 ± 1.65 mg/dl higher than Wild grasscutters (Opara et al., 2006), Captive reared grasscutters (Ogunsanmi et al, 2002), African Giant Rat (Oyewale et al, 1998a), Pangolin (Oyewale et al, 1998), West African Dwarf (WAD) goats (Opara et al., 2010) and Girgentana goat (Piccione et al., 2010), but still lower than mountain reedbucks (Reduncula fulvorufula) (Vahala et al., 1991), wild grasscutters (Opara et al., 2006) and cape hunting dogs (Vahala et al., 1990). In West African Dwarf (WAD) goats, urea and creatinine levels are not influenced by diet. This result is also showing the difference between species in regard to catabolism of protein's cell (Oduye and Adadevoh, 1976), activity of glukoneogensis so the level of urea in wide variety (Radostits *et al*. (1994) and variation of dietary protein (Tedesco et al., 1991). In conclusion, the value of urea nitrogen and creatinin for Civet is may help when interpreting laboratory of renal function in civet.

Acknowledgement

Thanks to Drh. Dwi Liliek Kusindarta, MP., Ph.D and drh. Woro Danur Wendo for the co-operation in providing the animal for the test. My gratitude also goes to Lusi Jayatingrum, Apriani for the co-operation in the research.

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Feeding Ecology of Mentawai langur (*Presbytis potenziani*) in Siberut, Mentawai Islands.

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Abstract

Mentawai langur, *Presbytis potenziani* are endemic to the Mentawai Islands. The species is categorized as an endangered primate. Loss of suitable forest habitat, through commercial logging and conversion of land to agriculture, and traditional hunting system are the main factor threatening populations of the species in all islands of Mentawai. Despite the urgent need for scientifically based conservation measures, there has been little documentation of their basic information on the ecology and behaviour of the species. Present study, we assessed feeding ecology of the species based on activity budgets, diet (food species, food preferences and food items) and niche breadth using focal animal sampling method. The study was carried out in Peleonan forest, the Mentawai island of Siberut from January 2008 through June 2009 and resulted in more than 2000 contact hours on one group (8 individuals). Results show that activity budget of *P. potenziani* was dominated by resting (45.7 events/ hour) and feeding (31.8 events/ hour). Meanwhile travelling, foraging, other activity and social behaviour were 6.2, 4.4, 1.3 and 0.6 events/ hour respectively. Regarding diet, *P. potenziani* used 118 food species in total, however, based on the top ten food species, accounting almost 50% of their overall diets. Average values on food preferences showed that the species mainly fed on fruit, (17.3 events/hour), followed by leaves, flowers and other items (10.8, 1.6 and 1.5 events/ hour respectively). Detailed analysis on food items (certain parts of different plant species eaten) revealed 256 food items in total were eaten by *P. potenziani* with different feeding proportion. Calculated niche breadths (Levin's index) of the species revealed a value of 0.22.

Keywords: feeding ecology, activity budget, diet, *Presbytis potenziani*, Mentawai islands

Introduction

Mentawai Langur (*Presbytis potenziani*) are endemic to the Mentawai Islands, an islands that situated about 100 km off the west cost of Sumatra. According to geological data, Mentawai has been separated from Sundaland since the Mid-Pleistocene, about 500.000 years ago (Verstappen, 1975). Due to its geographic isolation, Mentawai has undergone an evolutionary process that has resulted at least five primate species: the Kloss´s gibbon (*Hylobates klossii*), two species of macaques (*M. pagensis* on Sipora, North- and South Pagai, and *M. siberu* on Siberu), two species of langurs, the Pig-tailed langur (*Simias concolor)* and the Mentawai langur (*Presbytis potenziani*), which live sympatrically across the entire area of the archipelago. Among these, the Mentawai langur have been a subject of very little studied and particularly on their ecology and behaviour (Tilson and Tenaza, 1976; Watanabe, 1981; Fuentes, 1996; Sangchantr, 2004)

According to IUCN (2011), the Mentawai langur is considered to be Endangered (EN A2cd). Loss of suitable forest habitat, through commercial logging and conversion of land to

agriculture, and traditional hunting system are the main factor threatening populations of the species in all islands of Mentawai.

Despite the urgent need for scientifically based conservation measures, there has been little documentation of their basic information on the ecology and behaviour of the species. Present study, I assessed feeding ecology of the species based on activity budgets, diet (food species, food preferences and food items) and niche breadth based on Levin Index.

Materials and Methods

Study site

We conducted the study in the Peleonan forest, located at 0° 58' and 1° 03' S (latitude) and 98° 48' and 98° 51' E (longitude) in North Siberut, Mentawai, West Sumatra. The forest encompasses about 5,000ha. The area is confined by coastal forest in the North and the Tateiku river in the South, while the western and eastern borders are formed by the Peleonan and the Sigep rivers, respectively.

The Peleonan forest consists partly of peat swamp forest, making up the northernmost part, as well as of mixed forest representing the much larger part towards the South. The study area, located in the center of this mixed forest, is comprised of approximately 70 tree genera from 35 families, with Euphorbiaceae, Myrtaceae, Lauraceae and Moraceae being the dominant taxa. The forest structure consists of more than 50% trees with breast height diameters between 21-40 cm, and 50% of trees 6-15 m high and 35% 16-30 m in height (Hadi *et al*, 2009).

The climate at the study site is typically equatorial with high rates of precipitation throughout the year. The monthly rainfall recorded from January through September 2008 ranged from 120 – 568 mm, with fluctuation between months of low and high rates of precipitation being unpredictable. Temperatures recorded between March and December 2007, ranged between 20.6 °C and 33.6 °C (mean 25.6 °C) with a mean relative humidity of 89.4%.

Study animals

I used a group of *P. potenziani* consisted of seven individuals, including one adult male and three adult females. The number of group members increased to eight after a birth in 2008. For conducting focal animal sampling I used five individuals in total (one adult male, two adult females, one sub adult male and one sub adult female). All the individuals were fully habituated.

Data collection and analysis

I collected data daily between 06.00 am and 18.00 pm from January 2008 to June 2009 during more than 2000 contact hours. In total, we compiled 722 focal animal sampling protocols for *P. potenziani* (based on 5 individuals). Each focal animal protocol was composed of 20 second sampling intervals, covering a total length of 30 min, resulting in 90 data points (or events) per sample.

To gain representative behavioural data, we generated 12 focal animal sampling protocols per individual, per month, each representing a different hour of the daylight period (06:00-18:00). The behavioural data collected include six activity categories: resting, feeding, foraging, travelling, social behaviour and other (such as solitary play, autogrooming, agonistic and sexual behaviour). I recorded the food species and the parts of these species consumed, distinguishing leaves, flowers, fruit and others (bark, fungi, lichen, animal matter). For further identification of plants used by primates, we collected herbarium samples, which we sent to the Herbarium of Andalas University, Padang and to the Herbarium Bogoriense, LIPI Bogor, Indonesia.

I used the standardized Levin's index to calculate the values for niche breadth, applied to the proportions of food items consumed:

$$B = \frac{1}{\Sigma p_i^2}$$

where B is Levin's niche breadth and p_i is the proportion of a single resource category (food item) in relation to the overall consumption. Subsequently we calculated the standardized Levin's niche breadth (B_{sta}) by computing:

$$B_{sta} = \frac{B-1}{B_{max}-1}$$

where B is Levin's niche breadth and B_{max} is the total number of food items recognized. The standardized Levin's index varies between 0 (minimal niche breadth) and 1 (maximal niche breadth) (Levins, 1968; Colwell & Futuyma, 1971).

Results

Overall activity budgets for the species devoted the majority of their average daily time budget to resting (45.7 events/hour) and feeding (31.8 events/hour). Meanwhile travelling, foraging, other activity and social behaviour were 6.2, 4.4, 1.3 and 0.6 events/ hour respectively.

Regarding diet, *P. potenziani* used 118 food species in total, however, based on the top ten food species, accounting almost 50% of their overall diets. Average values on food preferences showed that the species mainly fed on fruit, (17.3 events/hour), followed by leaves, flowers and other items (10.8, 1.6 and 1.5 events/ hour respectively).

Detailed analysis on food items (certain parts of different plant species eaten) revealed 176 food items in total were eaten by *P. potenziani* with different feeding proportion. Calculated niche breadths (Levin's index) of the species revealed a value of 0.22.

Discussion

Activity budgets for *P. potenziani*, infact, follow patterns observed in other leaf eating monkeys in Africa [e.g. *Colobus guereza*, *C. satanas* and *Procolobus badius* (Rowe, 1996; Struhsaker & Oates, 1975)] and in other Asian colobines [such as *Presbytis comata* (Ruhiyat, 1983), *P. thomasi* (Gurmaya, 1994), *Trachypithecus cristatus* (Brotoisworo and Dirgayusa, 1991), *T. leucocephalus* (Li & Roger, 2004), *T. pileatus* (Islam & Husain, 1982) and *Nasalis larvatus* (Matsuda *et al*, 2009)], in which only small proportions of time are spent on social behavior, but more than 80% of mean daily activity is devoted to resting and feeding. This activity pattern is typical for colobines that consume diets largely based on leaves, seeds and unripe fruits, which requires prolonged periods of resting, to support digestion (fermentation) in their sacculated stomachs (Kuhn, 1964; Oates and Davies, 1994; Folk, 2000).

Compare with *S.concolor*, the Mentawaian Langur different in time spent of foraging that can be explained by the more efficient use of abundant food resources such as leaves and flowers by *S. concolor* (unpublished data) compared to the extensive foraging on scattered fruit resources by *P. potenziani*. Such a feeding strategy as observed in *S. concolor* requires an extra digestive stomach compartment, the presaccus, which assists in breaking down the great quantity of cellulose and hemicelluloses (Caton, 1998). This specific anatomical characteristic can be found in *S. concolor* but not in *P. potenziani,* further supporting a fruit dominated-diet in this species. Based on these in feeding ecology, *P. potenziani* gains much more energy from nutritious fruits, but has to spend more energy to explore these scattered food resources over wider ranges and in seasonally varying abundance.

In total, 118 food species were identified as being eaten by *P. potenziani*, however, based on the top ten food species, accounting almost 50% of their overall diets. All of which were considered to be abundant at the study site (Hadi *et al*, 2009). As comparation, *C.*

guereza use one species as 68% of their diet (Struhsaker & Oates, 1975) and *T. vetulus* use three species make up 70% of the diet (Hladik, 1977) indicated that Mentawai colobines relatively non-monotounous diet

Furthermore, when leaves, fruits, flowers, etc. of the same food plant species are considered as separate items, there are 176 food items accounted in total with different proportion. Based on the data, the Levin's niche breadth index of the species was 0.22. The value was slightly lower compare for example with *S. concolor* (0.34; unpublished data), reflecting a more specific use of the recorded food items of *P. potenziani* compared to the latter. Therefore *P. potenziani* seems spend more time for travelling and foraging to assort their diet.

Acknowledgement

I would like to thank the chief of Policoman village and local community, for permission to conduct long term field studies in the aboriginal communal forest of Peleonan.

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Green Tea Extract Protects Endothelial Progenitor Cells from Oxidative Damage Through Reduction of Intracellular Reactive Oxygen Species Activity

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A number studies have examined that tea consumption decreases cardiovascular risk, but the mechanisms remain undefined. Endothelial dysfunction has been correlated with coronary artery disease and circulating endothelial progenitor cells (EPCs) is contributed of this repair process. Endothelial dysfunction is associated with increased oxidative stress and may be reverse by antioxidant. Green tea is known as free radical scavenger which has a powerful antioxidant action. The aim of this study is to investigate whether green tea extract (GTE) can protect EPCs from oxidative stress through antioxidant protective mechanism. Total mononuclear cells (MNCs) were isolated from peripheral blood by Ficoll density gradient centrifugation. The cells were then plated on fibronectin-coated culture dishes. After being cultured for 7 d, EPCs were characterized as adherent cells double positive for DiLDL-uptake and lectin binding. Further characterizations were done by demonstrating the expression of CD34/45, CD133, and KDR. EPCs were then induced for oxidative stress using various concentrations of H_2O_2 (50, 100, 200 µM), and incubated with or without GTE (25 mg/L) and result showed that GTE ameliorated the cell viability of H_20_2-induced EPC at concentration 50, 100, 200 µM for about 28.72 ± 10.5%, 34.55 ± 7.64%, and 27.04 ± 3.42%, respectively, higher than that control. The level of intracellular reactive oxygen species (ROS) was quantified by fluorescence with 2',7'- dichlorofluorescein diacetate (DCFH-DA) using flow cytometry and showed that GTE decreased intracellular ROS level of H_20_2-induced EPC at concentration 50, 100, 200 µM for about 84.24 ± 8.59 %, 92.27 ± 1.08 %, and 93.72 ± 0.36%, respectively, compare to that control. The result showed that GTE may ameliorate cell viability by decreasing accumulation of intracellular ROS in H_20_2-induced EPCs.

Keywords: endothelial progenitor cell, oxidative stress, reactive oxygen species, green tea, antioxidant, endothelium, coronary diseases

Introduction

Tea, a product made from *Camellia sinensis*, is the second most widely consumed beverage in the world after water and well ahead of coffee, beer, wine, and carbonated soft drinks (Costa, et al., 2002; Macfarlane & Macfarlane, 2004; Rietveld & Wiseman, 2003). A number of studies have examined the relation between tea consumption and cardiovascular risk (Hertog, et al., 1993; Geleijnse, et al., 1999; Sesso, et al., 1999). The reduction of cardiovascular risk by tea consumption is suggested due to its flavonoid compound (Knekt et al., 1996; Hertgog et al., 1995). This suggestion is convincing by other studies that dietary intake of flavonoid from tea and other sources (onions, apples, red wine) is associated with reduced in cardiovascular risk (St Leger et al., 1979; Knekt et al., 1996; Yochum et al., 1999).

Green tea is a free radical scavenger and has abundant flavonoid, which has a powerful antioxidant action. Tea flavonoid contain of catechins (30 to 36% of dry weight) including Epigallocatechin-3-gallate (EGCG), which constitutes up to 63% of total catechins (Mannning & Roberts, 2003). The antioxidant activity of EGCG has been shown to be 25 to 100 times more potent than vitamins C and E (Doss, et al., 2005). One benefit of the dietary flavonoids is their antioxidant properties.

Flavonoids are well known for their free radical scavenger, such as reactive oxygen species (ROS) (Robak & Gryglewski, 1988). Other studies suggest that flavonoid may prevent LDL oxidation, a key early occurrence in atherosclerosis development (Diaz et al., 1997). Recent studies also suggest that flavonoid may favourably affect endothelial function (Fitzpatrick, et al., 1995; Andriambeloson, et al., 1997).

ROS are form of unpaired electron, such as superoxide anion ($O_2^{\cdot-}$) and hydroxyl radical (HO'), and also non-free radical species such as hidrogen peroxide (H_20_2) (Halliwell, 1995). In the cardiovascular system, ROS are recognized as important signaling molecules. Long-term exposure to ROS, can damage diverse macromolecules, including proteins, carbohydrates, lipids, and DNA. These damaging actions cause in vascular injury and result in endothelial dysfunction (Bauer & Bauer, 1999)

Endothelial dysfunction has been correlated with coronary artery disease and observed in patients with established coronary artery disease or coronary risk factors (Drexler, 1997). Endothelial progenitor cells (EPCs), a kind of stem cell that makes a vessel in peripheral blood, play important roles in maintaining the vessel tone and have role to repair the endothelial cell injury, an early stage of atherosclerosis caused by cardiovascular risk factor (Asahara, et al., 1997; Walter et al., 2002; Sata, 2003; Gill, et al., 2001). Recent a number of studies found that the number of circulating EPCs were reduced when there were more atherosclerosis risk factors causing the process of atherosclerosis (Vasa et al., 2001; Hill et al., 2003).

Endothelial dysfunction in atherosclerosis is close related with increased oxidative stress and might be reversed by antioxidant treatment (Diaz, et al., 1997). We hypothesized that green tea extract (GTE) protect EPC from oxidative stress through antioxidant protective mechanism, which contributes to protective effect on endothelial cells. To test this hypothesis, we assessed the protective effect and ROS-inhibiting effect of GTE on H_20_2-induced oxidative damage in human EPCs.

Methodology

Isolation and Cultivation of EPCs

EPCs were cultured according to the previously described method (Chen et al., 2004). Total mononuclear cells (MNCs) were isolated from peripheral blood of healthy young human volunteers by Ficoll-paque plus (GE Healthcare) using density gradient centrifugation method. MNCs were then plated on culture dishes coated with human fibronectin (Roche) and cultured using VascGrow^{TM} (Stem Cell and Cancer Institute). After 4 days in culture, new media were applied and the culture was maintained through day 7. Informed consent was provided from all volunteers. All of the procedures were done in accordance with ethical clearance board.

EPC Characterization

EPCs were characterized as adherent cells after 7 days in culture. Direct fluorescent staining was used to detect dual binding of Ulex europaeus agglutinin I conjugated with fluorescein isothiocyanate (FITC-UEA-I; Sigma) and 1, 1-dioctadecyl- 3, 3, 3, 3-tetramethylindo carbocyanine-labeled acetylated low density lipoprotein (DiI-acLDL;

Invitrogen). To detect the uptake Dil-acLDL, adherent cells were incubated with DiLDL (1mg/ml) at 37°C for four hours. Cells were then fixed with 3% paraformaldehyde for 10 minutes. After washing, cells were incubated with FITC-UEA-I (1 mg/ml) at 37°C for one hour. Cells also were nucleus-stained using DAPI (Invitrogen). After staining, cells were then observed using inverted fluorescent microscope (Axiovert 40 CFL, Zeiss). Cells that were double positive for DiLDL and lectin were defined as EPCs (Kalka et al., 2000; Vasa et al., 2001).

Further identified of EPCs were done using Fluorescence-activated cell sorting (FACS). Adherent cells were detached using 2 mM ethylene diamine tetra acetate (EDTA). 1 x 10^5 cells were pre-incubated for 15 minutes at room temperature with FcR Blocking (Miltenyi Biotech). Cells were then incubated at 4º C with FITC-conjugated anti-CD45/phycoerythrin -conjugated anti-CD34 (BD Biosciences) and phycoerythrin -conjugated anti-CD133 (Miltenyi Biotech) for 15 minutes, and PE-conjugated VEGF R2/KDR (R&D System) for another 40 minutes. Isotype-identical antibodies served as negative controls. Quantitative FACS was performed on a FACSCalibur *Flow Cytometer* (BD Biosciences).

EPC Cytotoxicity Assay

EPC cytotoxicity was done to determine the maximal tolerance concentration of GTE on EPC culture and to determine the optimal oxide damage concentration of H_20_2 for the following experiments. Cytotoxicity of EPC was determined by CellTiter® (Promega) based on quantitative colorimetric assay. After 7 days in culture and identification of EPC, cells were then digested with Trypsin-EDTA and were cultured at a density of 5 x 10^4 cells/mL on 96-well tissue culture plates using serum-free medium and cultured 24 h before treatment (Bickford et al., 2006; Chen, et al., 2004; Gu, et al., 2006). Cells were then treated with different concentration of GTE (3.13-100 mg/L) and H_20_2 (12.5 – 400 µM) for 24 h. EPCs were supplemented with 20 µL of CellTiter® each well and incubated for another 4 hours. OD value was measured at 490 nm using microplate reader (Bio-Rad)

Assessment Protective Effect of GTE on Oxidative Damage in EPC

After 7 days in culture, EPCs were then digested with Trypsin-EDTA and were cultured at a density of 5 x 10^4 cells/mL on 96-well plates using serum-free medium and cultured 24 h before treatment. Culture medium was replaced with fresh medium containing various concentration of H_20_2 (50, 100, 200 µM). GTE (25 mg/L) was added 1 h before treatment with H_20_2 for a subsequent 24 h (Jie, et al., 2006). Cell viability was measured by CellTiter® assay (Promega). Control was done by treated cells without H_20_2. The value of different absorbance was expressed as a percentage of control.

Measurement of Intracellular Reactive Oxygen Species.

Quantification of intracellular ROS level was done by fluorescence with 2',7'-dichlorofluorescein diacetate (DCF-DA; Invitrogen) using modification methods from Stolzing & Scutt (2006) and Jie et al. (2006). After 7 days in culture, EPCs were then digested with Trypsin-EDTA and 1 x 10^5 cells were incubated with 10 µM DCF-DA for 30 min at 37 °C. After the incubation, the excess probes were washed out with PBS+KCl, then incubated with GTE (25 mg/L) for 30 min. Cells were then incubated with H_2O_2 for final concentration 50, 100, 200 µM for another hour. The intracellular ROS levels were measured using FACSCalibur *Flow Cytometer* (BD Biosciences). Control was done by treated cells with H_2O_2 without GTE pre-treatment. The measured fluorescence values were expressed as a percentage of control cells.

Statistical analysis

Data were presented as mean ± standard deviation. Estimation of overall significance was statitiscally analized with one-way ANOVA and Duncan PostHoc test using SPSS V. 15.0. A probability level of 5% ($p < 0.05$) was considered significant.

Results and Discussions

EPC Characterization

When cultured in in vitro system, EPCs will attach to the fibronectin-coated dish and proliferate rapidly forming spindle-shaped cells within 4–7 days of culture (Hristov & Weber, 2004). Beside cell morphology, functional assay also be used to demonstrate that putative progenitors have endothelial cell potential including uptake of DiI-labeled acetylated-low density lipoprotein (Ac-LDL) (Voyta, et al., 1984) and binding of fluorescently labeled *Ulex europaeus agglutinin 1* (UEA-1) plant lectin (Suzuki, et al., 1990). Cell surface markers assay has been used for convincing the EPC identification.

In the present study, MNCs isolated and cultured for 7 days resulted in an attached cell with spindle-shaped morphology (Fig 1). EPCs were characterized as adherent cells double positive for DiLDL uptake and lectin binding (Fig 2). Further characterization were demonstrating the expression of CD34/45 (0.13 ± 0.041%), CD133 (0.14± 0.035%), and KDR (0.23±0.031%) (Fig 3)

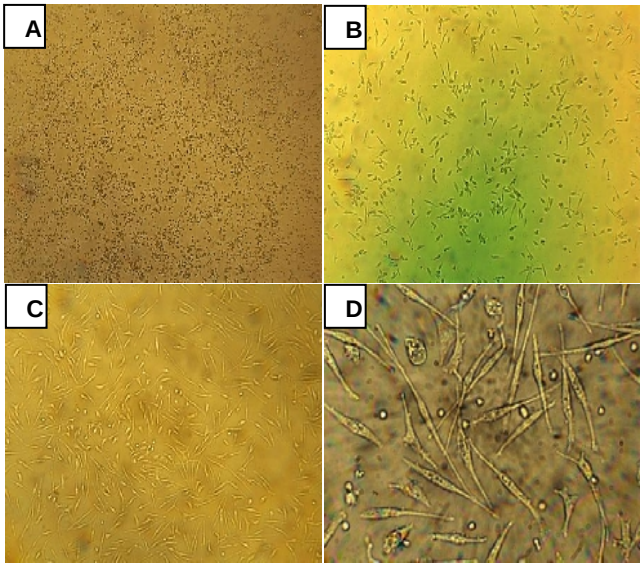

Figure 1. EPC morphology charac- terization. MNCs (A, 400 X) were cultured on VascGrow™ medium and start to exhibited a spindle-shaped on day 4 (B, 400 X) and more sharp in pattern on day 7 (C, 400X; D, 800X)

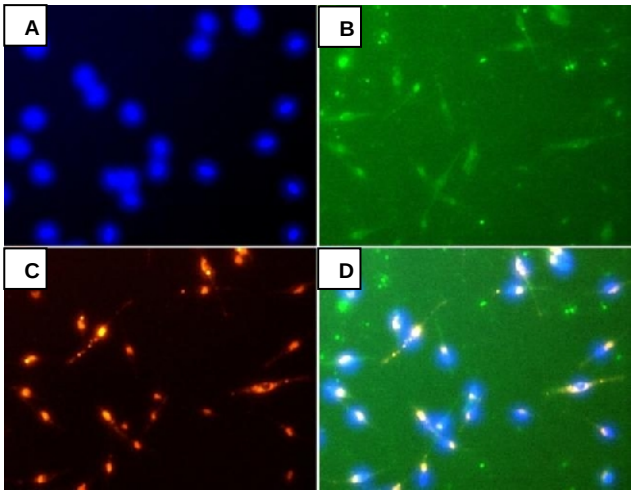

Figure 2. EPC functional characterization. Adherent cells were stained with DAPI (A), binding lectin (B) and taking up Dil-acLDL (C). Panel (D) was obtained by merging (A), (B) and (C). A-D were assessed under inverted fluorescent microscope, magnification 800X.

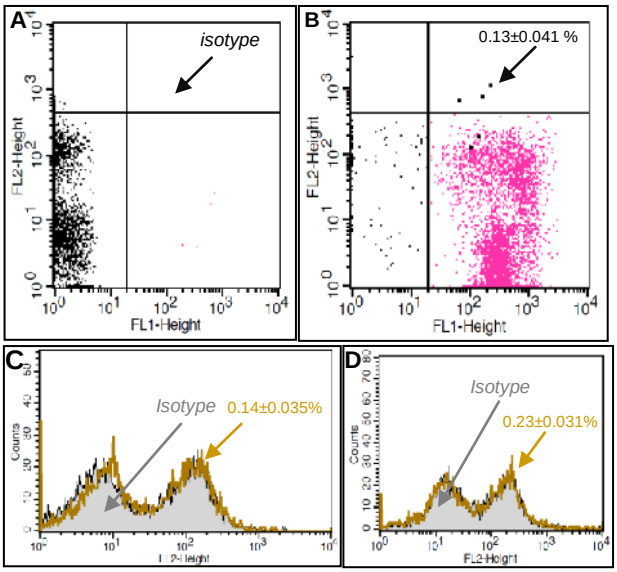

Figure 3. EPC marker characterization. Cells were demonstrating the expression of CD34/45 (A, isotype; B, marker CD34/45), CD133 (C), and KDR (D).

EPC Cytotoxicity Assay

The cell viability was measured using colorimetric method for determining the cytotoxicity assay. CellTiter® solution (Promega) has been used in this study. The solution contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl) -5- (3-carboxy methoxyphenyl)- 2 - (4-sulfophenyl)- 2H-tetrazolium (MTS). The MTS tetrazolium compound is bioreduced by cells into a colored formazan product due to conversion by dehydrogenase enzymes in metabolically active cells (Berridge & Tan, 1993).

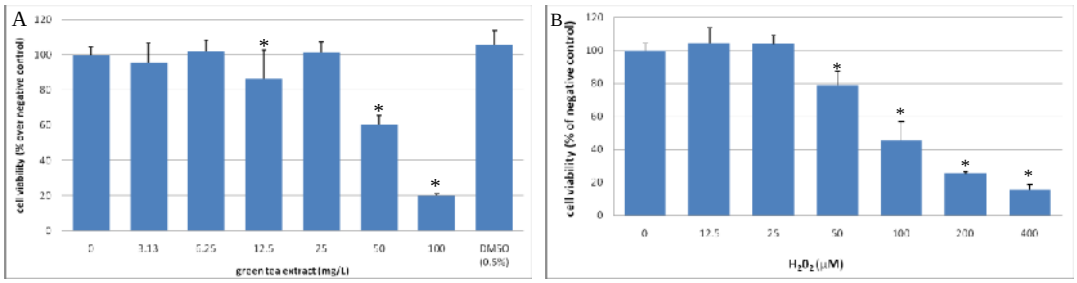

Figure 4. Effect of green te extract (A), and H_20_2 (B) on cytotoxicity of EPC. EPC were cultured on 96-well tissue culture plates (5 X 10^4 cells/well) and treated with GTE or H_20_2 at a wide range of doses for 24 hours. After treatment, cells were prepared for CellTiter® analysis of cell cytotoxicity as described in Methods. Data were expressed as mean (percentage over negative control) ± Standard Deviation (n = 3). *p < 0.05 when compared with that of the negative control (untreated cells).

The result of cytotoxicity effect from GTE and H_20_2 is shown in Figure 4 (A and B). After treatment for 24h, the cells that were treated with GTE at concentration 3.13-25 mg/L had relatively no effect on cell cytotoxicity. But after treated with GTE at concentrations 50, and 100 mg/L, the viability of cells start to decrease about 40 dan 80 %, respectively, relative to the negative control.

The cells that were treated with H_20_2 gave no oxide damage effect to the cells at concentrations of 12.5-25 µM and start to decreased the viability of EPC for about 21, 55, 75, and 84% relative to the negative control for the concentration of 50, 100, 200, and 400 µM, respectively. Concentration 25 mg/L of GTE and 50, 100, and 200 µM of H_20_2 have been choose for the following experiments for independent and dependent concentrations, respectively.

Protective Effect of GTE on Oxidative Damage in EPC

ROS have important role on oxidative stress which can damage diverse macromolecules and result in decreasing the cell viability. Thus the oxidative stress occurrence might be pressured by antioxidant treatment (Diaz, et al., 1997). EGCG, the most abundant component in green tea, has a potent antioxidant property and shown to be 25 to 100 times more potent than vitamins C and E (Doss, et al., 2005). ECGC actions are very diverse and include direct free radical scavenging, antioxidant, anticancer, antibacterial, and antiviral activities (Cabrera, et al., 2006). Several studies also have demonstrated that EGCG can protect heart, kidney, and brain from oxidative injury (Fu & Ko, 2006; Hirai, et al., 2007; Itoh, et al., 2005). EGCG provide protective effect from oxidative stress through a variety mechanisms (Chung, et al., 2003; Guo, et al., 1999).

The protective effect of GTE on H_20_2-induced EPC is shown in Figure 5. Pre-treatment with 25 mg/L GTE on H_20_2-induced EPC at concentrations 50, 100, 200 µM increased the viability cells about 28.72%, 34.55%, and 27.04%, respectively, relative to the control (treatment only with 50, 100, 200 µM H_20_2). These data showed that GTE protects the cells from oxidative damage and were ameliorated the H_20_2-induced loss of EPC cell viability.

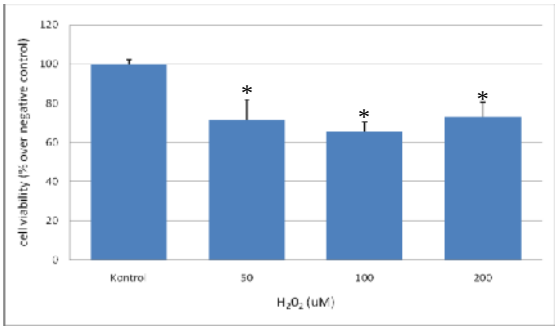

Figure 5. Effect of GTE on H_20_2-induced EPC. Viability of cell was estimated by CellTiter® assay after treatment with 50, 100, 200 µM H_20_2 only and control (50, 100, 200 µM H_20_2 with 25 mg/L GTE for 24 hours. Data were expressed as mean (percentage over negative control) ± Standard Deviation (n = 3). *p < 0.05 when compared with that of the negative control (treated only with H_20_2).

Measurement of Intracellular Reactive Oxygen Species.

In this study, intracellular ROS level measurement was done by fluorescence with 2',7'- dichlorofluorescein diacetate (DCF-DA; Invitrogen). More than decades, DCF-DA has been employed for several studies dealing with the effect of ROS in cell culture (Saez, et al., 1987; Scott, et al., 1988; Murphy, et al., 1989). DCF-DA is crosses membranes of viable cells and is enzymatically hydrolyzed by intracellular esterases to 2',7'- dichlorofluorescein (DCFH) without fluorescence. DCFH is rapidly oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS within the cells. DCF remains trapped within the cell and can be measured to represent the intracellular ROS level (Lebel et al., 1992; Jie, et al., 2006).

Representative dot blots of intracellular reactive oxygen species (ROS) levels in EPC is shown in Figure 6. The level of fluorescence intensity is an indicator of ROS production. The basal level of ROS with no exposure to H_20_2 was found about 7-16 % (Fig. 6B) compare to that control cells (unstained-DCFDA cells; Fig. 6A). After treatment with different doses of H_20_2 (50, 100, 200 µM) for 1 hour in EPC, the level of ROS in the cells increased for about 7-34 % (Fig. 6C,D,E) in comparison with that of negative control (untreated cells; Fig. 6B). When the cells were treated with 25 mg/L of GTE, the ROS levels were decreased dose-dependently for about 84.24 %, 92.27 %, and 93.72% compare to the control on H_20_2-induced EPC for the concentrations of 50, 100, 200 µM, respectively (Fig. 6F,G,H).

These results indicated that GTE treatment reduced the accumulation ROS level in H_20_2-induced cells. The graphic for fluoresence intensity of ROS level on EPC is shown in Figure 7.

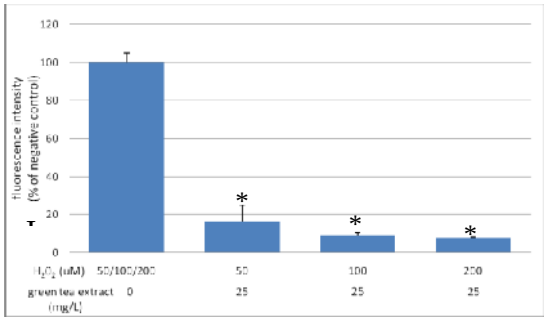

Figure 7. Fluorescence intensity of ROS from H_20_2-induced EPC. GTE showed its antioxidant capacity to reduce the ROS level in the cells. The cells were incubated with 10 µM DCF-DA for 30 min and exposure to several doses of H_20_2 with/without GTE treatment.

Data were expressed as mean (percentage over negative control) ± Standard Deviation (n = 3). *p < 0.05 when compared with that of the negative control (treated only with H_20_2).

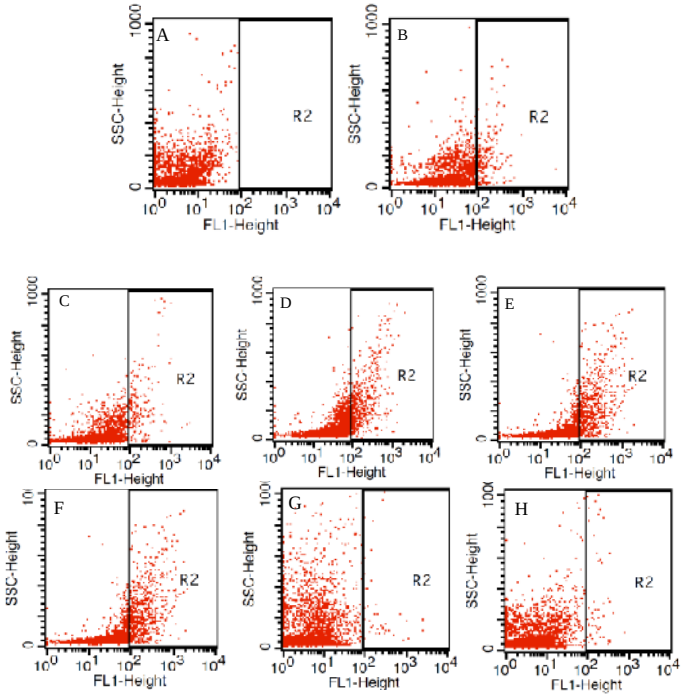

Figure 6. Intracellular ROS levels in EPC. The cells were incubated with 10 µM DCF-DA for 30 min and exposure to several doses of H_20_2 with/without GTE treatment. The basal level of ROS with no exposure to H_20_2 (B) was obtained by gating the ROS level with control unstained-DCFDA cells (A). The cells treated with of 50 µM (C), 100 µM (D), 200 µM (E) H_20_2 exhibited increasing in ROS level. Pararel samples were treated with 25 mg/L GTE and the ROS level in cells were decreased to the control (H_20_2-induced cells) for the concentrations H_20_2 of 50 µM (F), 100 µM (G), 200 µM (H).

Conclusion

In conclusion, GTE may protect EPC from oxidative damage by ameliorating the H_20_2-induced loss of EPC cell viability and decreasing its accumulation intracellular ROS

Acknowledgement

This work was funded by grant from Medicinal Faculty, Maranatha Christian University, Bandung and supported by Stem Cell and Cancer Institute (SCI), Jakarta.

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Potential Cytotoxic on Breast Cancer Cells Line and Antioxidant of Water Extract of *Catharanthus roseus* [L] G.Don., *Dendropthoe petandra* L., *Curcuma mangga* Val., *Piper betle* L.

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Breast cancer is the most common cancer among women and the second leading cause of cancer deaths in women today. The madagascar prewinkle (*Catharanthus roseus* [L] G.Don), mango parasite (*Dendropthoe petandra* L.), white saffron (*Curcuma mangga* Val), betel leaves (*Piper betle* L.) have been reported to exhibit antioxidant, antimutation and cytotoxic that suggested the chemopreventive potential against various cancer including breast cancer. This research was conducted to investigate cytotoxic activity on breast cancer cell line T47D, antioxidant activity of *C. roseus*, *D. petandra*, *C. mangga* and *P. betle* water extracts. The cytotoxic potency was determined with MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. The antioxidant activities were determined by using *in vitro* assay of 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity. *C. roseus* water extract was able to inhibit T47D cell proliferation with IC_{50} 4%, *D. petandra* with IC_{50} 1%, *C. mangga* with IC_{50} 14% and *P. betle* with IC_{50} 3%. The highest DPPH scavenging activity of *C. roseus* was 71.87%, *D. petandra* was 75.11%, *C. mangga* was 38.45% and *P. betle* water extract was 83%. We suggest that *D. petandra*, *P. betle* and *C. roseus* water extract have a potential cytotoxic and antioxidant activities compared with *C. mangga* water extract.

Keywords : cytotoxic, antioxidant, *Catharanthus roseus*, *Dendropthoe petandra*, *Curcuma mangga*, *Piper betle*, water extract, breast cancer, T47D

Introduction

The three most commonly diagnosed types of cancer among women in 2010 were cancers of the breast, lung, and colorectum, accounting for 52% of cancer cases in this group. Breast cancer alone accounted for 28% (207,090) of all new cancer cases among women (Kaghani *et al*., 2011). Breast cancer (BC) is one of the most important causes of morbidity and mortality representing the first tumor in the female sex in terms of incidence and the third in terms of mortality in the western world (Andreetta *et al*., 2010).

The chemotherapeutic drugs including etoposide, camptothecin, vincristine, cis-platinum, cyclophosphamide, paclitaxel (Taxol), 5- fluorouracil and doxorubicin have been observed to induce apoptosis in cancer cells (Kaufman *et al*., 2000; Johnstone *et al*., 2002; Abdolmohammadi *et al*., 2008). Lipid peroxidation is a free radical mediated phenomenon in biological tissues where poly unsaturated fatty acids are generally abundant and is used parameters for assessing the involvement of free radicals in cell damage (Sinha *et al*., 2009), as evidenced by the formation of thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides (LOOH) and conjugated dienes (CD) as well as the status of the antioxidants

superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) in breast cancer tissues was enhanced compared to control (Kumaraguruparan *et al*., 2002). Antioxidant CAT, SOD also act as anti-carcinogens and inhibitors at initiation and promotion/transformation stage in carcinogenesis. Mutation caused by potassium superoxide in mammalian cells is blocked by SOD. Plasma DNA strand scission caused by xanthine/xanthine oxidase is prevented by SOD and CAT enzymes (Sinha *et al*., 2009). The leaves extract of *P. betle* is reported to exhibit biological capabilities of detoxication, antioxidation and antimutagenic activities that suggested the chemopreventive potential of the extract against various ailments including liver fibrosis (Shun *et al*., 2007; Fatahilah *et al*., 2010). Ethanolic extarct of *P. betle* leaves is promising source as natural antioxidant and antiproliferative in breast cancer T47D cell line (Widowati et al., 2011).

Herbal medicines are usually very easily accepted by women, as many as 80% of women with breast cancer use some form of complementary or alternative medicine, the most common using herbs, lessen the side effects of treatment, improve quality of life, provide a greater sense of control, and reduce stress (Roberts, 2010; Kaghani *et al*., 2011). *C. roseus* was used as a remedy in cancer related diseases. Aerial part of the plant contains about 90 different alkaloids. Crude extract of *C. roseus* using 50 and 100% methanol had significant anticancer activity against different cell types in vitro at <15 µg/mL) (Ueda *et al*., 2002). *D. petandra* is traditionally used as cancer medicine. Its flavonoids content can inhibit growth of *Artemia salina* Leach as anticancer activity assay *in vivo* (Sukardiman *et al*., 1999). White saffron rhizome is a spice commonly used in traditional medicine. Compounds from *C. mangga* showed high cytotoxic activity against a panel of human tumor cell lines, such as human leukemia (HL-60), breast cancer (MCF-7) and liver cancer (HepG2) (Abas *et al*., 2005). Water extract of *C. mangga* exhibit antioxidant activity (Pujimulyani *et al*., 2004).

Materials and Methods

Plants materials

Materials were aerialas and roots of *C. roseus* [L] G.Don., small branches of *D. petandra* L. and rhizomes of *C. mangga* Val., levaes of *P. betle* were collected from from plantation located in Bogor, West Java, Indonesia (May, 2009). The plants were identified by staff of herbarium, department of biology, school of life sciences and technology, Bandung institute of technology, Bandung, west Java, Indonesia. The aerials and roots, leaves and branches, leaves and rhizomes were collected, chopped finely and kept under drier tunnel ($40\text{-}45^0$ C).

Preparation of extract

Ten gram of dried and chopped materials were boiled with 100 ml distilled water (aquadest) with $75\text{-}90^0$C until the remained water was 50 ml, and filtrated. The water extracts were stored at 4 ^{o}C. The water extracts of *C. roseus, D. petandra, P. betle* and *C. mangga* were dissolved in 10% dimethy sulfoxide (DMSO-Merck) and subsequently diluted to appropriate working concentrations with Dulbecco's Modified Eagle's Medium (DMEM-Sigma Aldrich) culture for proliferation inhibitor proliferative (Tan *et al.,* 2005).

Cell culture

The human breast cancer T47D cell line was obtained from the Indonesian institute of sciences, research centre for chemistry, division of natural products, food and pharmaceuticals, Bandung, West Java, Indonesia. The cells were grown and maintained in DMEM supplemented with 10% (v/v) foetal bovine serum (FBS-Sigma Aldrich), 100

units/ml penicillin (Sigma Aldrich) and 100 µg/ml streptomycin (Sigma Aldrich), and incubated at 37^0 C in a humidified atmosphere and 5% CO_2 (Mooney *et al.,* 2002; Tan *et al*., 2005).

DPPH scavenging activity assay

The DPPH assay was carried out as described by Unlu *et al* (2003). Pipette 50 µl of ethanol extracts of *C roseues, D petandra, P. betle, C. mangga*. To obtain the IC_{50} value, a range of various final concentrations was used e.g. 100, 50, 25, 12.5; 6.25, 3.125, 1.563, 0.781, 0.391 and 0.195 µg/ml introduced at the microplate and then were added 200 µL of 0.077 mmol/l DPPH (Sigma Aldrich) in methanol and the reaction mixture was shaken vigorously and kept in the dark for 30 min at room temperature, furthermore DPPH scavenging activity was determined by microplate reader at 517 nm.

The radical scavenging activity of each sample was expressed by the ratio of lowering of the absorption of DPPH (%), relative to the absorption (100%) of DPPH solution in the absence of test sample (negative control).

$$\text{scavenging \%} = \frac{A_c - A_s}{A_c} \times 100$$

As: absorbance of samples, Ac: negative control absorbance (without sample)

IC_{50} determination

The IC_{50} (median inhibition concentration) is the concentration of toxic extract that reduces the biological activity by 50 %. The IC_{50} value for cytotoxicity was obtained from the MTS assay and calculated using linear regression analysis in Microsoft Excel software. Optical density (OD) at 515 nm of cells number without treatment was established as standard curve function. Read OD of sample was converted to number of cells using standard curve equation, linear graphic of % living cells in function of extract concentrations was traced. The IC_{50} value was the concentration of toxic extracts reduced the biological activity by 50 %.

Results

Cytotoxic activityof C. roseus, D. petandra, P. betle* and *C. mangga extracts

Figure 1. shows the cell viability of T47D cells treated by P. *C. roseus, D. petandra, P. betle* and *C. mangga* extracts, the *C. roseus, D. petandra, P. betle* extracts exhibited a decrease in viability in a concentration dependent-manner. Higher concentration extracts will increase the cytotoxicity. The IC_{50} of *C. roseus, D. petandra,P. betle* and *C. mangga* extracts in T47D cells respectively were 1% ; 4%; 3% and 14% concentrations.

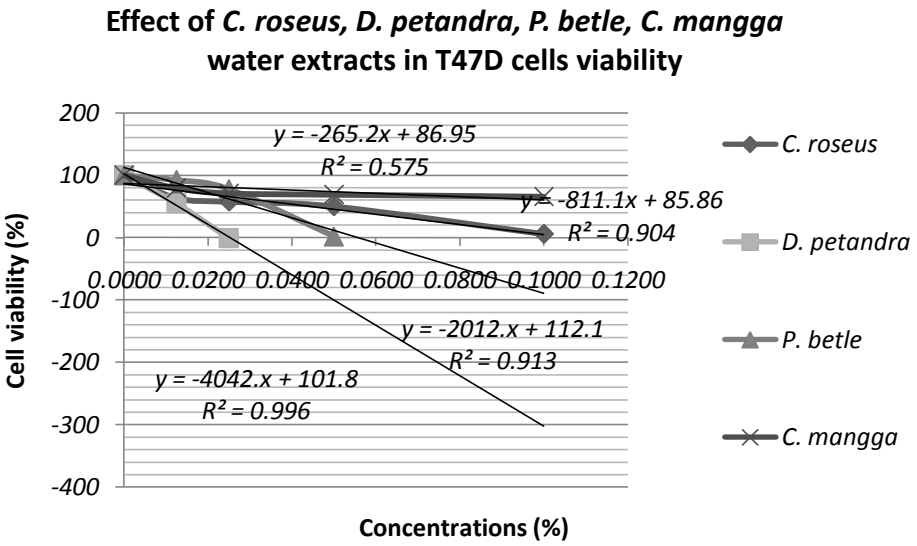

Figure 1. *C. roseus, D. petandra, P. betle* and *C. mangga* water extracts in T47D cells viability

Antioxidant activity of C. roseus, D. petandra, P. betle* and *C. mangga extracts

The DPPH free radical scavenging activity of *C. roseus, D. petandra, P betle* and *C. mangga* water extracts of various concentration were measured to examine the antioxidant activity. The IC_{50} is the concentration of antioxidants activity to scavenge DPPH free radical 50 %. Figure 2. shows the DPPH scavenging activity of *C. mangga* extract showed the lowest activity compared to *C. roseus, D. petandra, P. betle* water extract. The highest of *P. betle, C. roseus, D. petandra and C. mangga* water extracts towards DPPH scavenging activity can be seen at Table 1.

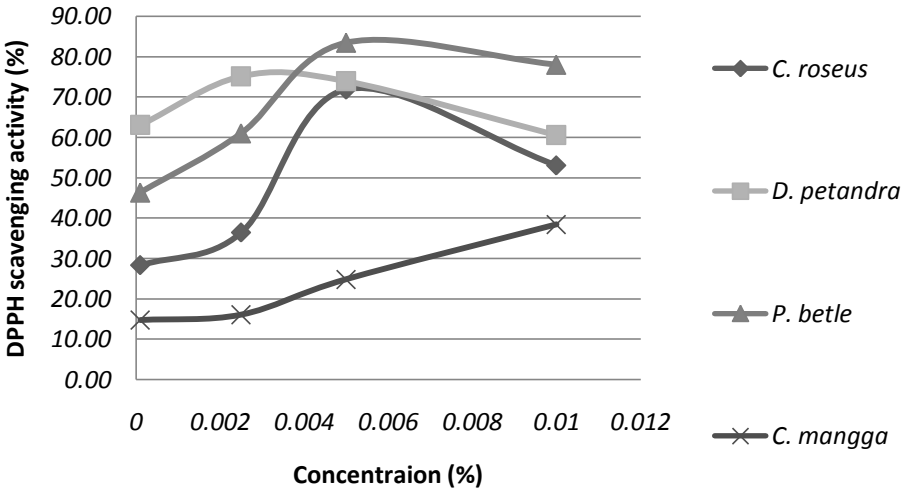

Figure 2. The DPPH scavenging activity of *C. roseus, D. petandra, P. betle* and *C. mangga* water extracts

Table 1. The highest DPPH scavenging activity

Samples	The highest DPPH scavenging activity (%)	Concentration (%)
C. roseus	71.87	0.5% (0.005)
D. petandra	75.11	0.25% (0.0025)
P. betle	83.46	0.5% (0.005)
C. mangga	38.46	1% (0.01)

Discussion

Base on the data (Figure 1.) showed that *C. roseus*, *D. petandra* and *P. betle* water extracts had cytotoxic activity with IC_{50} 4%, 1% and3%. This results were validated with previous study by Widowati *et al*. (2010a) and Widowati *et al*. (2011) that *C. roseus* ethanolic extract has cytotoxic activity, can induce apoptosis in T47D cell line. This results are consistent with previous studies the *C. roseus* extract is able to induce DNA fragmentation by gel electrophoresis. In each case, DNA fragmentation was characterised by oligonucleosomal size fragments of about 180-200 base pairs (bp), a well-known feature indicative of programmed cell deat (Compton 1992; Ahmad *et al*., 2010). Crude extract of *C. roseus* using 50 and 100% methanol had significant anticancer activity against different cell types in vitro at <15µg/mL) (Ueda *et al*., 2002). Crude decoction (200 mg and 1 g herb/mL water) showed moderate *in vitro* antiangiogenesis effects (Ghosh and Gupta, 1980; Chattopadhyay *et al*, 1991, 1992). *D. petandra* water extract showed cytotoxic activity. This results are validated with previous research that *D. petandra* is traditionally used as cancer medicine. Its flavonoids content can inhibit growth of *Artemia salina* Leach as anticancer activity assay *in vivo* (Sukardiman *et al*., 1999), but this results was not validated with previous study that water and ethanolic extracts of *D. petandra* leaves has not cytotoxic activity in melanoma cancer B16 cell line (Artanti *et al*., 2006), ethanolic extract of *D. petandra* has no cytotoxic activity in breast cancer T47D cell line with IC_{50} 728.05 µg/ml (Widowati *et al*., 2011). *P. betle* water extract showed cytotoxic activity. This results were validated with previous study by Widowati *et al*. (2011) that *P. betle* ethanolic extract has cytotoxic activity with IC_{50} 55.2 µg/ml. This results are consistent with previous studies that *P. betle* aqueos extract has antiproliferative activity towards nasopharyngeal epidermoid carcinoma cells (Fatahilah *et al*., 2010). Cytotoxic effect of *P. betle* aqueous extract on KB cells, exhibit strength antiproliferative activity towards KB cells with IC_{50} 29,5 µg/mL and do not show any cytotoxic activity even at 100 µg/ml on HeLa cells. Biologically active in the *P. betle* extract is identified as chlorogenic acid and kills myeloid and lymphoid cancer cells but normal cells are unaffected (IICB Report, 2004). The chlorogenic acid is shown to induce program cell death in human cancer cells transplanted in experimental nude mice and at the same time, shows no effect on the growth of non-cancerous cells. Those previous studies showed that *P. betle* extract has great potential to be developed as a target-specific, therapeutic drug for blood cancer (Fatahilah *et al*., 2010). *P. betle* aqueous leaves extracts have found to exhibit stronger antiproliferative activity towards human nasopharyngeal epidermoid carcinoma (KB) cells compared to their essential oils (Manosroi *et al*., 2006; Fatahilah *et al*., 2010). *C. mangga* water extract exhibited no anticancer activity in T47D cell line, with IC_{50} resulted 14%, this result was validated with previous research that ethanolic extract of *C. mangga* has no anticancer activity in T47D cell line with IC_{50} 404.76 µg/ml (Widowati *et al*., 2011).

Base on data Table 1. and Figure 2. showed that *C. roseus* water extract had antioxidant activity to scavenge DPPH free radical at level concentration 0.5% resulted71.87%. This results were not validated with previous studies that ethanolic extract of

C. roseus has no antioxidant activity (Widowati *et al*., 2010a; Widowati *et al*., 2011). *D. petandra* water extract had antioxidant activity to scavenge DPPH free radical at level concentration 0.25% resulted 75.11%. This results were validated with previous research that *D. petandra* ethanolic extract exhibit highest antioxidant activity is comparable with ascorbic acid and quercetin (Widowati *et al*., 2011). The crude decoction of *D. petandra* has high antioxidant activity (Maria, 1996), Water and ethanol extract of *D. petandra* exhibit DPPH free radical scavenging activity with IC50 < 50 µg/ml (Fajriah *et al*., 2006). Quercetin is one of the compound in *D. petandra* has high antioxidant activity (Dewiyanus, 1996; Gordon, 2001). *P.betle* water extract had antioxidant activity to scavenge DPPH free radical at level concentration 0.5% resulted 83.46%. This results were validated with previous research that *P. betle* ethanolic extract exhibit high antioxidant activity with IC_{50} 3.48 µg/ml (Risdian *et al*., 2010) and IC50 of ethanolic extract of *P. betle* is 5.49 µg/ml (Widowati *et al*., 2011). *P. betle* ethanolic extract is higher DPPH scavenging activity than *C. roseus* and C. mangga extract . Therefore, we assume that DPPH free radical scavenging activity is related to the presence of bioactive compounds such as phenolic compounds in extract. Our previous work showed that phenolic contents using kaempferol as standard, *P. betle* ethanolic extract contains high polyphenol 548.667 µg KE/mg (Widowati *et al*., 2010b), using *Epigallo Catehin Gallate* (EGCG) as standard, *P. betle* ethanolic extract contains high polyphenol 269.97µg EGCGE/mg (Risdian *et al.*, 2010). Polyphenol-rich extracts are potent DPPH scavengers offering overall protection against various stresses. P. *betle* extract shows activity similar to quercetin and protects LDL from oxidation in a dose dependent manner at concentrations higher than 10 µg/ml (Kumar *et al*., 2010). Polyphenols are one of the major plant compounds with antioxidant activity. The –OH groups in phenolic compounds are thought have a significant role in antioxidant activity (Arumugam *et al*., 2006). The antioxidant activity of phenolic compounds is reported to be mainly due to their redox properties (Rahman *et al*., 2008). Aqueous extract of *P. betle* leaves is also shown to be a scavenger of H_2O_2, superoxide radical and hydroxyl (*OH) radical (Kumar *et al*., 2010). *C mangga* water extract had no antioxidant activity to scavenge DPPH free radical at level concentration 1% resulted 38.46%, this research is very contradictory with previous research by Ruangsang *et al.* (2009) which *C. mangga* rhizomes have antioxidant, anticancer and anti-inflammatory activities. Water extract of white saffron (*C. mangga*) exhibit antioxidant activity using ß-carotene bleaching and DPPH scavenging method. Higher concentration of white saffron extract will increase the antioxidant activity, it may be due the curcuminoid content (Pujimulyani *et al*., 2004). Curcuminoid is one of the compounds in Curcuma exhibit antioxidant activity as free radical scavenger (Majeed *et al*., 1995; Pujimulyani *et al*., 2004). The antioxidative activity of curcuminoid compounds (curcumin, demethoxy curcumin and bisdemethoxy curcumin) is 20, 9 and 8 times higher compared with a-tocopherol using modified active oxygen method (Toda *et al*., 1985; Pujimulyani *et al*., 2004).

Conclusions

Water extracts of *C. roseus, D. petandra, P. betle* have cytotoxic activity in breast cancer T47 D cell line and have antioxidant activity to scavenge DPPH free radical activity. Water extract of *C. mangga* has no cytotoxic activity and antioxidant activity.

Acknowledgment

We gratefully acknowledge for the financial support of Directorate General for Higher Education, National Ministry of Republic Indonesia for research grant of Hibah Bersaing 2009-2010.

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ISSN: 2088-9771

Preference of *Apis cerana* to Six Pollen Substitutes

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Adequate substitutes for pollen are necessary for maintaining healthy honeybee colonies during periods of pollen dearth. The research aimed to make pollen substitutes preferred by honeybee *Apis cerana*. Basic ingredients of pollen substitutes (PSs) were defatted soy flour and skim milk. There were six pollen substitutes prepared for the honeybee colonies. Three PSs contained yeasts associated with the honeybee, one PS contained commercial baker yeast, and two PSs without adding dry yeasts. PS1 contained basic ingredients, *Candida hawaiiana* CR015, and honey; PS2 contained basic ingredients, *C. parapsilosis* CR057, and honey; PS3 contained basic ingredients, *Debaryomyces hansenii* CR133, and honey; PS4 contained basic ingredients, commercial baker yeast, and honey; PS5 contained basic ingredients and sugar syrup; PS6 contained basic ingredients and honey. The PSs were fed daily in paste form. No pollen substitute was given to the control colonies. The pollen substitutes were fed to colonies of *A. cerana* for 20 days, and they were allowed to forage on flowers. Amount of pollen substitutes consumed by the honeybee were weighted daily. The consumption data of each pollen substitute per day were used to determine the preference of the honeybee for a particular pollen substitute. The results showed that the pollen substitutes containing *C. hawaiiana* CR015 (PS1) and commercial baker yeast (PS4) were more preferred by A. cerana compare to other pollen substitutes (PS2, PS3, PS4, and PS6). The pollen substitute made of basic ingredients and sugar syrup (PS5) was the least consumed or not preferred by *A. cerana.*

Key words: *Apis cerana*, honeybee, pollen substitutes, preference, yeasts

ISSN: 2088-9771

Cytotoxic Activity Prescreen of Leaves of Primate Consumed Plants Subclassis *dilleniidae* and *hamamelididae* Using Brine Shrimp Lethality Test

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ABSTRACT

Brine Shrimp Lethality Test (BSLT) is a prescreened method of cytotoxic activity which one of its aim is finding a new cancer agent. The closest relatives between human and nonhuman primate emerge an idea to utilize a primate consumed plant as an alternative medicinal. In this experiment, five plants extracts from each subclassis (*Dilleniidae* and *Hamamalididae*) were tested using BSLT. The result showed that nine of ten extract were toxic (LC_{50} < 1000 μg/mL) and the highest activity was given by Tangkolo (*Kleinhovia hospita*) extract whose LC_{50} was 88,025 μg/mL. Furthermore, Tangkolo extract was fractionated by Liquid-Liquid Extraction using n-hexane, ethyl acetate and water. All fractions then tested by BSLT. The result showed that ethyl acetate fraction gave the highest activity. The LC_{50} value of n-hexane fraction, ethyl acetate fraction and water fraction respectively were 93,998 μg/mL, 53,333 μg/mL and 757,424 μg/mL.

Keywords: Brine Shrimp Lethality Test (BSLT), cytotoxic, primate consumed plant, *Dilleniidae* and *Hamamalididae,* Tangkolo

International Conference on Bioscience and Biotechnology (ICBB) 2011

October 11th-12th, 2011, Yogyakarta, Indonesia

Oral Paper Cluster 3

Tree Species Diversity of Kerinci-Seblat National Park and Its Potentials for Natural Substances-Based Medicines

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Abstract

Indonesia has long been known as a country with enormous species diversity, which can be sustainably explored its various functions. Unfortunately such a natural treasury has been neglected, even though it contains many natural substances for medical uses that can generate significant economical advantages and increase human welfare. Plants generally develop defense mechanisms against herbivores and pathogens through both mechanical structures and biochemical substances. The latter can be explored for their pharmaceutical uses. The objective of the study is to conduct a preliminary assessment on the potential of Kerinci-Seblat as a source for natural substances for medical uses. A plot of 1 ha was established and all trees with > 5 cm were measured, and collected their herbarium specimens, then identified. Their pharmaceutical potentials were determined through various literature reviews. The results show 27, 147, and 11 tree species respectively potentially contain Benzyl-isoquinoline (BI), Ellagic acid dan proanthocyanins (EL&P), dan Iridoid (Ir). These substances can be used to fight heart-related disorder, diabetics, and to develop natural-based drugs for antioxidants, antivirus, and anti depressants. Natural substances within sixteen families and 60 species can be further developed into anti cancer drugs, while 13 families and 30 species contain potentially substances that can be used to fight HIV.

I. Introduction

Indonesia has long been recognized as one of megadiversity area and therefore can serve as many both tangible and intangible services. However, many people have narrow views on defining this enourmous natural resources. They treat forest resource as a source of both timbers and land that can be later converted to other purposes such as plantation, agricultural area, and housing. These financial-driven purposes eventually conceal the intangible values which may serve to maintain life supporting systems, and have much higher values. The perception that heavily emphaizes only on tangible services indeed causes very cheap valuations on the environmental services, which further accelerates the deforestation and conversion of forest to the other purposes.

Non-timber aspects are growing to be more important in the near future. One of them is natural substances that can be extracted from forest vegetation. The use of plants for pharmaeutical purpose has been known to significantly influence the human health. The role of plants in the human health is even much greater in the developing countries, where the accesses to health system seems to be a main constraint. In general, the various parts of plants are being widely used by local people in many developing counties for preventing and curring various deseases, and maintaining health status. On the other hand, natural substances from various plants have been further explored to find new drugs to cure many deaceses in the developed countries. Batubara et al (2009) stated that many plant species in the nature have many potensial for pharmaceutical purposes. Data released by US National Cancer Institute in 2004 showed that more than 1400 tropical plants have potentials to be further extracted and developed into drugs againts cancer. In the tropics, more than 600 plant species have been explored, and yet need to pharmacological tests for further uses.

Due to the difficulty to collect plant specimens, it is only a considerably small number of plant species that is actually explored their drug-related potentials (Wiart 2006).

Plant is a renewable natural resource that has produced primairy and secondary compunds. Secondary compounds are parts of defence mechanism for plants to survive and protect them from herbivores and pathogens (Cronquist 1988). They also play important and complex roles both in the plant evolution and the raise of new families, orders, genera, dan species in the flowering plant (Ehrlich and Raven 1964). These secondary compunds are further used by pople for various purposes, especially for medical purposes (Leny 2006). The main objectives of the research are to know tree plant diversity at Tambang Sawah lower lowland forest of Kerinci-Seblat National Park, and to conduct a preliminary assesment its potential for medical purposes.

II. Methods

The research was conducted in Tambang Sawah tropical forest of Kerinci-Seblat National Park. Tambang Sawah site had been briefly selectively logged in early 80's. The research site was situated in the Kerinci-Seblat National Park and nearby a private land planted by mixed coffee crops and durian trees. The site had an altitude of 540 m, and was considered as lower montane forest formation (Susatya 2007). Data collected by Susatya (2007) from 1 ha plot at the site were used for the analysis. All plants with diameter at breast heigh (DBH) more than 5 cm were tagged and measured, and collected their herbarium specimens. Specimens were identified at Herbaria of Universitas Bengkulu (HUB), Universiti Kebangsaan Malaysia (UKMB), and Forest Research Institute of Malaysia (KEP). Species nomenclature was followed IPNI, Turner (1995), Ng (1979), and Whitmore (1972) (Susatya 2007). The potentials of tree species for medical purposes were determined through literature reviews, mainly from Qronquist (1988).

III. Results and discussions

Fourty two families was found in the site, where Euphorbiaceae. Meliaceae, Sapotaceae, Moraceae, and Lauraceae were dominant families. The forest structure itself was assembled by species with Importance Value Index (IVI) less than 10 %. This structure indicated that the forest eventually consists of many species with few individual trees. This could lead to the difficulty in conservation. The research site was assembled by 94 genera and 185 species (Table 1, Susatya, 2007).

Table 1: Family, genus, tree species and its secondary compounds.

No	Family	genus	%	Species	%	Indv.	%	compounds
1	Euphorbiaceae	15	15.98	35	19.02	115	19.90	ELP
2	Lauraceae	8	8.51	12	6.53	24	4.15	ELP
3	Annonaceae	5	5.32	8	4.35	12	2.08	BI
4	Flacourtiaceae	4	4.26	8	4.35	16	2.77	ELP
5	Meliaceae	4	4.26	15	8.15	63	10.90	ELP
6	Rubiaceae	4	4.26	5	2.72	20	3.46	IR
7	Sapindaceae	4	4.26	5	2.72	21	3.63	ELP
8	Theaceae	4	4.26	5	2.72	14	2.42	BI
9	Burseraceae	3	3.19	5	2.72	13	2.25	ELP
10	Moraceae	3	3.19	15	8.15	42	7.27	ELP

11	Myristicaceae	3	3.19	7	3.80	17	2.94	BI
12	Sapotaceae	3	3.19	5	2.72	24	4.15	ELP
13	Sterculiaceae	3	3.19	3	1.63	6	1.04	ELP
14	Anacardiaceae	2	2.13	2	1.07	4	0.69	ELP
15	Fagaceae	2	2.13	4	2.17	9	1.56	ELP
16	Actinidiaceae	1	1.06	1	0.54	3	0.52	BI
17	Apocynaceae	1	1.06	1	0.54	3	0.52	IR
18	Bombacaceae	1	1.06	1	0.54	1	0.17	ELP
19	Celastraceae	1	1.06	1	0.54	3	0.52	ELP
20	Cornaceae	1	1.06	1	0.54	1	0.17	ELP
21	Dilleniaceae	1	1.06	1	0.54	1	0.17	ELP
22	Dipterocarpaceae	1	1.06	2	1.09	4	0.69	BI
23	Ebenaceae	1	1.06	6	3.26	8	1.38	ELP
24	Elaeocarpaceae	1	1.06	3	1.63	9	1.56	ELP
25	Guttiferae	1	1.06	1	0.54	1	0.17	BI
26	Lecythidaceae	1	1.06	1	0.54	6	1.04	ELP
27	Leguminosae	1	1.06	1	0.54	1	0.17	ELP
28	Melastomaceae	1	1.06	1	0.54	5	0.87	ELP
29	Myrtaceae	1	1.06	10	5.43	15	2.60	ELP
30	Oleaceae	1	1.06	2	1.09	3	0.52	IR
31	Poligalaceae	1	1.06	2	1.09	2	0.35	ELP
32	Proteaceae	1	1.06	1	0.54	1	0.17	ELP
33	Rhamnaceae	1	1.06	1	0.54	2	0.35	ELP
34	Rhizophoraceae	1	1.06	1	0.54	1	0.17	ELP
35	Staphyleaceae	1	1.06	1	0.54	4	0.69	ELP
36	Styracaceae	1	1.06	1	0.54	1	0.17	ELP
37	Symplocaceae	1	1.06	2	1.09	5	0.87	ELP
38	Tiliaceae	1	1.06	2	1.09	15	2.60	ELP
39	Ulmaceae	1	1.06	1	0.54	3	0.52	ELP
40	Urticaceae	1	1.06	1	0.54	31	5.36	ELP
41	Verbenaceae	1	1.06	3	1.63	17	2.94	IR
42	Violaceae	1	1.06	1	0.54	32	5.54	ELP
	Total	94	100	184	100	578	100	

(Susatya 2007)

BI =Benzyl-iso Benzyl-isoquinoline (BI), Ellagic acid and proanthocyanins (EL&P), and Iridoid (Ir)

Four subclasses of the flowering plants was found in the site, and consisted of Magnoliidae, Dilleniidae, Rosidae, Asteridae, and Hamamelidae. Magnoliidae was characterized by the presence of Benzyl-isoquinoline (Cronquist 1988). Benzyl-Isoquinoline is one of alkaloid compounds that has various potentials to be further developed into anti-cancer, antibiotics, anti depressant drugs, and also for relieving Alzheimer's dan Parkinson's (Wiart 2006). This subclass consisted of Annonaceae, Myristicaceae, and Lauraceae (Table 1). These three families composed 8, 7, dan 12 tree species. Meanwhile, Dilleniadae, Hamamelidae, and Rosidae were characterized by the presence of Ellagic acid and Proanthocyanins (EL&P), which have function as anti oxydant. Proanthocyanin can be extraxted to develop drugs for lowering the risk of the heart attact, lowering the blood pressure, improving blood circulation,

and is also known to have stronger anti oxydant than that of vitamin C (Murphy et al 2003). Within this group, it was found 35 families (83%) consisting of 147 tree species (79%) (Fig. 1 and 2). The last subclass was Asteridae, which was characterized by the abundance of Iridoid. The isolation and purification of this secondary compound can be further developed for making cardiovascular-releted drugs, preventing and healing liver damaged by chemical substances (antipheptotoxic), anti tumor and virus, analgesic, and preventing swollen (Didna et al, 2007). This catagory contains Apocynaceae, Verbenaceae, Oleaceae, dan Rubiaceae with the total of 11 trees. In the family level, there were 3 (7.1%), 35 (83%), and 4 (9.4%) families with the presence of Benzyl-Isoquinoline, Ellagic acid and Proanthocyanins (EL&P), and Iridoid, respectively (Fig 1). Meanwhile in the species level, there were 27 (21%), 147 (79.5%), and 11 (5.4%) of tree species (Fig 2).

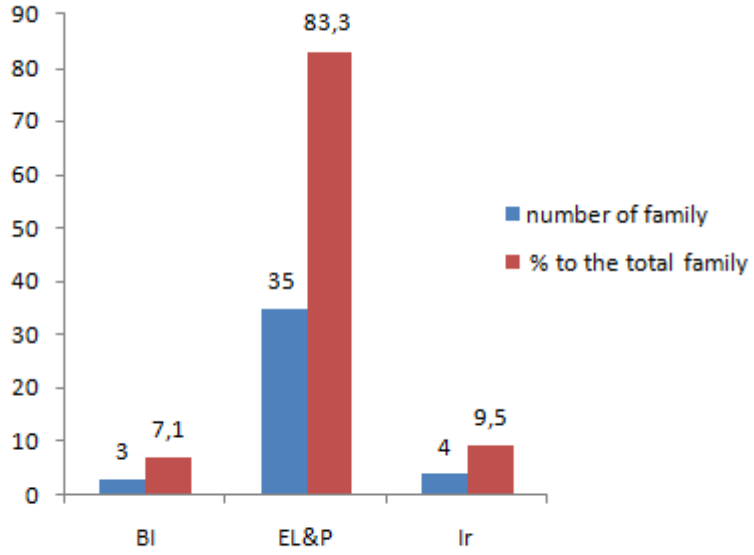

Fig 1: The number of family with the presence of Benzyl-isoquinoline (BI), Ellagic acid and proanthocyanins (EL&P), and Iridoid (Ir).

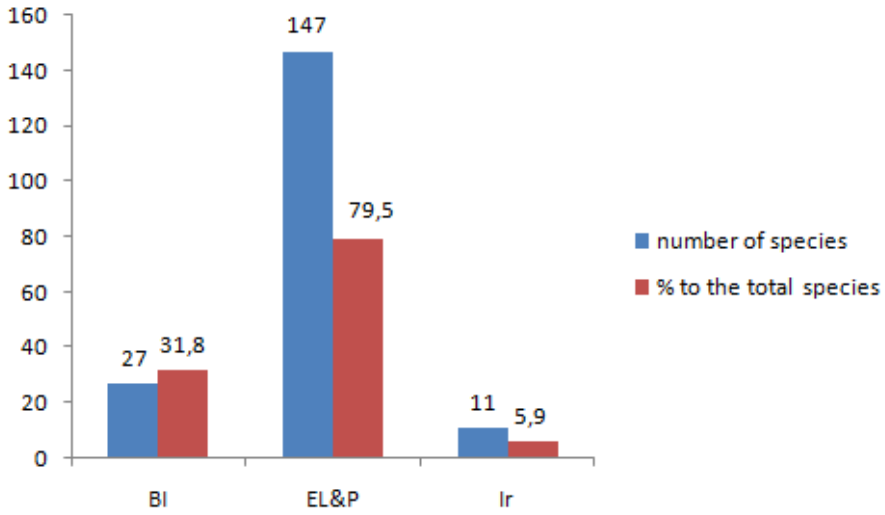

Fig 2: Number of species with the presence of Benzyl-isoquinoline (BI), Ellagic acid and proanthocyanins (EL&P), and Iridoid (Ir)

Detail reviews in the family level showed an interesting result concerning the potency of tree plants to be tapped their substances for anti tumor and cancer. Myristicaceae was known to have indole compound (Wiart, 2006). Indole and its derivates can be used to fight carcinogens induced by pesticides and othe chemical compounds (http//:www.phytochemicals.info/). Wiar (2006) also showed that phenolic compounds extracted from the bark of *Knema globerata* can inhibit the development of tumor cell. The substances from the member of Myristicaceae can also generate the hallucination effects which can be further used for developing antidepressant drugs, and healing the problem of central neural system (Wiart, 2007). Species of Myristicaceae found in the research site were *Gymnacranthera eugenifolia, G. farguhariana, G. forbesii, G. bancana, Horfieldia punctatifolia,* and *Knema glauca* (Susatya, 2007). Another subclass of Magnoliidae was Annonaceae consisting of 8 species distributed in the genus of *Cyathocalyx* (2 species), *Phaeanthus* (2 species), *Polyalthia* (1 species), *Pseduvaria* (1 species), and *Goniothalamus* (2 species). Annonaceae has been known to have substances for anti canter, anti bacteria, and reducing the risks of high blood pressure (Wiart, 2006).

Saponins can be found in the families of Sapindaceae and Sapotaceae, and can be used to inhibit colon cancer, and controlling collesterol level (Arnellia 2009). Furthermore, Sapindaceae contains Triterpenoid compound that can be used to reducing the risks of bone cancer or osteosarcome (Yasumasa et al. 2001). Spesies of Sapindaceae in the site were found in the genus of *Nephelium* (2 species), *Paranephelium* (1 species), *Pometia* (1 species), and *Ganophyllum* (1 species), while species of Sapotaceae consisted of the genus of *Palaquium* (2 species), and *Madhuca* (2 species).
Various species of Lauraceae were found in the research site and distributed in the genera of *Alseodaphne, Beilschmiedia,Cryptocarya, Endiandra,Litsea, Neolitsea, Persea,* and *Phoebe.* Secondary compounds in this family have characteristics of cytotoxics which can be used to against the development of tumor cells, , and also of neuroactives which can help to solve insomnia (Wiart 2006).

Species within Ebenacea are characterized to have naphathoquinones dan saponin. Naphathoquinones will generate black or brown effects on the various parts of plants when exposed to the direct sunlight. Naphathoquinones have capabilities for anti virus (Okuyama 1999, cytotoxis (Kuo et al 1997), anti oxydant (Wiart. 2006), and can be further developed to fight tumor (Kapadia et al. 1997) and leukemia (Maher et. al. 2009). Other uses of the secondary compounds within Ebanaceae were for anti inflammatory, and anti irritation. Furthermore, the saponins can be utilzed to fight colon cancer, and to lower the collesterol level (Arnelia 2009). Species of Ebenaceae found in the research site were *Diospyros apiculata, D. buxifolia, D. cauuliflora, D. pendula, D. pilosanthera,* and *D. sumatrana* (Susatya 2007).

Flacourticaceae consisted of 8 species in the genera of *Caesaria,* (3 species*), Hypnocarpus* (2 species), *Osmelia* (1 species), dan *Ryparosa* (2 species) (Susatya, 2007). Generally, Flacourticaceae can be sources of natural substances for inti inflammatory and anti irritation (Wiar, 2006). Different genera in this family have different substances for various purposes. Species of *Caesaria* have cytotoxic substantes, anti oxydant, anti bacteria, and anti HIV (Mossadik et al. 2004). Furhermore, the species of *Hypnocarpus* have secondary compounds that can be extracted to pruduce substances to cure leprosy (Wiart 2006).

Species from Melastomataceae, Rhizaophoraceae, Euphorbiacae, Sapindaceae, Anacardiaceae, and Meliaceae have tannin substances that can be utilized to control high

blood pressure, to cure fever, and to stop diarrhea (Wiart 2006). Species of *Croton* (Euphorbiacaea) can be further developed into anti tumor and HIV drugs (Wiart 2006). Futhermore, species of both Melastomaceae (Wiart 2006) and Rhizophoraceae (Premanathan et al. 1996) have potentials for anti HIV. Species found in the research site were *Croton argyratus*, *C. laevifolius* (Euphorbiaceae), *Ptenandra tessalata* (Melastomaceae), dan *Carallia brachiata* (Rhizophoraceae) (Susatya 2007).

Species of both Apocynaceae and Verbenaceae have various substances that can be utilized for developing for analgesic, anti pyretic, and anti inflammatory drugs. Especially, for the species of Apocynaceae, it has long been investigated their alkaloid substances for fighting cancer (Wiart 2009). One of species of Apocynaceae found in the site, *Wrightia pubescens*, has been examined for developing anti leukemia drug (Kawamoto et al. 2003). Elaeocarpaceae consisting of *Elaeocarpus floribundus*, *E. petiolatus* dan *E. stipularis* was known to have both cucurbitacins which has can be used againt cancer and indolizidine alkaloid which can be utilized againt HIV and diabetics (Fang et al. 1984). Furthermore, in the family level, it was 16 and 13 families that have potentials for anti cancer and HIV respectively, while inn the species level, it was 60 and 30 tree species respectively (Fig.3).

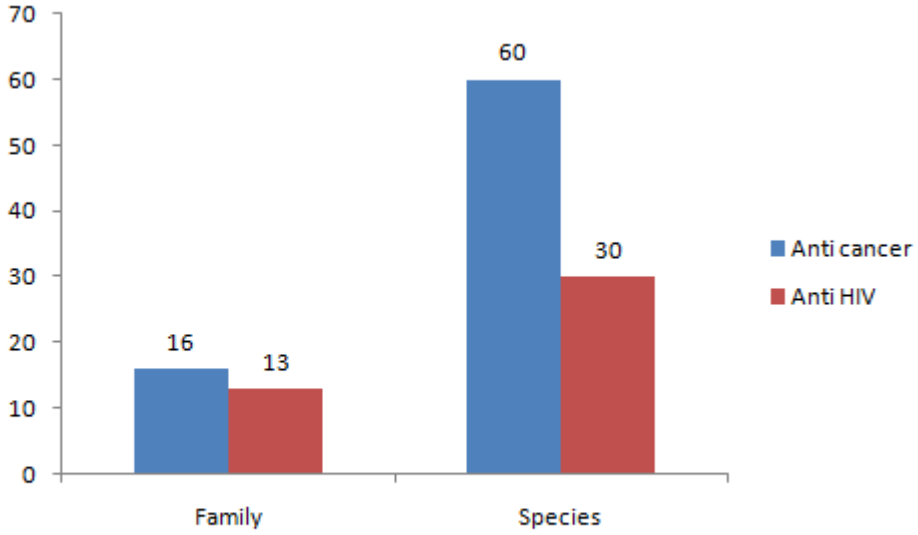

Fig 3: The number of family and species with anti cancer and HIV potentials

IV. Conclusion

Kerinci-Seblat National Park is known to have high tree species diversity. Within 1 ha plot, it was found 42 families, 94 genera and 185 species. All these tree species have evolved their defence mechanisms against herbivores through out developing various secondary compounds. Based on their secondary compounds, they can be examined their potentials as natural sources for bioactives, and further developed into drugs for againts depression, bacteria, tumor, cancer, oxydant, and for reducing the risk of heart attact, and protecting the liver. Sixteen families and 60 tree species have potentials for anti caner, while 13 families and 30 tree species can be used againts HIV.

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Morphological Variation and Phenetic Relationship of Hyacinth Bean (*Lablab purpureus* (L.) Sweet) in Lombok, West Nusa Tenggara

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Hyacinth bean (*Lablab purpureus* (L.) Sweet) is a member of Fabaceae which has high economic potential but is still treated as an underutilized crop. Research on hyacinth bean in Indonesia so far is only focused on bio-chemical content and protein isolate. However, the study on morphological characters of hyacinth bean has never been conducted. The aimed of this research was to study morphological variation and the phenetic relationship of hyacinth bean germplasm (*Lablab purpureus* (L.) Sweet) in Lombok, West Nusa Tenggara. The research was conducted in 4 regencies (East Lombok, Central Lombok, West Lombok and North Lombok) and samples (stem, leaf, flower, fruit, and seed) were collected using exploration method.The result showed that based on the morphological characters, the 26 collected samples showed that there were morphological differences on stem color, pod morphology (color, edge, texture), and seed morphology (color, shape, and spot existence). Furthermore, phenetic relationship of 26 OTU's (Operational Taxonomy Unit) based on 78 morphological characters showed that the hyacinth bean was divided into 2 large groups. The groups was differentiated by pod color. Group I had white or green pod with light or dark green edge while group II had white pod with purple edge. Member of group II was a dominant germplasm of hyacinth bean.

Keywords: hyacinth bean, morphological variation, phenetic relationship, Lombok

Introduction

Hyacinth bean (*Lablab purpureus* (L.) Sweet) is a member of Fabaceae originated from Afrika and widely distributed in tropics and subtropics (Konduri *et al.*, 2000). Over past 50 years, hyacinth bean has become an important legume crops particularly in Australia and America (Maass *et al.*, 2005). In Indonesia hyacinth bean were found in East Java and West Nusa Tenggara (Setyorini, 2008). Hyacinth bean has a high economic potential but is still treated as an underutilized crop. Their seeds can be used as an alternative resources in making tahu and tempeh, ketchup, composite flour, delicacy (emping), and protein isolate (Subagyo & Morita, 2008; Anonim, 2009). Smartt (1985) in Pengelly & Maass (2001) stated that this legume should be a priority in developing legumes potential in tropical agriculture.

Developing a crops potential needed basic information about their phenotype (morphology) and genetic diversity. Radford (1986) said that morphological characters traditionally were useful evidence in whole taxonomic levels particularly in species and genus. The appearance of morphological characters were affected by genetic and environmental factor. Researches on plant diversity usually started from the existence of morphological variation indicated in certain plant population.

Hyacinth bean shows diversity on their morphological characters particularly in flower colour, seed characteristics, and pod characteristics (Yuan *et al.*, 2008). In Lombok, people called hyacinth bean as *komak* and named it based on their growth form and pod appearance. Generally hyacinth bean divided into 2 types namely wild type and cultivated type. Wild type were characterized by erect and bushy growth form, relatively small seed and existence of spots on the seed. Cultivated type were characterized by climbing growth form,

relatively large seed, and usually no spots on the seed (Maass, 2006; Hall & Naidu, 2008). Researches on morphological variation in hyacinth bean were done by Pengelly & Maass (2001), Maass (2006), and Gowda (2006). Pengelly & Maass (2001) found that seed weight, seed colour, and pod length were distinguished character between wild and cultivated type. Gowda (2006) and Maass (2006) found that seed characteristics especially seed weight was the most varied character of hyacinth bean.

Material and Method

The research was conducted in 4 regencies (West Lombok, Central Lombok, East Lombok, and North Lombok) and samples (stem, leaf, flower, fruit, and seed) were collected using exploration method. Twenty six samples whose morphologically completed were collected during the exploration. Scoring and standarization were done on 78 morphological characters based on descriptor used by Pengelly & Maass (2001) and Gowda (2006). List of morphological characters that had been used in this research are in Table 1.

Table 1. Qualitative and quantitative characters of hyacinth bean used for this research

Characters			
	Qualitative characters	40	Appendage position on raceme
1	Habitus	41	Fruit type
2	Growth form	42	Pod constriction
3	Leaf type	43	Pod colour
4	Leaf vernation	44	Pod edge
5	Leaf venation	45	Pod serrate colour
6	Leaf colour	46	Pod edge colour
7	Leaf vein colour	47	Down edge pod colour
8	Leaf hairiness on down surface	48	Pod rod colour
9	Petiole colour	49	Pod attachment (young)
10	Pulvinus colour	50	Pod attachment (mature)
11	Leaf stalk shape	51	Pod widest part
12	Leaf stalk hairiness	52	Pod surface
13	Terminal leaflet shape	53	Seed colour (young)
14	Terminal leaflet margin	54	Seed colour (mature)
15	Leaf apice type	55	Seed colour (dry)
16	Terminal leaflet petiole colour	56	Spot existence on the seed
17	Terminal leaflet base	57	Spot,s dispersal
18	Left leaflet shape	58	Seed shape
19	Left leaf let margin	59	Hilum colour
20	Left leaflet apice	60	Inner seed colour
21	Left leaflet petiole colour		**Quantitative characters**
22	Left leaflet base	61	Pulvinus length
23	Right leaflet shape	62	Leaflet lenght
24	Right leaf let margin	63	Terminal leaf ratio
25	Right leaf apice	64	Petiole length of terminal leaf
26	Right leaf base	65	Stipule number of terminal leaf
27	Stem colour	66	Left leaf ratio
28	Stem hairiness	67	Petiole length of left leaflet
29	Stem shape	68	Stipule number of left leaflet
30	Branch orientation	69	Right leaf ratio
31	Raceme type	70	Petiole length of right leaflet
32	Raceme stalk shape	71	Stipule number of right leaflet
33	Raceme stalk colour	72	Raceme length
34	Raceme position	73	Pod ratio
35	Flower bud colour	74	Number of seeds/pod
36	Petal colour	75	Seed ratio (fresh)

Result and Discussion

This research showed that from 26 samples collected based on morphological characters, the hyacinth bean varied on stem colour, leaf characteristics (venation colour and leaf shape), pod characteristics (pod colour, pod edge colour, and pod texture), seed characteristic (seed shape, seed colour, and the existence of spots on the seed).

Table 3. List of samples and morphological characteristic of hyacinth bean (*Lablab purpureus* (L.) Sweet) in Lombok, West Nusa Tenggara

No	Collection name	Local name	Morphological Characteristics	Location
1	LOBAR 1	*Komak IR*	Terminal leaf round,purple stem, purple flower, short raceme, white pod with purple edge, black seed	Kediri Village, Subdistrict Kediri, West Lombok
2	LOBAR 2	*Komak Beaq Bokon*	Temninal leaf *deltoid*, purple stem, purple flower, long raceme, white pod with purple edge, dark brown seed	Narmada village, Subdistrict Narmada, West Lombok
3	LOBAR 3	*Komak Putiq*	Terminal leaf *deltoid*, light green stem, white flower, long raceme, white pod with light green edge, dark brown seed	Medana village, Subdistrict Tanjung , West Lombok
4	LOTENG 1	*Komak Ejo*	Terminal leaf *deltoid*, dark green stem, white flower, long raceme, light green pod with green edge, brown seed	Pujut village, Subdistrict Pujut, Central Lombok
5	LOTENG 2	*Komak Putiq*	Terminal leaf *deltoid*, light green stem, white flower, long raceme, white pod with light green edge, dark brown-black seed	Segala Anyar village, Subdistrict Pujut, Kabupaten Central Lombok
6	LOTENG 3	*Komak Bangket*	Terminal leaf *deltoid*, dark green stem, white flower, long raceme, green pod with dark green edge, cream seed with spots near hilum	Segala Anyar Village, Subdistrict Pujut, Central Lombok
7	LOTENG 4	*Komak Ejo*	Terminal leaf *deltoid*, dark green stem, white flower, short raceme, green pod with dark green edge, dark brown seed	Kawo Village, Subdistrict Pujut, Central Lombok
8	LOTENG 5	*Komak Putiq*	Terminal leaf *deltoid*, light green stem, white flower, long raceme, white pod with light green edge, brown seed	Barejulat Village, Subdistrict Jonggat, Central Lombok
9	LOTENG 6	*Komak Ejo*	Terminal leaf *deltoid*, dark green stem, purple to reddish flower, long raceme, green pod with dark green edge, black seed	Barejulat Village, Subdistrict Jonggat, Central Lombok
10	LOTENG 7	*Komak Ejo*	Terminal leaf *deltoid*, green stem, white flower, short raceme, light green pod with green edge, dark brown seed	Barejulat Village, Subdistrict Jonggat, Central Lombok
11	LOTENG 8	*Komak Beaq Bokon*	Terminal leaf *deltoid*, purple stem, purple flower, long raceme, white pod with purple edge, black seed	Barejulat Village, Subdistrict Jonggat, Central Lombok
12	LOTENG 9	*Komak Beaq Bokon Belo*	Terminal leaf rhomboid, purple stem, purple flower, short raceme, white pod with purple edge, black seed	Jago Village, Subdistrict Praya Tengah, Central Lombok
13	LOTENG 10	*Komak Lanter*	Terminal leaf rhomboid, green stem,white flower, short raceme, light green pod with dark green edge, black seed	Pengenjek Village, Subdistrict Pringgarata, Central Lombok
14	LOTENG 11	*Komak Putiq*	Terminal leaf *deltoid*, light green stem, white flower, long raceme, white pod with light green edge, brown seed	Aik Bukak Village, Subdistrict Batukliang Utara, Central Lombok
15	LOTENG 12	*Komak Beaq Bokon*	Terminal leaf round, purple stem, purple flower, long raceme, white pod with purple edge, black seed	Aik Bukak Village, Subdistrict Batukliang Utara, Central Lombok

No	Collection name	Local name	Morphological Characteristics	Location
16	LOTENG 13	*Komak Beaq Bokon Belo*	Terminal leaf *deltoid*, purple stem, purple flower, short raceme, white pod with purple edge, black seed	Aik Bukak Village, Subdistrict Batukliang Utara, Central Lombok
17	LOTENG 14	*Komak Beaq Bokon Belo*	Terminal leaf *deltoid*, purple stem, purple flower, short raceme, white pod with purple edge, black seed	Karang Sidemen Village, Subdistrict Batukliang Utara, Central Lombok
18	LOTENG 15	*Komak Beaq Bokon*	Terminal leaf *deltoid*, purple stem, purple flower, long raceme, white pod with purple edge, brown seed	Karang Sidemen Village, Subdistrict Batukliang Utara, Central Lombok
19	LOTIM 1	*Komak Putiq*	Terminal leaf rhomboid, light green stem, white flower, long raceme, white pod with light green edge, dark brown seed	Montong Bunut Village, Subdistrict Tanjung Luar, East Lombok
20	LOTIM 2	*Komak IR*	Terminal leaf *deltoid*, purple stem, purple flower, tlong raceme, white pod with purple edge, black seed	Montong Bunut Village, Subdistrict Tanjung Luar, East Lombok
21	LOTIM 3	*Komak Leles*	Terminal leaf rhomboid, dark purple stem, purple flower, long raceme, white pod with purple edge, dark brown seed	Prian Village, Subdistrict Montong Gamang, East Lombok
22	LOTIM 4	*Komak Putiq*	Terminal leaf *deltoid*, light green stem, white flower, long raceme, white pod with light green edge, dark brown seed	Prian Village, Subdistrict Montong Gamang, East Lombok
23	LOTIM 5	*Komak Putiq*	Terminal leaf *deltoid*, light green stem, white flower, short raceme, white pod with light green edge, brown seed	Bat Man, Subdistrict Selaparang Village, East Lombok
24	LOTIM 6	*Komak Leles*	Terminal leaf *deltoid*, dark purple stem, purple flower, short raceme, white pod with purple edge, black seed	Bat Man Village, Subdistrict Selaparang, East Lombok
25	LOTAR 1	*Komak Beaq Bokon*	Terminal leaf *deltoid*, dark purple stem, purple flower, short raceme, white pod with purple edge, dark brown seed	Senaru Village, Subdistrict Bayan, North Lombok
26	LOTAR 2	*Komak Beaq Bokon*	Terminal leaf *deltoid*, dark purple stem, purple flower, long raceme, white pod with purple edge, black seed	Senaru Village, Subdistrict Bayan, North Lombok

Pod and seed characteristics were the most varied character among hyacinth bean population in Lombok. Based on pod characteristics, hyacinth bean in Lombok were divided into 3 large groups : (1) member of hyacinth bean which had white pod with purple edge, it was the most common hyacinth bean found in Lombok; (2) member of hyacinth bean which had white pod with light green edge; and (3) member of hyacinth bean which had green pod with dark green edge, this kind of bean were rare and hard to found.

Morphological variation in hyacinth bean showed many characters were polymorphic in both their vegetative and generative organs. Of 78 characters, 65 were polymorphic consist of 18 quantitative characters and 47 were qualitative characters. Phenetic-numerical analysis of hyacinth bean showed morphologically they were clustering based on certain characters and showed no reflect on geographic.

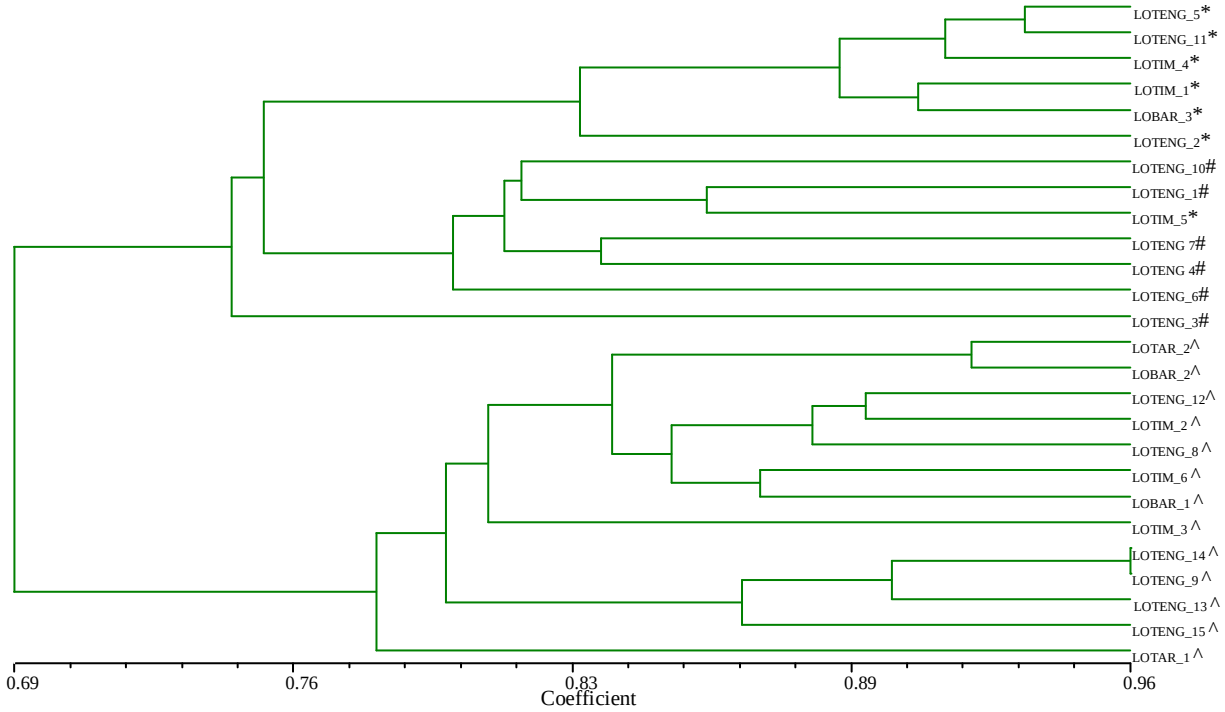

Figure 1. Dendrogram showed phenetic relationships based on morphological characters on hyacinth bean (*Lablab purpureus* (L.) Sweet) in Lombok, West Nusa Tenggara

Note :

* : white pod with light green edge

^ : white pod with purple edge

\# : green pod with dark green edge

Dendrogram (Figure 1) showed phenetically hyacinth bean in Lombok were divided into 2 large clusters with similarity index ranging between 69-96%, LOTENG 9 and LOTENG 14 were the closest OTU with similarity index 96%. Stem colour, flower colour, pod edge colour, and seed colour were distinguished character of both clusters. Cluster I were characterized by green series on stem colour (light green or dark green), vein colour, and pod edge colour (light green or dark green); white flowers; and brown seed on almost their member. Cluster I were divided into 3 subclusters with stem colour, pod colour, and raceme length were distinguished each subclusters. Clusters II were characterized by purple series on their stem colour, vein colour, pod edge colour, and flowers colour; seed colour varied from black (70%) to dark brown (30%). Cluster II were divided into 3 subclusters with raceme length were characterized each subclusters.

Those clustering pattern and distinguished characters were differ with clustering pattern of hyacinth bean found in Africa, India, and Australia. Pengelly & Maass (2001) found that hyacinth bean in India, Africa, and Australia were clustered based on wild and cultivated type and not reflected geographic origin. Members of the clusters were determined by seed characteristics (seed weight and colour) and pod size. Meanwhile, members of subclusters were determined by raceme length, time to flower, no.of nodes/raceme, plant height, leaf and pod length. The same results were also obtained by Maass (2006) and Gowda (2006) stated that seed weight were the most varied character.

Morphological diversity of hyacinth bean in Lombok had similarity index ranging from 69-96%. This value was lower than Pengelly & Maass (2001) which showed similarity index of hyacinth bean in India, Africa, and Australia ranging from 89-99%. It might be because the differences in number, characters type, and samples collected method that had been used in their research. Pengelly & Maass (2001) were collected samples for core collection candidate so not all hyacinth bean germplasms which showed morphological variation in both their vegetative and generative organs were chosen as a sample. Meanwhile, samples collected on analysis of morphological variation of hyacinth bean in Lombok were conducted by exploration method where each morphological variation that found in each location were collected to characterized.

Although it does not reflect geographic pattern but certain hyacinth bean which had white pod with purple edge tend to had wide geographic distribution and considered as a dominant. The other hyacinth bean which had green pod tend to had narrower distribution area. This hyacinth bean with narrow distribution area needed to consereved immediately to prevent them from extinction because of habitat lost and lack of people concerned to conserved (Maass *et al*., 2005; Hall & Naidu,2008).

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Diversity of Birds in Tepus Village of Gunungkidul District of Yogyakarta

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ABSTRACT

Tepus village of Gunungkidul District is one of the coastal regions of karst in the Daerah Istimewa Yogyakarta. Coastal karst area generally has its own species of avifauna that typical considering several factors supporting the life of birds as well as the existence of the reef, the typical vegetation of karst and others. This research aims to discover the diversity species of birds in the Tepus village of Gunungkidul District. The study was conducted during two months in May - June 2011. We retrieval the data with *Explore Method* and analyze it with an *Encounter Rates* Method. Observers are divided into 3 groups with different observation spot but are in one location. The types of birds are observed will be identified, count the number of individual preference, and recorded in a table of observations.

The results showed there are 42 species of birds included into 22 families. Types with the highest abundance are in Cave Swiftlet (*Collocalia linchi*) with a value of 193.548387. Species of birds endemic to the island of Java that was found was the Sunda Coucal (*Centropus nigrorufus*), Flame-Fronted Barbet (*Megalaima armillaris*), Plain Prinia (*Prinia inornata*), and the Brown Prinia (*Prinia polychroa*). The Sunda Coucal is recorded VU (Vulnerable)/ threatened in Red list of IUCN. Based on the Status of the rules of international trade in CITES, Peregrine Falcon (*Falco peregrinus*) which was recorded in the area are listed in Appendix I. Beside of that there are eight species of birds are listed as protected species Act No. 5/1999 and PP No. 7/1999.

Key words: *Diversity, Birds, Karst, Tepus, Gunungkidul.*

INTRODUCTION

Indonesia is known as a country that has the highest biodiversity of Southeast Asia, at the level of world Indonesia was ranked second after Brazil, there are even says "world's mega biodiversity nation" which is included in the group "biodiversity hotspots" nations ". This is not the geographical layout of Indonesia. Geographically Indonesia has thousands of islands that lies between the two continents of Asia and Australia, and crossed the equator.
With this position, Indonesia is one of the countries which have great biodiversity in the world. With a total area of 1.3% of the Earth, as a country, Indonesia has mega biodiversity wildlife is very high which consists of mammals 515 species (12% of the mammals of the world), the reptiles 511 species (7.3% of the species of reptiles in the world), amphibians 270 species of animals are vertebrates 2800 species, 35 species of primates, 1400 species of freshwater fish and 121 species of butterflies (Status Lingkungan Hidup, 2008). In addition, Indonesia is one of the important areas for biodiversity bird; Indonesia had a 1598 bird species (17% of the world). From 1598 was scattered across Indonesia, the island of Sumatra has 628 species, the Java-Bali 507 species, Lombok and surrounding 426 species, Papua 671 species, Borneo and Sulawesi species 522.416 species (Brando, 2011).

Birds are an important component in the food chain cycles in a region. Birds play a role in assisting the process of dispersal of seeds, assisting the process of pollination to the flowers,

and as indicators of environmental pollution. Because of that, bird conservation is needed. For that purpose, collecting the data about the diversity and abundance of bird in certain area was very important to do, particularly in the Tepus Village of Gunungkidul District that consist of three beaches, Sundak Beach, Indrayanti Beach and Watulawang Beach. Along that beaches are a constituent composition of natural attractions with karst rocks and white sand beaches. With the composition of karst rocks and white sand in the region must determine the diversity of birds. For that, there is need for data collection diversity and abundance of birds in Tepus Village.

METHODS

The method that we used to retrieval the data is *Explore Method*. The area divided in to three of trek observation. Then the observers divided too for take the data of each trek in the same time. The method for analyze the data research is *Encountered rates*. Encounter rate data can be split into crude ordinal categories of abundance (e.g. abundant, common, frequent, uncommon and rare) (Bibby et.al, 2000), making these terms much more useful as they have some definition and allowing a species list to be annotated in such a way that future surveys might detect large scale changes in the abundance of individual species. The analysis of encounter rate data is very straightforward. There are three observers to collecting the data. Each observer has a different observation trek. The encounter rate for each species is equal to the total number of individuals recorded by all three observers divided by the period of observation and multiplied by ten to give a result in units of number of individuals recorded per ten hours of survey.

STUDY AREA

Picture 1. Map of the Area of Research (Anonim, 2011)

Tepus Village of Gunungkidul District consists of three beaches, Sundak Beach at 08,146150 Indrayanti Beach at 08, 149683 and Watulawang Beach at 08,149683. Those beaches are centre of tourism where the birds live also. According to the physics view (Picture 1), observation area divided in to two kinds of area, The Coastal Shrub and Karst Forest. Type of The Coastal Shrub dominated with underbrush and shrubs on row of reefs that have low density of vegetation. The substrate is white-sand so that can not save water well. The vegetation consists of genus *Casuarina* that known as introduction plants, *Pandanus* spp, Acacia, and row of Coconut trees (*Cocos nucifera*).

The Karst Forest is range of hills. This area consists of caves and river that flows under the land surface. Water distribution move faster based on the type of vegetation that dominating. Commonly, the composition of plants is dominated by *Tectona grandis, Acacia nilotica,* and *Acacia longifolia*.

RESULTS

During the data retrieval, there are 42 species of birds around the location of observation that is included in to 22 families (table 1). Types of waterfowl found amounted to 5 species in the Family Ardeidae, Sternidae, Stercorariidae, and Phaetonidae. Rusila-Noor et.al. (1999) wrote that the water can be defined as a type of birds ecologically dependent on wetlands. Wetlands are referred to in this research area is the coral and white sand beaches along the Sundak Beach - Indrayanti Beach - Watulawang Beach which are characteristic of karst area. The land is used by waterfowl as a nesting and foraging. The other is the seventeenth tribal land birds that many tribes inhabiting the forests of lime and Sandy bush land.

Table 1. Bird abundance in Tepus, Wonosari, Gunung Kidul DI Yogyakarta

Species		Number of individuals by each observer (3 observers)			Number of individuals	Number of individuals/10 hours	Class or relative abundance
Family	**Scientific Name**	**1**	**2**	**3**			
Ardeidae	*1. Egretta sacra*	1	1	1	3	1.935483871	uncommon
Sternidae	*2. Gygis alba*	6	9	6	21	13.5483871	common
	3. Sterna sumatrana	1	2	10	13	8.387096774	frequent
Apodidae	*4. Collocalia linchi*	100	100	100	300	**193.5483871**	abundant
	5. Apus affinis	7			7	4.516129032	frequent
	6. Collocalia maxima	2			2	1.290322581	uncommon
Nectarinidae	*7. Nectarinia jugularis*	7	2	3	12	7.741935484	frequent
	8. Anthreptes malacensis	1	1		2	1.290322581	uncommon
Columbidae	*9. Streptopelia chinensis*	3	1	1	5	3.225806452	frequent
	10. Macropygia ruficeps	2			2	1.290322581	uncommon
	11. Geopelia striata			3	3	1.935483871	uncommon
	12. Macropygia emiliana		1		1	0.64516129	uncommon
	13. Treron sp.		1		1	0.64516129	uncommon
	14. Streptopelia bitorquata		1		1	0.64516129	uncommon
Alcedinidae	*15. Halcyon chloris*	13	7	4	24	15.48387097	common
Lanidae	*16. Lanius schach*	8	6	3	17	10.96774194	common
	17. Lanius cristatus	1	2		3	1.935483871	uncommon
Ploceidae	*18. Passer montanus*	23		1	24	15.48387097	common
Estrildidae	*19. Lonchura leucogastroides*	60	45	23	128	82.58064516	abundant
	20. Lonchura punctulata		2		2	1.290322581	uncommon
Pycnonotidae	*21. Pycnonotus aurigaster*	14	43	33	90	58.06451613	abundant
	22. Pycnonotus goiavier	15	2		17	10.96774194	common
Falconidae	*23. Falco peregrinus*	2	1		3	1.935483871	uncommon

Silviidae	*24. Orthotomus palpebrosus*	4		2	6	3.870967742	frequent
	25. Prinia polychroa	2	1		3	1.935483871	uncommon
	26. Prinia inornata			5	5	3.225806452	frequent
	27. Orthotomus ruficeps		1		1	0.64516129	uncommon
	28. Prinia sp.		2		2	1.290322581	uncommon
	29. Cisticola juncidis	6	1		7	4.516129032	frequent
Acanthizidae	*30. Gerygone sulphurea*		2		2	1.290322581	uncommon
Cuculidae	*31. Centropus nigrorufus*	2			2	1.290322581	uncommon
	32. Centropus sinensis			1	1	0.64516129	uncommon
	33. Cocomantis sepulcralis	1	1	1	3	1.935483871	uncommon
	34. Cocomantis merulinus	1		1	2	1.290322581	uncommon
Alaudidae	*35. Mirafra javanica*		1		1	0.64516129	uncommon
Stercorariidae	*36. Stercorarius parasiticus*			1	1	0.64516129	uncommon
Phaetontidae	*37. Phaethon lepturus*			1	1	0.64516129	uncommon
Dicaeidae	*38. Dicaeum trochileum*			2	2	1.290322581	uncommon
Capitonidae	*39. Megalaima armillaris*	1			1	0.64516129	uncommon
Aegithinidae	*40. Aegithina tiphia*	1		4	5	3.225806452	frequent
Hirundinidae	*41. Dalycon dasipus*	5		1	6	3.870967742	frequent
Artamidae	*42. Artamus leucorhyncus*	5	2		7	4.516129032	frequent

Description: Relative abundance in the table above refers to the standards established by Lowen et al (1996) as follows:

Table 2. Standard Value Abundance

Abundance category (Number of individuals per 100 field hours)	Abundance score	Ordinal scale
< 0,1	1	rare
0,1 - 2,0	2	uncommon
2,1 - 10,0	3	frequent
10,1 - 40,0	4	common
40,0 +	5	abundant

The highest abundance values are the type with a value of 193.5483871 Cave Swiftlet (*Collocalia linchi*). The lowest abundance is 0.64516129 which are occupied by several species of birds that is Island Collared Dove, Horsfield's Bush Lark, Ruddy Cuckoo Dove, Pigeon (not identified), Ashy Tailorbird, Parasitic Jaeger, White-tailed Tropicbird, Sunda Coucal, and Flame-fronted Barbet. In this study also successfully found four bird species have limited distribution (endemic), is the only bird species found in Java. The four species of birds are Sunda Coucal (*Centropus nigrorufus*), Flame-fronted Barbet (*Megalaima armillaris*), Plain Prinia (*Prinia inornata*), and Brown Prinia (*Prinia polychroa*). Nine species of birds in this study also entered into the record some of them Wildlife protection status according to IUCN (*International Union Conservation of Nature and Natural Resources*), the international status according to CITES (*Convention on International Trade of Endangered*

Species of Wild Fauna and Flora) trade regulations, and protection status of the Law of the State of Republic Indonesia (Table 3).

DISCUSSION

The high value of abundance of a species of bird can be interpreted that these birds can adapt to environmental conditions, both physical conditions such as weather and landscape as well as other supporting conditions such as availability of food, type of vegetation and other animal species that live in the same environment. Several groups of birds can live sustainably until today due to have succeeded in creating a special niche for itself to reduce competition for resources and needs as a form of adaptation to environmental conditions.

The high value of abundance of a species of bird can be interpreted that these birds can adapt to environmental conditions, both physical conditions such as weather and landscape as well as other supporting conditions such as availability of food, type of vegetation and other animal species that live in the same environment. Several groups of birds can live sustainably until today due to have succeeded in creating a special niche for itself to reduce competition for resources and needs as a form of adaptation to environmental conditions. The southern coastal region dominated Limestone Mountains has fewer sources of food and thus affects the rate of population of a species, especially birds. Some species become difficult to find because it is very sensitive to changing environmental conditions and human existence, allowing species is very difficult to find or even not found.

Several bird species that enter the lowest abundance value caused less able to utilize the resources that exist around the environment. Bird family Columbidae (*Streptophelia bitorquata*, *Macropygia Emiliana* and *Treron* sp.) is the fruit and seed-eating birds. While the intensive search areas are dry areas with open composition of vegetation *Tectona grandis*, *Acacia nilotica*, and *Acacia longifolia*. Ashy Tailorbird (*Orthotomus ruficeps)* an insectivorous bird, the bird is rarely found in the area of research. This is because in addition to the size of 11 cm is also sensitive to the presence of observers. Parasitic Jaeger *(Stercorarius parasiticus)* is a habitat for water birds; these birds are rarely found because the activity of these birds flew low over the sea. This species is found on the offensive Black-naped Tern *(Sterna sumatrana)* on cliffs (Mackinnon, 2010). White-tailed Tropicbird *(Phaeton lepturus)* is a habitat for water birds, these birds are rarely found because in addition to his activities were flying over the ocean surface to look for pelagic fish meal (fish floating), assumed to be a little individual. Sunda Coucal (*Centropus nigrorufus)* is a bird-eating caterpillar (Mackinnon, 2010), this bird is rarely found because in addition to the limited spreading of endemic Java, has a tolerance level which a narrow habitat. This bird has the only coastal habitats and wetlands. Flame-fronted Barbet *(Megalaima armillaris)* is eating fruits, Ficus (Banyan tree) and insects. This bird is rarely found because of the color that resembles a leaf and is a quiet bird.

Table 3. Species Protection Status

Species		Distribution	Status
Common Name	**Scientific Name**		
Pacific Reef Egret	*Egretta sacra*	SKJCMTP	AB[3]
Angel Tern	*Gygis alba*	SKJMTP	AB[3]
Black-naped Tern	*Sterna sumatrana*	SKJCMTP	AB[3]
Cave Swiftlet	*Collocalia linchi*	SJT	*

House Swift	*Apus affinis*	SKJCT	*
Black-nest Swiftlet	*Collocalia maxima*	SKJ	*
Olive-backed Sunbird	*Nectarinia jugularis*	SKJCMTP	AB[3]
Brown-throated Sunbird	*Anthreptes malacensis*	SKJCMT	AB[3]
Spotted Dove	*Streptopelia chinensis*	SKJFFT	*
Little Cuckoo Dove	*Macropygia ruficeps*	SKJT	*
Zebra Dove	*Geopelia striata*	SFJFFT	*
Ruddy Cuckoo Dove	*Macropygia emiliana*	SKJT	*
Pigeon	*Treron sp.*		*
Island Collared Dove	*Streptopelia bitorquata*	FKJCMT	*
Collared Kingfisher	*Halcyon chloris*	SKJCMTP	AB[3]
Long-tailed Shrike	*Lanius schach*	SKJCMT	*
Brown Shrike	*Lanius cristatus*	SKJCMTP	*
Javan Munia	*Lonchura leucogastroides*	SJTE	*
Eurasian Tree Sparrow	*Passer montanus*	SJ	*
Scaly-breasted Munia	*Lonchura punctulata*	SKJCT	*
Sooty-headed Bulbul	*Pycnonotus aurigaster*	FFJF	*
Yellow-vented Bulbul	*Pycnonotus goiavier*	SKJFMT	*
Peregrine Falcon	*Falco peregrinus*	SKJCMTP	I[2] – AB[3]
Common Tailorbird	*Orthotomus palpebrosus*	FFJFMTF	*
Golden-bellied Gerygone	*Gerygone sulphurea*	SKJCMT	*
Brown Prinia	*Prinia polychroa*	J	*
Plain Prinia	*Prinia inornata*	J	*
Ashy Tailorbird	*Orthotomus ruficeps*	SKJ	*
Prinia	*Prinia sp.*		*
Zitting Cisticola	*Cisticola juncidis*	SKJCMTP	*
Sunda Coucal	*Centropus nigrorufus*	J	VU[1] – AB[3]
Greater Coucal	*Centropus sinensis*	SKJ	*
Rusty-breasted Cuckoo	*Cocomantis sepulcralis*	SKJCMT	*
Plaintive Cuckoo	*Cocomantis merulinus*	SKJC	*
Horsfield's Bush Lark	*Mirafra javanica*	KJTP	*
Parasitic Jaeger	*Stercorarius parasiticus*	JCT	*
White-tailed Tropicbird	*Phaethon lepturus*	SKJCMTP	*
Scarlet-headed Flowerpecker	*Dicaeum trochileum*	SKJT	*
Flame-fronted Barbet	*Megalaima armillaris*	J	AB[3]
Common Iora	*Aegithina tiphia*	SKJ	*
Asian House Martin	*Dalycon dasipus*	SKJ	*
White-breasted Woodswallow	*Artamus leucorhyncus*	SKJCMTP	*

Description: [1]Status IUCN protection; [2]Status CITES protection; [3]Status Republic Indonesia Protection based on Act No. 5/199 and PP No. 7/1999; * Status Unknown

Based on the results of observation, there are 42 species of 22 families with different of habitat conditions and habits. Of the 42 species found 1 of them according to the IUCN is Sunda Coucal (*Centropus nigrorufus*). One of them according to CITES included in Appendix I is Peregrine Falcon (*Falco peregrinus*). All types in Appendix I are endangered species and will be impact if traded. Trading is only permitted only in certain conditions such as for scientific research). While five of them including protected species Law no. 5 / 1990 (*On the Conservation of Natural Resources and Ecosystems*) and PP. 7 / 1999 (*Concerning the Preservation of Type Plants and Animals*) are Pacific Reef Egret (*Egreta sacra*), Black-naped Tern (*Sterna sumatrana*), Olive-backed Sunbird (*Nectarinia jugularis*), Collared Kingfisher (*Halcyon chloris*), Peregrine Falcon (*Falco peregrinus*), Angel Tern *(Gygis alba),* and Flame-fronted Barbet (*Megailama armillaris).* Because a lot of bird that protected with Law, There is a need to continue the monitoring and conduct a more intensive survey at Tepus Village for the conservation of bird there.

CONCLUSION

There are 42 species of bird that conclude in 22 families in Tepus Village of Gunungkidul District Yogyakarta. High or low abundance value of a bird species can be interpreted that these birds can adapt to environmental conditions. The environmental condition of Tepus Village is karst that supports some specific bird to life with all of the excesses and lacks.

Tepus Village of Gunungkidul District Yogyakarta is importans site of bird.

ACKNOWLEDGEMENT

We say thank you especially for BIOLASKA over the infinite place of learning. The highest appreciation to all members of BIOLASKA, Joko, Dini, Nurdin, Avid, Ita, Marvi, Tutut, mas Imam, mas Slem who has been faithful help in data retrieval. Thanks especially to the Mas Untung for guidance in terms of research methods, Mas Nasir on GPS and map coordinates, mas Irhan and mas Ari Fauzi for their willingness in reviewing this manuscript. We are also very grateful to Mr. M. Jafar Luthfi on his motivation to continue learning to write.

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Detection of Immunoglobulin Geneheavy Chain Binding Protein in *Eimeria tenella* Collected from Yogyakarta Using One Step Reverse Transcriptase PCR

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ABSTRACT

Coccidiosis is an infection disease caused by *Eimeria tenella* in chicken intestine. The disease causes the economic lost in poultry industry. This disease is very difficult to be controlled for some reasons, *e.g.*the lack of accurate diagnostic tools, expensive vaccinationcost, and coccidiost at resistance in Eimeria. Immunoglobulin heavy chain binding protein (IHC-BiP) is an immunogenic agent for internal parasite which involves 70 families of Eimeria in the heat shock protein. Gene sequence of the binding protein(IHC-BiP) was found in *E. tenella* and *E. maxima* from United Kingdom, however there is no information about this gene in Eimeria from Indonesia. This research was aimed to find IHC-BiP gene in four developmental stages of *E. tenella* collected from Yogyakarta. Samples were collected from the field and propagated in 50 chickens. Each chicken was infected with 10.000 *E. tenella* oocysts. The oocyst of *E. tenella* from propagation were then sporulated and prepared for sporocyst and sporozoite stages. Total RNAs were isolated from each stage of *E. tenella* development (unsporulated oocyst, sporulated oocyst, sporocyst, and sporozoite) and the gene was detected by one step reverse transcriptase PCR(RT-PCR). The result showed that the IHC-BiP gene was only found in sporocyst stage of *E. tenella* and did not in other stages. The gene has size of 714 bp. This study suggests that the IHC-BiP protein of *E. tenella* from Yogyakarta is produced during sporulation, especially in sporocyst stage.

Keywords: IHC-BiP, *Eimeria tenella*, reverse transcriptase PCR, Yogyakarta

INTRODUCTION

Eimeria tenella is one parasitic coccidian of seven species of Eimeria that causes coccidiosis in chicken. This parasite is the most pathogenic species of Eimeria as well as *E. maxima*. Coccidiosis is an economically important disease in poultryindustry. Prevention by in-feed medication and treatment contribute a major portion of the losses in addition to mortality, malabsorption, inefficient feed utilization and impaired growth rate in broilers, and a temporary reduction of eggs production in layers. The prevention strategies to control coccidiosis are the usage of anti-coccidial drugs and vaccination. However, there ~~is~~ still needs to develop the alternative control strategy due to rapid emergence of drug resistant field strain of Eimeria and high cost associated with development of a new drugs and vaccine (Lillehoj, 2005). Moreover, the lack of accurate diagnostic tools for Eimeria also contributes to the difficulty to manage this disease (Subramanian *et al*., 2008).

Nowadays many recombinant vaccine and molecular diagnoses have been developed. It is cost-effective but the difficulty remains in identification of the antigens or genes which are specific and responsible for eliciting protective immunity. Since Eimeria is a parasite with multiple stages of life cycle, it is also important to develop drug or vaccine which targeted on the disruption of the parasite reproduction as earlier as possible (Subramanian *et al*., 2008). Immunoglobulin heavy chain binding protein (IHC-BiP) is a binding protein which is

included in the heat shock protein 70 (HSP 70) in *E. tenella*. This protein is considering as immunogenic protein since it plays an important role in parasite invasion and can induce the immune response of the host. The sequence of this protein in *E. tenella* and *E. maxima* shares homology with the chaperone in reticulum endoplasmic (Dunn *et al*., 1996).

There is still no sufficient information regarding the IHC-BiP gene or protein in Eimeria from Indonesia, especially *E. tenella* which is the most abundant species in Indonesia. Therefore, this research aim was to find out in which developmental stages in *E. tenella* collected from Yogyakarta that bears the IHC-BiP gene. The method use in this identification of the gene is by implementation of the one step reverse transcriptase polymerase chain reaction or one step RT-PCR.

MATERIALS AND METHODS

Oocyst sample collection

The *E. tenella* oocysts were isolated from the field (paddy field) in Yogyakarta. These isolated oocysts were propagated in chicken, and the caecum of the chicken was collected after 7 days of incubation. The feces in the caecum were taken and filtered using 325 mesh (45 μm) filter. The filtrates which consisted of unsporulated oocyst were then washed with sterile water three times for 90 minutes of sedimentation process. Some of the unsporulated oocyst suspension was then kept at freezer as unsporulated oocyst sample. The rest of the oocysts were then sporulated using 2.5% potassium dichromate. Sporocyst sample was made from the sporulated oocysts that were broken by glass beads and vortexing.Finally, the sporozoites were created from sporocyst excystation using 2.5% sodium taurocholate and 1.5% tripsin. All the samples were kept in the freezer for further analysis.

mRNA isolation

Prior to mRNA isolation, each of the cells sample were ruptured by glass beads grindingas amodified procedure of Haug *et al*. (2007). After grinding process, the mRNA was isolated using the Total RNA Isolation Kit from SBS Genetech. The total RNA samples were kept at -20ºC for further analysis.

Reverse transcriptase PCR

One step reverse transcriptase PCR (One-step RT-PCR) was applied for amplification of total RNA samples. The PCR procedure was based on the protocol of the One-step RT-PCR Kit (SBS Genetech). The modification of procedure during PCR-was only to get melting temperature at 48.7ºC. The primers for PCR were designed as followed, the forward primer was 3’ CGATCTTGGTGGTGGTACC 5’ and the reverse primer was 3’ AGCGGACTGGTTGTCAGAGT 5’.

Visualization of PCR product

The PCR products were then visualized using 1.5% agarose gel. The electrophoresis was run at 50 Volt electrical powers for about 1 hour. The gel was stained in Good View Nucleic Acid Stain (SBS Genetech) then visualized using UV illuminator.

RESULTS

The four developmental stages of unsporulated oocysts, sporulated oocysts, sporocysts, and sporozoites in *E. tenella* were used in this research. The unsporulated oocyst was the stage of this parasite which came out to the environment together with the feces. This stage then sporulated in the environment to become the infective stage of the sporulated oocyst. In the intestinal lumen of the chicken, the oocyst ruptures and releases the sporocyst which then burst and release sporozoite. Sporozoite is the invasive stage that invades the

intestine epithelial cell to reproduce (Allen and Fetterer, 2002). The morphological feature of these four stages is presented in Figure 1.

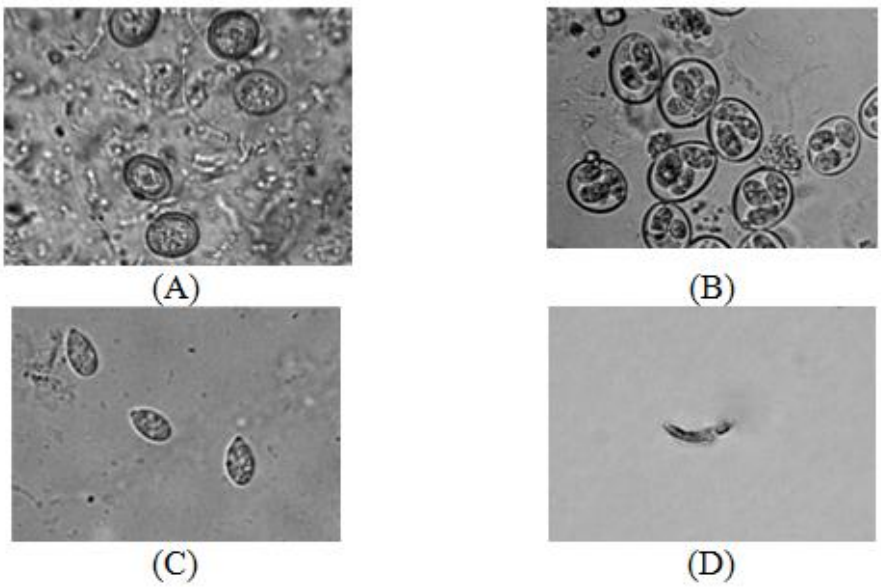

Figure 1. The four developmental stages of *E. tenella*
(A. unsporulated oocyst, B. sporulated oocyst, C. sporocyst, and D.Sporozoite)

The detection of IHC-BiP gene in four developmental stages of *E. tenella* collected from Yogyakarta showed that only sporocyst stage which bore the gene. The gene size is predicted in about 714 base pairs (bp). Detection of the IHC-BiP gene was conducted by one step RT-PCR and the result showed in Figure 2.

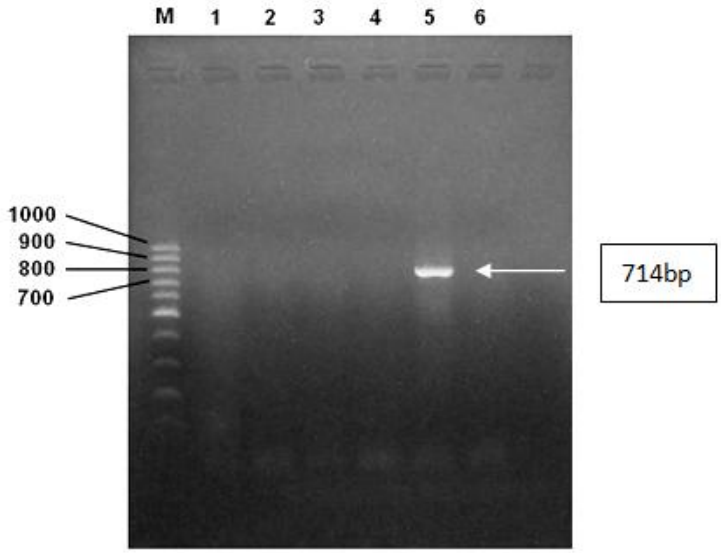

Figure 2. The detection of IHC-BiP gene. The gene was only detected in sporocyst stage (M= marker, 1 and 2= unsporulated oocyst, 3 and 4= sporulated oocyst, = sporocyst, and 6= sporozoite)

DISCUSSION

Immunoglobulin heavy chain binding protein (IHC-BiP) is a binding protein found in *E. tenella* and *E. maxima* which is homologue to chaperone in reticulum endoplasma. It is included in the third member of the heat shock protein 70 (HSP70). Many parasites produce the HSP70 protein during their invasion process as a response to an increase of the host's temperature or due to stress from the invasion process (Dunn *et al*., 1996). In general, HSP70 are among the dominant antigens recognized by the immune system in a large spectrum of parasites. Therefore, it can be assumed that their role in the host-pathogen interaction, this protein is an immunological important (Maresca and Carratu, 1992). In addition, the HSP70 in parasitic protozoa also plays an important role during stages of conversion, infectivity, and virulence. In Eimeria, this protein has been found as cellular and molecular events which occur during sporulation phase, the phase that forms the sporocyst and sporozoite (Del Cacho *et al*., 2008).

Eimeria tenella is the parasitic protozoan which has multiple stages in its life cycle. Many proteins are produced and secreted during its invasion process in the gut epithel (Subramanian *et al*., 2008). Therefore, it is very important to find the proteins which involve in this process as the materials for vaccine development, drugs, or diagnostic tools so that life cycle of the *E. tenella* can be stopped as earlier as possible.

This research showed that *E. tenella* which isolated from Yogyakarta had the gene that coded the IHC-BiP protein. This gene was only found in sporocyst stadium with the size of about 714 bp. This figures that the gene is only active during sporulation process to produce the protein. The finding is similar to the previous research which was conducted by Del Cacho *et al*. (2008). This research concluded that the IHC-BiP gene found in *E. tenella* collected from Yogyakarta is the gene that encoded the binding protein. This protein isinvolved in HSP70 protein, and therefore the protein is an immunogenic protein.

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Amplification and Sequencing Growth Hormone Genes in the Nurseri Center for PO Cattle on Balai Besar Inseminasi Buatan (BBIB) Singosari and Unit Perusahaan Aliansi (UPA) Pasuruan

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Abstract

This study aims to amplify the bovine growth hormone gene and learn the sequence of bases as well as the homology of growth hormone gene from the gene bank. This is important because as a Indonesia local cattle in this study will be a sperm-producing parental stock which will then be used by the farmers through insemination techniques. Related to our research goal, DNA sampels of 3 superior bull PO at BBIB Singosari, East Java and 10 PO cow of the parental stock at UPA Pasuruan, East Java had been analyzed. DNA isolation had been conducted by salting out technique on the total samples, followed by PCR-sequecing procedure. The process of amplification of bovine growth hormone gene carried by one pair of primer. The process of DNA amplification by PCR generating 329 bp long. Results showed that correct amplicon sequencing is the bovine growth hormone gene showed 99% homology with the GH gene Bos indicus Breed Butane that has been published in GenBank.
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Keywords: PO cattle, growth hormone gene, PCR, sequencing

INTRODUCTION

In recent times, it was developed the use of molecular technology in various field of science, for example field of animal husbandry in case of increasing of the productivity of PO Cattle. Related to the increasing of production of the meat, it is needed a gen-level study which control the growth characteristic (meat production). One of genes, that is indicated, influenced the meat production (growth) is Bovine Growth Hormone.

Bovine Growth Hormone is a single peptide with 22 KDa molecul-weight and codified by 191 amino acid (Wallis, 1973) with sequence length of nucleotide 2856 pb (Gordon et. al., 1983). Bovine growth homone gene consist of 5 exon dan seperated by 4 intron (Gordon et. al., 1983) and located at chromosome 19 (Hediger et. al., 1990). Growth hormone gene is growth hormone-marker which is produced by somatotropes, located in fore pituitary gland dan have some physiology activities. GH Gen has important role in case of regulate growth characteristics, reproduction, metabolism, lactation, and mamary glands growth (Cunningham, 1994; Hoj et. al., 1993).

Various research reported the relevance between GH gen with the milk production and the meat of foreigh cow (Lucy et. al., 1991, Hoj et. al., 1993, Schlee et. al., 1994a, Lagziel et. al., 1996, Sutarno et. al., 2000). In Indonesia, as reported Sutarno (2000) that GH gene can be a marker in process of selection of local cattle, including PO cattle by various research of GH gene.

Based on the analyze above, the analyze at the level of gene in this sense growth hormone gene need to be done by the offices which have duties as seed cattle supplier in

Indonesia in order to increase the quality and quantity of Indonesian cattle. One of the offices that has duty as supplier of seed cattle, including PO cattle is BBIB Singosari. BBIB Singosari is a technical implementer unit which carry out male replacement and production of superior seed sustainability by supplying frozen semen. In doing so, BBIB Singosari works with local state firm to supply the female cattle for the people. One of firms which collaborate with BBIB Singosari is UPA Pasuruan. UPA Pasuruan is a state firm which supply the female PO Cattle that will give descent and then will be used by the people. Until now, the effort of this two units in order to supply superior stock parental is selection based on morphology-marker. This marker still has many restrictiveness for the characteristic is more influenced by the surroundings and it is not generated to its descents (Sutarno et. al., 2005). Then, it is needed a study at the level of gene which control the growth characteristic (meat production) completing the morphology-marker.

The study at level of gene that control the growth characteristic (meat production) can be done by PCR-sequencing technique. Recently, PCR technique is one of methods which is easy relatively besides sequencing is a more powerful technique of measuring genetic diversity that has been innovated an automated recently, has been widely used in genetics to forensic. This research aims to detect the existence of growth hormone gene of PO cattle of all research samples by using PCR technique and sequencing.

MATERIALS AND METHODS

Sample

The sample used in this research is all bull PO (superior catlle) in BBIB Singosari. Those are amount 3 bull PO Cattle and 10 cow PO. The latest are used as cow-mother in the process of reproduction of PO Cattle in UPA Pasuruan. The blood samples were taken 3-5 ml through jugular vain by means vacutainer 9 ml contained EDTA (anti-coagulant) and shaken until homogeneous. The blood samples are kept in cold temperature.

Leucocyte Isolation of Blood Sample

Isolation is made by saltingout technique. The blood sample from vacutainer are moved as much as 3 ml of each and put into polypropylene tube 15 ml sized, added 9 ml solution lysis RBC as much as one time and the tube then is inverted 2-3 times and incubated in room temperature during 10 minutes. Then, it is centrifuged with a speed of 1500 rpm during 10 minutes until the pellets and supernatant formed. The formed supernatant then disposed of then added 9 ml of RBC as much as one time and incubated in room temperature during 10 minutes then it is centrifuged with a speed of 1500 rpm during 10 minutes and supernatant is disposed of.

DNA Isolation from Leucocyte

The pellet that form leucocyte is added 750 µl cell lysis solution and then homogenized by the way of pipeting. It is incubted at level of 37^oC temperature during 15 minutes then it is added precipitation protein as much as 500 µl then the vortex process is made, centrifuged with a speed of 7000 rpm, temperature level 40^oC, during 15 minutes. The formed supernatant is moved to the new tube, added 2250 µl cool ethanol then the tube is inverted 25 to 30 times until the white DNA band showed. After that, it is centrifuged with a speed of 10.000 rpm, 4^0C temperature during 15 minutes and then supernatant disposed of. Continued by adding 3 ml of ethanol with 70% cold temperature, inverted for a few times, centrifuged with a speed of 10.000 rpm, 4^0C temperature during 15 minutes and then ethanol disposed of. Next treatment is the remain pellet then wind-dried at level of room

temperature then added 100 µl TE buffer, placed at the level of oven temperature 37^0C, during 10 minutes and kept at the level of -20^0C temperature.

Quantitative Measurement of DNA using Spectrofotometer

Purity measuring and DNA Concentration is isolated using spectrofotometer (Genesys 10). DNA Concentration is known by the way of measuring the absorbance value at length of wave 260 nm and 280 nm. The formula, that is used to measure the DNA consentration using spektrofotometer, is (Fatchiyah et., al., 2007):

Consentration (µg/ml) = A260 . fp . 50 µg/ml
A260 = OD 260 value in the measured DNA solution
fp = dilution factor
50 = OD 260 same with 1 mol then equal with 50 µg/ml

Whereas, the purity of DNA is measured by the way of comparing the absorbance at length of wave 260 nm and 280 nm.

Qualitative Measurement of DNA using Agarose Electrophoresis 2%

Agarose is weighed as much 0,3 g and fused into 15 ml of TBE and then heated until all fused. The solution gel is refregerated until warm (+45^{o}C) then added EtBr as much as 0,8 µL and moulded using gel mould which is set tool comb-like. Then let the gel condensed dan the tool like a comb is dropped, after that the gel is moved to electrophoresis chamber then it is filled with TBE until the gel damped. As much as 4 µL of DNA sample, the result of isolation, is added 3 µl loading dye, put into gel well. The electrophoresis is carried out using voltage 100 volt more or less 1 hour. The result of running then outstretched upon UV transluminator.

DNA Amplification using PCR

Amplification of hormon gene of PO Cattle growth is made by Polymerase Chain Reaction (PCR) method. PCR is made by mixing aquadest steril 4 µl and PCR mix 10 µl. The reaction is started by adding 2 µl DNA sample as template and 2 µl for each primer. Amplification is executed to PCR tool (gene cycler), programmed in accordance with used paired primer. The used primer is 5'-CCCACGGGCAAGAATGAGGC-3' as forward and 5'-TGAGGAACTGCAGGGGCCCA-3' as reverse, in which each primer can recognize gene sequence located in 2054-2074 bp for primer and gene sequence located in 2457-2337 bp (Sutarno, 2010). Gene cycler is programmed in accordance with used primer, that is hot start at 94^{o}C temperature during 1 minute, denaturation at 94^{o}C temperature during 1 minute, annealing at 60^{o}C temperature during 1 minute, extension at 72^{o}C temperature during 1 minute and ended with post extention at 72^{o}C temperature during 5 minutes. The amount of amplification are 30 cycles.

Sequencing PCR Result

The sample of the result of amplification is continued to the stage of sequencing, beforehand purified using ethanol method/EDTA precipitation. Then it is continued to the stage of sequencing with reagent ABI PRISM BigDye Terminator v3.1 cycle sequencing kit. This sequencing process is made by the analysis merit of the laboran of State Islamic University, Malang.

Data Analysis

The data of PCR result is used to know the existence of growth hormone gene, by observation of the photo of agarose electrophoresis result, whereas the sequencing data is used to confirm the sequence of PCR result and each sample is traced and confirmed

between the result of forward primer sequence and reverse primer sequence to each gene by using software sequence scanner v.10 and software Bioedit. Then, the result of sequencing is confirmed by using BLAST (Basic Local Aligment Search Tool) programme which can be found in NCBI website. It is used to know whether growth hormone gene in GenBank has similarity with gene sequence of growth hormone of cattle which is the sample of this research.

RESULT AND DISCUSSION

The result of amplification of GH Gen fragment is showed figure 1, whereas the result of sequencing is showed figure 2. Growth Hormone Gene is a single peptide with nucleotide sequence length 2856 pb, consist of 5 exon and seperated by 4 intron (Gordon et al., 1983). Based on the used primer pair, the length of the product of the result of gen GH fragment amplification is 329 pb, located at exon 3 and 4. The length of this fragmen, that has similarity with the result of amplification (Zhou et al., 2005 and Sutarno et al., 2010), is 329 pb with formation of a single DNA band. The success of GH gene amplification is determined by the annealing conditions on the target gene and condition of thermocycler (denaturation temperature, annealing, and extention). Beside, it is determined by the interaction of the component of PCR reagent in appropriate concentration (Viljoen et al., 2005). Annealing temperature that is used in this research is 60^0C during 1 minute. It is different as suggested by Mitra et al. (1995) and Sutarno (2005) that the primer annealing occurs at 60^oC temperature during 40 and 45 seconds. That annealing time can not be used in this research for if it is used the level of success of amplification of growth hormone gene to this cattle show less optimum results because of there are some DNA bands.

Then the result of PCR is continued by sequence process using reagent ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit. The data of sequence result of each sample is traced and confirmed between the result of forward primer sequence with reverse primer to each gen by using software sequence scanner v.10 and Bioedit. Then, based on that data, the homology is confirmed with the growth hormone gene of cattle by using BLAST (Basic Local Aligment Search Tool) programme which can be found in NCBI website. It is used to know whether growth hormone gene in GenBank has similarity with gene sequence of growth hormone of cattle.

The result of alignment (table 1) shows that gene sequence of growth hormone of PO cattle in BBIB Singosari and UPA Pasuruan have homology value that reach 99% with 5 samples of growth hormone gene in GenBank. Based on this homology value, it is concluded that the gen of amplification result is a correct growth hormone gene of cattle and can be used for further research.

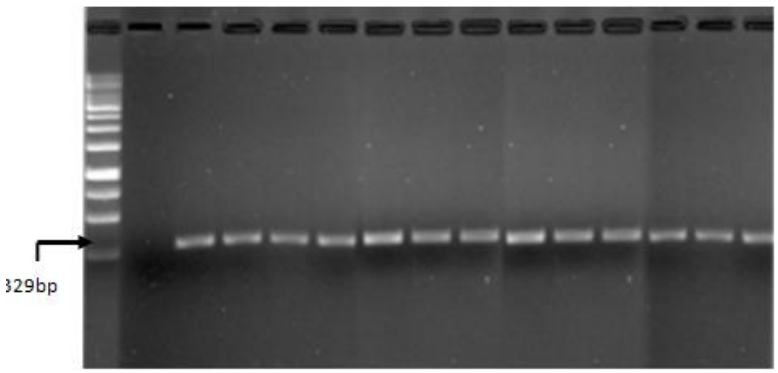

Fig. 1. Result of amplification of growth hormone gene of PO cattle using Gel Agarose 1,5%
Explanation: M: Marker; K: Kontrol (Control); 1-13: Samples

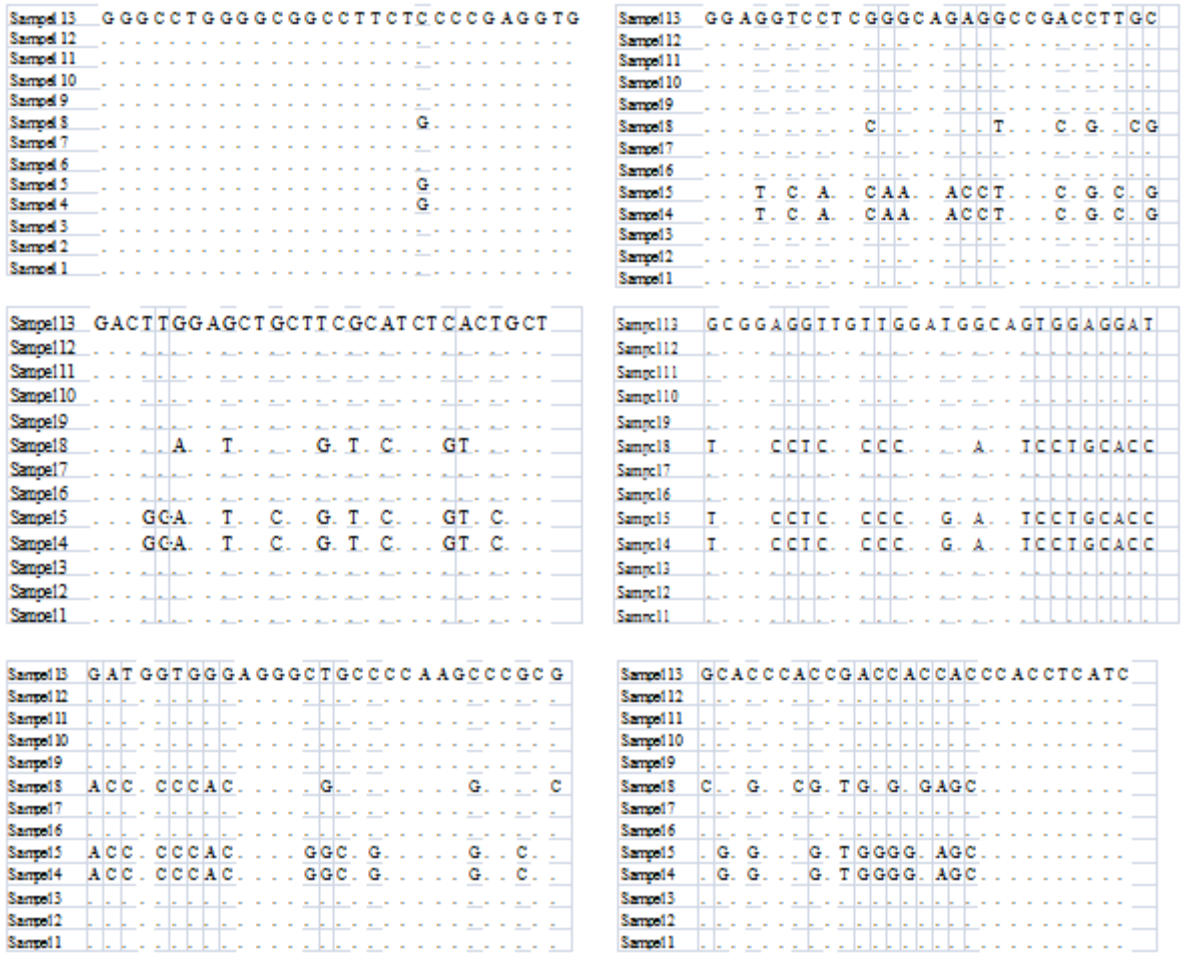

Fig. 2. The result of sequencing of growth hormone gene
Explanation: samples numb. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10: from UPA Pasuruan and numb.11, 12, 13: from BBIB Singosari

Table 1. Sequence similarity value of growth hormone gene of superior bull PO Cattle in BBIB Singosari and mother-cattle in UPA Pasuruan with growth hormone gene

Sample	Sequence of Growth Hormone Gene (Source: *NCBI*)				
	J00008.1	EF592534.1	EF592533.1	AF529184.1	AY271297.1
Sample A	99%	99%	99%	99%	99%
Sample B	99%	99%	99%	99%	99%
Sample C	99%	99%	99%	99%	99%
Sample D	99%	99%	99%	99%	99%
Sample E	99%	99%	99%	99%	99%
Sample F	99%	99%	99%	99%	99%
Sample G	99%	99%	99%	99%	99%
Sample H	99%	99%	99%	99%	99%
Sample I	99%	99%	99%	99%	99%
Sample J	99%	99%	99%	99%	99%
Sample K	99%	99%	99%	99%	99%
Sample L	99%	99%	99%	99%	99%
Sample M	99%	99%	99%	99%	99%

Note. Sample A-C: superior bull PO Cattle from BBIB, sample D-M: mother-PO cattle from UPA, J00008.1: bovine growth hormone (presomatotropin), EF592534.1: Bos indicus breed Kenana growth hormone gene, EF592533.1: Bos indicus breed Butana growth hormone gene, AF529184.1: Bos grunniens growth hormone precursor, AY271297.1: Bos grunniens growth hormone gene.

ACKNOWLEDGEMENT

Thanks to State Islamic University which allows the implementation process in their sequencing laboratory and to Higher Education Directorate, the National Education Department of Indonesia financing this research through a doctoral research grant program

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Isolation, Characterisation and Identification of Sea Urchin-Associated *Bacillus* in Mentigi Beach, West Lombok

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The genus *Bacillus* is gram-negative bacterium that can be found in the variety of habitat and can be isolated from many sources. This genus have many potential, mainly as a new source of antibiotic substance. The objectives of the study are to isolate, characterize and identify sea urchin-associated *Bacillus* in Mentigi Beach, West Lombok. Sea urchin-associated *Bacillus* were isolated from the external and internal part of the body of sea urchin that had been heated at 80^{o} C. This research was able to obtain four *Baclllus* isolates, namely 1A, 2J, and 3L. On the basis of phenotypic characterizationed by using profile matching method isolates 1A was assigned to *Bacillus spahericus*, isolate 2J to *Bacillus carotarum*, and isolate *3L* to *Bacillus cereus.*

Key words: Sea urchin, *Bacillus*, Mentigi beach

INTRODUCTION

The genus *Bacillus* is gram-negative bacterium that widely distributed in the nature. This group can be found in the variety of habitat and can be isolated from many sources. This genus have many potential, mainly as a new source of antibiotic substance.

Bacillus species produce many kinds of antibiotics which share a full-range of antimicrobial activity such as bacitracin, pumulin and gramicidin (Todar, 2009). *Bacillus* species polypeptide antibiotics which constitute the *Bacillus* bacteria have been gaining importance as a result of studies.The *Bacillus* species that produce antibiotics are *B. subtilis, B.polymyxa, B. brevis, B. licheniformis, B. circulas,* and *B. cereus*. The *bacillus* species have a wide range of antimicrobial activities since they are used as anti-fungal agents, anti-viral agents, anti-ameobocytic agents, and anti-mycoplasma agents (Yilmaz *et al*., 2006).

Marine microbial live in a biologically competitive environment with unique condition of pH, temperature, oxygen, light, nutrient and salinity. There in no wonder that marine microbial metaboliets exhibit special biological activities compared with terrestrial bacteria (Zhang *et al.*, 2005). Researched by Kanagasabhapathy *et al.* (2005) found *Bacillus* that live on the surface of marine invertebrates produce chemicals that are having potential antibacterial and antifouling activities. In this study we isolate sea urchin-associated *Bacillus*, characterize and identify them. This *Bacillus* isolates will be used in our next research to study their antibiotic potential.

MATERIALS AND METHODS

Sea Urchin Collection

Sea urchin samples were collected from depth of 2-5 metre along the coastal waters of Mentigi beach, West Lombok . The collected specimens were washed with sterile water to remove adhering debris and associated biota. Then the sea urchin samples were dissected after cut their prickles. Intestinum and prickles of the sea urchin were crushed into smaller pieces, after that 5 ml of NaCl was added to it.

Isolation of *Bacillus*

The extracts were heated in water bath at 80^0C for 30 minutes to induce endospore forming. Serial dilution ($10^{-1} – 10^{-7}$) was done using sterile physiologic water. 100 µL of the bacterial suspensions (10^{-3}, 10^{-5}, 10^{-7}) were poured on Nutrien Agar (NA) medium plates. Plates were incubated at 30^0C for 24-48 hours.

Characterisation and Identication of *Bacillus*

The isolated bacteria were characterized according to Gram characteristics, cell and spore morphology, and motility. In addition, the following characterized tests were carried out: urea hydrolis, catalase, H_2S, sugar fermentation, citrate, oxidase,Voges-Proskauer, mehyl red, and indol tests.

Identification by using *Profile matching* method based on *Bargey's Manual of Determinative Bacteriology* (Holt et al., 1984) and Pibwin Ver. 1.9.2 Build 29 software with *Bacillus* matrix (downloaded from University of Sothampton, UK website).

RESULT AND DISCUSSION

This research was found three species of sea urchin from Mentigi beach, West Lombok, namely: *Echinotrix calamaris*, *Echinometra mathei*, and *Diadema setosum*. These species is the general type of sea urchin and abundance in this beach.

From external and internal parts of three sea urchin specien that was collected, we successfully isolated three species of *Bacillus.* These three isolates grew slowly (3-4 x 24 hrs) at 25-30^0C incubation temperature but showed faster growth rate at 33-37^0C (only 1-2x 24 hrs)

The unique characteristic of that three isolates that they are can not growth in NA medium supplemented by sea water. It just resistence to maximal 10% NaCl and grew very well in standard NA media (without supplemented by sea water). This case maybe because the isolated bacteria live in the tissue of sea urchin (not in sediment) that have rather low salinity.

The characteristics of colony and cell morphological is showed in Table 1 and Figure 1. All isolates have circle form colony and entire edge but they have vary elevation and colour.

Table 1. Colony and cell morphology of bacterial isolates

Characters	**Isolate**		
	1A	2J	3L
A. Colony morphology			
Form	Circle	Circle	Circle
Edge	Entire	Entire	Entire
Elevation	Flat	Convex	Convex
Colour	White	Yellow	Yellow

B. Cell morphology			
Form	Rod	Rod	Rod
Arrangement	Chain	Single	Single
Gram stain	Positive	Positive	Positive
Spore form	Oval	Oval	Oval

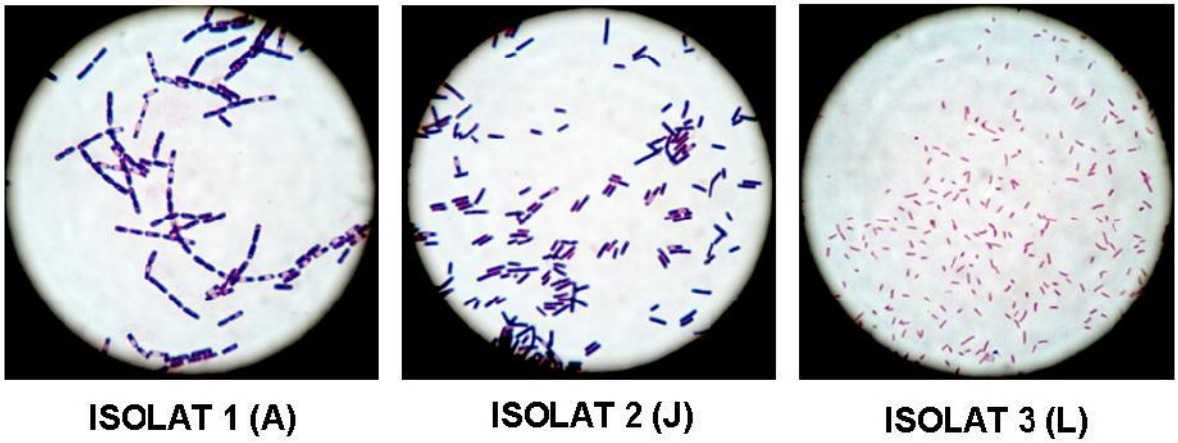

Figure 2. Gram stain of bacterial isolates (1000x)

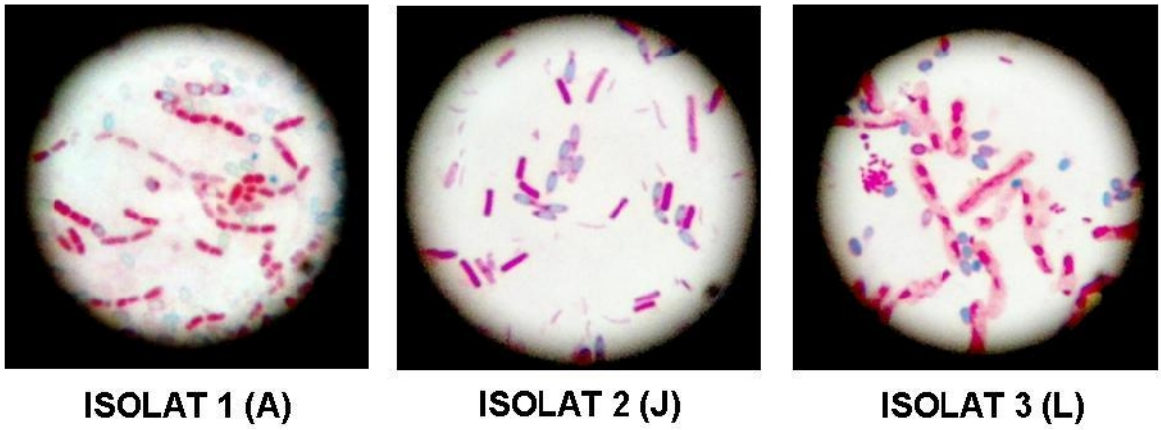

Figure 3. Endospore stain of bacterial isolates (1000x)

We can see from the table and figures that all isolates are the gram positive bacteria, rod form and have oval endospore.These characteristics make sure that all bacterial isolates are member of *Bacillus* genus.

Biochemistry characteristics of bacterila isolates are showed in Table 2.

Table 2. Biochemistry characteristics of bacterial isolates

Characteristics	**Isolate**		
	1A	**2J**	**3L**
Carbohydrate fermentation: – Glucose	+	+	+
- Maltose	+	+	+
- Manitol	+	+	+
- Sucrose	-	-	-
Motility	+	+	+
Catalase	+	+	+

Simmons Citrate	-	-	-
Urease	+	+	+
TSI	+	+	+
Indol	+	+	+
Methyl Red	-	-	+
Voges Proskauer	-	+	+
Oxidase	+	+	-
Cloramphenicol	+	+	+
Nalidixic Acid	+	+	+
Polymyxin	+	+	+
Growth in NaCl 10%	+	+	+
Growth in anaerobic condition	-	+	-

Identification by using *Profile matching* method based on *Bargey's Manual of Determinative Bacteriology* (Holt et al., 1984) and Pibwin Ver. 1.9.2 Build 29 software with *Bacillus* matrix (downloaded from University of Sothampton, UK website) identify that isolates 1A was assigned to *Bacillus spahericus*, isolate 2J to *Bacillus carotarum*, and isolate *3L* to *Bacillus cereus.*

Bacillus sphaericus is member of *Bacillus* genus that produce toxin for mosquito larvae (larvasidae). This species mainly found in soil and freshwater sediment (Anonim, 2009). *B. carotarum* found in the soil that was used as ornament in Spanyol (Heyrman *et al.*, 2005). *Bacillus cereus* produce exotoxin that gave occasion to intoxication (Todar, 2009)

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Isolation and Identification of *Rhizoctonia* Associated with *Phalaenopsis amabilis* (L.) Blume Roots

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ABSTRACT

Most of orchid mycorrhizal fungi are from *Rhizoctonia* genus and these fungi have known can enhance the growth of orchid. This study aimed to isolate and identify *Rhizoctonia* associated with *Phalaenopsis amabilis* roots. Isolation of *Rhizoctonia* from healthy roots was carried out using a modification of Yuan method. Identification was based on the macroscopic and microscopic characteristics. Nuclei were stained with safranin O and KOH. Four *Rhizoctonia* isolates isolated from root of *Ph. amabilis* were identified as *Ceratorhiza* sp1, *Ceratorhiza* sp2, *Epulorhiza repens*, and *Moniliopsis* sp. Nuclear staining revealed that *Ceratorhiza* sp1, *Ceratorhiza* sp2 and *Epulorhiza repens* were binucleate and *Moniliopsis* sp. was multinucleate.

Key word : *Rhizoctonia*, *Phalaenopsis amabilis*, isolation, identification, orchid

INTRODUCTION

Phalaenopsis amabilis (L.) Blume or moon orchid is one of Indonesia native orchid. This orchid was determined as Indonesian National Flower called Puspa Pesona. Naturally, all orchids are associated with endophytic fungi. All orchids utilize endophytic fungi to initiate seed germination, to enhance growth and to protect orchid from pathogen (Batty *et al*., 2001; Chang, 2007; Dearnaley, 2007; Lee, 2002).

Based on earlier studies, it was known that most isolated fungi from orchid roots were classified as *Rhizoctonia* or *Rhizoctonia*-like fungi (Otero *et al*., 2002; Shimura *et al*., 2009). *Rhizoctonia*-like fungi included the anamorphic genera *Ceratorhiza*, *Epulorhiza*, *Moniliopsis*, and *Rhizoctonia* and the teleomorphic genera were *Ceratobasidium*, *Sebacina*, *Tulasnella* and *Thanatephorus* (Dearnaley, 2007; Mursidawati, 2007; Rasmussen, 2002)

Research by Wu *et al*. (2010) showed that inoculation of *Rhizoctonia* spp. could enhance fresh biomass, plant height and leaf number of *Cymbidium goeringii* seedlings. Shan *et al*. (2002) also reported that *Epulorhiza* isolates were strongly stimulated the germination and development of three orchid's species (*Arundina chinensis*, *Spathoglottis pubescens* and *Spiranthes hongkongensis*).

In Indonesia, research on *Rhizoctonia* has been done by Sudanta and Abadi (2006) and Irawati (2005) on *Vanilla*. There are still limited studies on the association between *Rhizoctonia* and *Phalaenopsis* roots, therefore, it is necessary to further examine the isolation and identification of *Rhizoctonia* from *Ph. amabilis* roots. This study aimed to isolate and identify of *Rhizoctonia* associated with *Ph.amabilis* roots.

MATERIAL AND METHODS

Healthy *Ph. amabilis* roots were collected from Mekarlestari Orchid Nursery Yogyakarta and Handoyo Budi Orchid Nursery Malang. Isolation and identification was done at Laboratory of Plant Taxonomy, Faculty of Biology, Gadjah Mada University from October 2010 – January 2011.

Rhizoctonia isolation and culture

Healthy roots were rinsed with tap water slightly, immersed in ethanol 75% for 40s, immersed in sodium hypochlorite 4% for 10 min and finally rinsed in sterile distilled water with three times. Roots were cut into 0.5-1 cm length section and transferred to a plate with PDA medium supplemented with chloramphenicol to avoid bacteria growth. Plates were sealed with Parafilm to avoid desiccation and cultured at room temperature in dark. Hyphae emerging from segments were subcultured onto fresh PDA for purification (Yuan, 2009)

Identification

The identification of the isolates was based on the characteristic of the hyphal branching and septation pattern. Hyphal and moniliod cells were measured. The number of nuclei per cell was determined by examination after staining with safranin O and KOH. Literatures used in the fungal identification were (Athipunyakom *et al*., 2004; Shan *et al*., 2002; Currah and Zelmer, 1992).

RESULT AND DISCUSSION

Four isolates were recovered from *Ph. amabilis* roots. Based on morphological characteristics, four isolates showed *Rhizoctonia* characters such as branching near the distal septum in young vegetative hyphae, formation of a septum in the branch of near of the point of origin, constriction of branch hyphae at the point of origin, right angle (90º) branches of hyphae and produced monillioid cells on old culture. Further identification showed that the isolates were identified as *Ceratorhiza* sp1, *Ceratorhiza* sp2, *Epulorhiza repens*, and *Moniliopsis*. Nuclear staining revealed that *Ceratorhiza* sp1, *Ceratorhiza* sp2 and *Epulorhiza repens* were binucleate and *Moniliopsis* sp. was multinucleate. Four isolates are described below :

Ceratorhiza sp1

Host : Healthy roots of *Ph. amabilis* collected from Mekarlestari orchid nursery, Yogyakarta

On PDA, colony grew rapidly, 9 cm in diameter after 4 days incubation at room temperature, white when young turned to white to cream at maturity (Figure 1a). Observation under microscope showed that fungi produced septa and branched hyphae. Branches arise in right angles (90º) from the main hyphae (Figure 1b). Hyphae was Hyaline on color, with 2.5 – 7.5 µm in width. On mature colony (>7 days) hyphae produced monilliod cells. The monillioid cells were ellipsoidal or elongate barrel shape, the widht and the length were 5 – 12.5 x 20.0 – 50.0 µm (Figure 1c). Each cell of the hyphae and monillioid cells contained two nuclei or binucleate (Figure 1d).

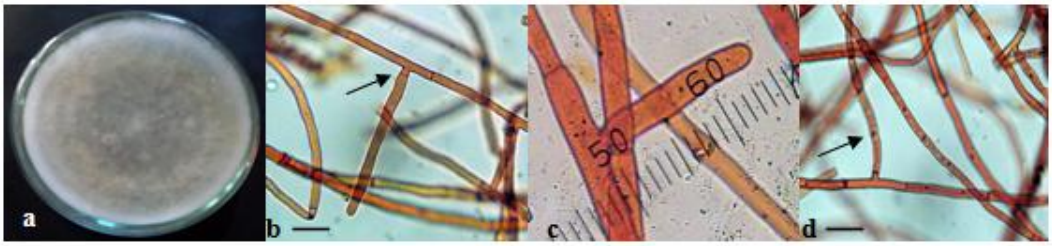

Figure 1. *Ceratorhiza* sp1 (a) colony on PDA 7 days, (b) 90º branched hyphae (arrow head), (c) monillioid cell and (d), binucleate hyphae (arrow head), Bar 10 µm

According to characteristic described before, the isolate have similar characteristics to the genus *Ceratorhiza* sp. that has been described by Currah and Zelmer (1992) such as colony color, aerial hyphae widht, branched hyphae and the number of nuclei. Athipunyakom *et al*. (2004) reported that one of the most abundant mycorrhiza associated with orchid is *Ceratorhiza* sp.

***Ceratorhiza* sp2**

Host : Healthy roots of *Ph. amabilis* collected from Handoyo Budi orchid nursery, Malang

On PDA, colony grew rapidly, 6.91 cm in diameter after 4 days incubation at room temperature, white when young turned to white to cream at maturity (Figure 2a). Observation under microscope showed that fungi produced septa and branched hyphae. Branches arise in right angles (90º) from the main hyphae (Figure 2b). Hyphae was Hyaline on color, with 5 µm in width. On mature colony (>7 days) hyphae produced monilliod cells. The monillioid cells were ellipsoidal or elongate barrel shape, the widht and the length were 7.5 – 10.0 x 20.0 – 25.0 µm (Figure 2c). Each cell of the hyphae and monillioid cells contained two nuclei or binucleate (Figure 2d).

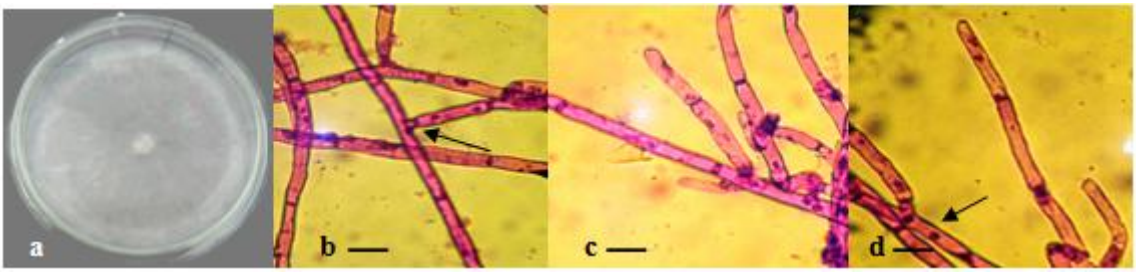

Figure 2. *Ceratorhiza* sp2. (a) colony on PDA 7 days, (b) 90º branched hyphae (arrow head), (c) monillioid cell and (d), binucleate hyphae (arrow head), Bar 10 µm

Based on the characteristic described before, the isolate showed similar characteristic to *Ceratorhiza* sp. by Shan *et al*. (2002) such as colony color, width and color hyphae, and dimension of monilioid cells, but different in colony growth rate. The growth rate of isolate was faster than *Ceratorhiza* sp. by Shan *et al*. (2002). Shan reported that the growth rate of *Ceratorhiza* sp. was 0.42-0.52 mm/h, but the isolate of this study was 0,72 mm/h. Currah (1992) reported that *Ceratorhiza* sp. had fast growth rate, furthermore Athipunyakom *et al.* (2004) reported that after 4-5 days incubation *Ceratorhiza* sp. growth could reach 9 cm in diameter and it was not differ much to the isolate of *Ceratorhiza* sp.2. Comparison based on the characteristic of *Ceratorhiza* sp.2. with *Ceratorhiza* sp. by Shan *et al.* (2002), Currah *et al*. (1992) and Athipunyakom *et al.* (2004), confirmed that the identity of isolate was*Ceratorhiza* sp.

Ceratorhiza sp. is an anamorphic genera for binucleate *Rhizoctonia* with dolipore septa and perforate parenthosomes, with telemorph *Ceratobasidium* (Shan *et al*., 2002; Garcia *et al*., 2006). *Ceratobasidium* spp. were known as pathogens of turfgrasses and cereals and had been reported as orchid endophytes in Australia, North America, and tropical Asia (Otero *et al.*,2002).

Epulorhiza repens

Host : Healthy roots of *Ph. amabilis* collected from Mekarlestari orchid nursery, Yogyakarta

On PDA, colony grew rapidly, 8.9 cm in diameter after 4 days incubation at room temperature, white to cream and sub merged (Figure 3a). Observation under microscope showed that the fungi produced septa and branched hyphae. Branches arise in right angles (90º) from the main hyphae (Figure 3b). Hyphae was hyaline on color, with 2,5 – 5 µm in

width. On mature colony (>7 days) hyphae produced monilliod cells. The monillioid cells were ellipsoidal to spherical shape, the width and the length were 10.0 – 15.0 x 15 – 25.0 µm (Figure 3c). Each cell of the hyphae and monillioid cells contained two nuclei or binucleate (Figure 3d).

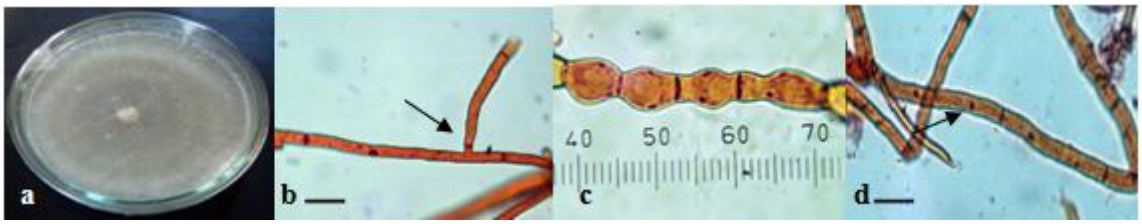

Figure 3. *E. repens* (a) colony on PDA 7 days, (b) 90º branched hyphae (arrow head), (c) monillioid cell and (d) binucleate hyphae (arrow head), Bar 10 µm

Currah and Zelmer (1992) reported that *Epulorhiza repens* produced cream-coloured colony and usually submerged, hyphae's colour was hyaline and monillioid cells ellipsoidal nearly spherical, these characteristics were similar to the characteristics of isolate described previously. Based on the comparison between characteristics of isolate and *E. repens* described by Currah and Zelmer (1992), it was confirmed that the identity of the isolate was *E. repens*, which is the anamorph of *Tulasnella calospora*.

The research carried out by Athipunyakom *et al*. (2004) found that there were several *E. repens* associated with *Spathoglottis plicata* in Chiang Mai and Chanthaburi. These fungi were tested to promote the germination and development of *S. plicata* seeds *in vitro*. The result showed that *E. repens* had the capability to stimulate growth and development of *S. plicata*.

***Moniliopsis* sp.**

Host : Healthy roots of *Ph. amabilis* collected from Mekarlestari orchid nursery, Yogyakarta

On PDA, colony had a moderately fast growth rate with 5.36 cm in diameter after 4 days incubation at room temperature, white to yellowish (Figure 4a) and turned to brownish with age. Observation under microscope showed that the fungi produced septa and branched hyphae. Branches arise in right angles (90º) from the main hyphae (Figure 4b). Hyphae was hyaline on color, with 5 – 7.5 µm in width. On mature colony (>7 days) of hyphae produced monilliod cells. The monillioid cells were barrel shaped, the width and the length were 10 x 20 – 25 µm (Figure 4c). Each cell of the hyphae and monillioid cells was multinucleate (Figure 4d).

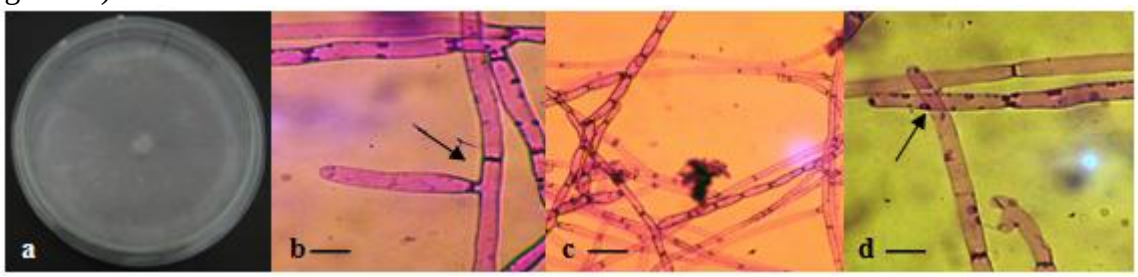

Figure 4. *Moniliopsis* sp. (a) colony on PDA 7 days, (b) 90º branched hyphae (arrow head), (c) monillioid cell and (d) multinucleate hyphae (arrow head), Bar 10 µm

Based on similar characteristic of the isolates to *Moniliopsis* sp. by Currah (1992), the isolate was identified as *Moniliopsis* sp. Athipunyakom *et al.*, (2004) stated that *Moniliopsis* sp. wasan anamorphic genera for multinucleate strains with perforate parenthosomes of *Thanatephorus* or *Waitea* telemorphs. *Rhizoctonia solani* (teleomorphs : *Thanatephorus*), the member of *Moniliopsis* sp., was known as important plant pathogens (Otero, *et al.*,2002)

CONCLUSION

Three genera from four isolates of *Rhizoctonia* fungi which were isolated from healthy *Phalaenopsis amabilis*'s roots, were identified as *Ceratorhiza* sp.1 and *Ceratorhiza* sp.2, *Epulorhiza repens* (binucleate *Rhizoctonia)*, and *Moniliopsis* sp. (multinucleate *Rhizoctonia*).

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ISSN: 2088-9771

The Variation of Diversity of Cave Bats Dweller in Tuban and Menoreh Karstic Area Indonesia

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ABSTRACT

Indonesia has wide and segmented karstics landscape. The climate characteristics and the locations (the altitude and the latitude) give the possibility for the high level of biodiversity and uniqueness to the each area, both exokarstic (aboveground) area and endokarstic (underground) area. The variation is not restricted in species within the different area but also in species in the same area. The two compared area in this research are Tuban and Menoreh Karstic area. These two areas have some differences based on the location. Tuban's karstics area is located near the dry coastal but as the contrary Menoreh karstics area is located on the high damp cold plateau.

The total species found within those two areas are 17 species that belong to 7 different families. 9 species were found within those two areas, 4 species were only found within the Tuban's karstic area, and 4 species were only found within Menoreh's karstics area. The species distribution that is different from one are to others is affected by the condition of the karstics area itself both exokarstics and endokarstics. Furthermore, environment causes variation within species on the different karstics area.

Keyword: Diversity, Bat, Karst, Tuban, Menoreh

ISSN: 2088-9771

Identification of Pheromone Binding Protein Gene of Yellow Rice Stem Borer *Scirpophaga incertulas* (Walker) (Lepidoptera: Crambidae)

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The yellow rice stem borers (YRSB) moth, *Scirpophaga incertulas* (Walker) (Lepidoptera: Crambidae), is known as the most major rice stem borer in tropical Asia. Pesticides are not effective to control the population of these insects due to almost entire larvae phase and pupae are in the rice stem. Hence, other control technique is needed such as based on the mating behaviour. Pheromone binding protein (PBP) in male *S. incertulas* antennae plays a role in the recognition of sex pheromone produced by the female, therefore influenced in their mating behavior. The aim of this study was to identify PBP gene of *S. incertulas*. Touchdown PCR and touchdown-nested PCR were the main techniques conducted to identify genomic of PBP gene from *S. incertulas* and revealed 700 and 600 bp amplicons, respectively. Those amplicons strongly expected as PBP gene. Sequence analysis of *S. incertulas* from touchdown-nested amplicon identified 575 bp which was consisted of 169 bp of exon 3 and 406 bp of intron 2. This study revealed putative amino acid sequences of exon 3 from *S. incertulas* has one conserved cysteine while other Lepidopterans PBP have three conserved cysteine. In phylogenetic analysis, the putative amino acid sequences obtained, showed a phylogenetic signal i.e. by clustering with PBPs from other Crambidae moths. The result of this study is important as a basic data for PBP expression analysis in female or male *S. incertulas* as the initial step to develope new insect biocontrol.

Keywords: *moth, sex pheromone, mating behavior, touchdown-nested PCR, conserved cysteine*

International Conference on Bioscience and Biotechnology (ICBB) 2011

October 11th-12th, 2011, Yogyakarta, Indonesia

Oral Paper Cluster 4

Banana (*Musa paradisiaca* L.) and Corn (*Zea mays* L.) Waste as a Biosorbent for Cu Metals from Wastewater of Textile Industry

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ABSTRACT

Copper is one of the heavy metals contained in wastewater of textile industry that can be wasted and accumulated in the environment that pose a dangerous risk for living beings. Therefore we need an adsorbent to lowering the levels in textile wastewater. In this research the adsorbent would be made from natural resources, that commonly called biosorbent. Biosorbent would be made from the banana peels and banana stem (Musa x paradisiaca L.) and also from skin covering the corn and corn's tree trunk (Zea mays L.) to reduce the metal content of copper in textile industry wastewater. Biosorbent was made by heating using direct sunlight and then activated with sodium hydroxide and formaline. The research were carried out by adsorption capability batch system using a variation of activating substances, biosorbent particle size, pH and contact time. Copper content in the sample was measured by atomic absorption spectrophotometry (AAS). The results indicated that biosorbent have the ability to adsorb copper metal on the basis of adsorption capacity and adsorption percentage. The optimum adsorption capacity of a banana peel was 13.24 mg / g biosorbent with adsorption percentage 59.81% in a banana peel which are activated with sodium hydroxide; mesh 20 particle size; pH 5 and the contact time of 24 hours. The optimum adsorption capacity of banana stem was 19.7 mg / g biosorbent with adsorption percentage 89.02% in banana stem which are activated by formaline; mesh 30 particle size; pH 4 and the contact time of 12 hours. Optimum adsorption capacity of biosorbent for skin covering the corn was 14.84 mg / g biosorbent with adsorption percentage 67,04 % which are activated by formaline; mesh 30; pH 5 and the contact time of 24 hours .the optimum adsorption capacity of corn's tree trunks was 11.42 mg / g biosorbent with adsorption percentage 51,62 % which are activated by formaline ; mesh 20; pH 5 and the contact time 12 hours. From the results of research can be concluded that the banana peel, banana stem, skin covering the corn and the corn's tree trunks can be used as biosorbent of metallic copper in the wastewater of textile industry.

Keywords : Banana peel, Banana stem, skin covering the corn, corn's tree trunks, Biosorption, Copper metal

INTRODUCTION

Wastewater from industrial process are the dominant environmental issue. Unprocess wastewater could give bad effect to the waterways especially water resource (Junaidi and Hatmanto, 2006). Textile are one of the wastewater resources in Indonesia. Textile industry wastewater consist of heavy metals such as As, Cd, Cr, Pb, Cu, Zn; halogenized hydrocarbon (from dressing and finishing process); coloring agent pigment and organic solvent(Shanty, 2010).

Heavy metal ions such as Cu, Cd, Hg, Zn, Pb, Cr etc., are natural components of the earth's crust and cannot be degraded and have been recognized as ecotoxicological hazards. Their chronic toxicities and ability to accumulate in living organism have been of great interest (Cay et al., 2004). Copper (Cu) is one of the heavy metals kinds that has toxic effect. Copper are essential in certain concentration (0,6 mg/days for women and 0,7 mg/days for

men), but if high concentration was taken intoxication could be happened and cause liver damage, brain congest and bleeding (Vanessa *et al*., 2010).

Few methods was developed for heavy metals handling in textile industry wastewater. The methods are chemical precipitation, membrane filtration, electrolytic process, adsorption and biological sorption. Adsorption technic for wastewater treatment becaming popular in recent years because the effiency to diminish pollutant especially heavy metals, coloring agent and organic pollutant (Malakootian *et al*., 2008). Adsorption usually done using activated carbon. Beside activated carbon few natural materials could be used as adsorbent, the process had been known as biosorption.

Biosorption is emerging as an effective alternative for the removal for heavy metals from polluted water. Various plant and microorganism including sawdust (Shukla et al., 2002), solid residue of olive mill products (Gharaibeh et al., 1998), straw xanthate (Kumar et al., 2000), yeast (Huang and Morehart, 1990; Volesky et al., 1993), inactivated bacteria (Chang et al., 2003), fungus (Tunali et al., 2006), algae (Vilar et al., 2006) and aquatic plants, seaweeds (Mohanty et al., 2006; Suzuki et al., 2005) have been used as an efficient biosorbents.

Materials containing cellulose can be used for heavy metals treatment in wastewater and one of the biomaterial that has been proven as heavy metals biosorbent is sugar bargasse that contain 36% cellulose(Vanessa *et al*., 2010). Another biomaterial that contain cellulose are banana peel, banana stem, skin covering the corn and corn's tree trunk. Banana peel contain crude fiber 31,7% containing cellulose 39,12%, holocellulose 72,71%, lignin 8,88% and acid dissolve lignin 1,9%(Anhwange *et al*., 2009; Li *et al*., 2010). Corn's tree trunck has cellulose content about 45% and skin covering the corn's about 23,3% (Puspitasari,2008; Nurcholis and Wardhani,2010).

Banana peel, banana stem, skin covering the corn and corn's tree trunk are part of the plants that usually thrown as a waste. This research conducted for enhancing utilization of banana peel, banana stem, skin covering the corn and corn's tree trunk by using it as biosorbent for copper content in wastewater of textile industry.

RESEARCH METHODS

Materials

Banana peel and banana stem, wastewater of textile industry, aquadest, aquabidest (PT IKA Pharma), hydrochloric acid (HCl) (Merck), nitric acid (HNO_3) (Merck), formaline (Brataco), base fuchsin (Merck), sodium hydroxide (NaOH) (Brataco), sodium sulfite anhydride (Na_2SO_3) (Merck), copper nitrate trihydrate ($Cu(NO_3)_2.3H_2O$) (Merck), Atomic Absorption Spectrophotometer(Shimadzu, AA-6501S), moisture balance(Ohaus, MB35), analytical balance(Mettler Toledo), mechanical agitator(IKA, HS260 Basic), pH Meter (Mettler Toledo), Milling tools with *Screen* 2 (Hammer Mill), Mesh 20 size, Mesh 30 size.

Research Methods

Collection and Plant Determination

Banana peel, banana stem, skin covering the corn and corn's tree trunk were collected by separating it from the fruit and chopped. Determination to know the identity of the plants were done at Laboratorium Taksonomi Department of Biology, Universitas Padjadjaran.

Preparation of Biosorbent

Banana peel, banana stem, skin covering the corn and corn's tree trunk were cut and wash with water in order to diminish dust and other impurity. The materials then dryed by using sunlight. After drying process, biosorbent then milled using screen 2 and was sieved using mesh 20 and mesh 30 to homogenize the particle size.

Activation of the Biosorbent

Biosorbent that has been prepared then activated using two different activator which are sodium hydroxide 0,1 M and formaline 1 N.

A. Activation of biosorbent using sodium hydroxide 0,1 M

1 gram biosorbent was mixed with 20 mL NaOH 0,1 M. the mixture then stirred about 2 hours and the supernatant separated. The materials was rewashing with aquadest and was dryed at 55^{o}C for 24 hour (Vanessa *et al.,* 2010).

B. Activation of biosorbent using formaline 1 M

50 g biosorbent was mixed with 500 mL formaline 1 N for 24 hours and placed in *waterbath* (70^{o}C) for 1,5 hours. Biosorbent then cooled and neutralized with 250 mL NaOH (Karthika *et al.,* 2010). Qualitative analysis of formaline content was done toward the biosorbent to make sure the biosorbent free from formaline. Formaline qualitative analysis was done using Schiff reagent.

Determination of biosorbent loss on drying before and after activation

Loss on drying determination was done using moisture balance.

Adsorption activity assay of the biosorbent

Adsorption activity assay are consist of copper content analysis in wastewater, biosorbent activity assay toward wastewater, copper content analysis after adsorption. Copper content analysis was done using atomic absorption spectrophotometer (AAS).

1. Copper content analysis in wastewater by using AAS

   5 mL textile industry wastewater was mixed with 1 mL concentrated nitric acid. Sample was stilled for 24 hours and filtrated. Filtrate was inserted into 10 mL volumetric flask and added with aquadest until mark.

2. Preparation of Standard copper solution 1000 ppm

   0.1900 gram Cu(NO3)2.3H2O was inserted into 50 mL volumetric flask and mixed with 1 mL KNO3 1 M and aquadest was added until mark.

3. Standard Curve Determination

   Cu solution 10 ppm was made by transferring 0,1 mL Cu solution 1000 ppm into 10 mL volumetric flask and add aquadest until mark. Cu standard solution 0,1; 0,2; 0,4; 0,6 ppm and 0,8 ppm was made by transferring 0,1; 0,2; 0,4; 0,6 and 0,8 mL of Cu solution 10 ppm into 10 mL volumetric flask and add aquadest until mark. Standard curve of Cu solution was made from 0,1; 0,2; 0,4; 0,6 and 0,8 ppm.

A. Biosorbent acitivity assay by using batch methods

Biosorbent activity assay on copper content in textile industry wastewater with batch methods was done by variating 4 parameter :

1. Particle size : mesh 20 and mesh 30
2. Activator : formaline 1 N and NaOH 0,1 M
3. Adsorption pH : 4 and 5

4. Contact time : 12 hours and 24 hours using mechanical agitator with speed 150/minute

B. Copper content analysis after adsorption process by using AAS

After adsorption process, the wastewater was filtered and prepared with the method for determining copper content in wastewater.

Statistical data analysis

Adsorption capacity data of the biosorbent was analyze by using UNIANOVA (*univariate analysis of variance*).

RESULT AND DISCUSSION

Collection and Plant Determination

Fresh banana peel and banana stem was taken from banana plantation in Majalaya, West Java. Skin covering the corn's and corn's tree trunck was taken from corn's plantation in Jatinangor, West Java. Determination process was confirmed that the plant were banana and corn's.

Biosorbent activation

Biosorbent that activated with NaOH and formaline has different colour comparing before. Activation was done to increase adsorption capacity of biosorbent toward metals by modifying the functional group. (Vanessa *et al*., 2010).

Loss on drying determination before and after activation

Table 1 Loss on drying before activation

Biosorbent	Loss on drying (mean) (%)
Banana stem	8,43
Banana peel	4,46
Skin covering the corn	7,27
Corn tree trunck	8,81

Table 2 Loss on drying After activation

Biosorbent	Activator	Particle Size	Loss on Drying (mean) (%)
Banana stem	NaOH	Mesh 20	17,99
		Mesh 30	17,42
	Formaline	Mesh 20	10,41
		Mesh 30	10,57
Banana peel	NaOH	Mesh 20	11,73
		Mesh 30	6,42
	Formaline	Mesh 20	8,7
		Mesh 30	8,93
Skin covering the corn	NaOH	Mesh 20	7,98
		Mesh 30	7,54
	Formaline	Mesh 20	3,34
		Mesh 30	3,59
Corn tree trunck	NaOH	Mesh 20	9,6
		Mesh 30	10,6
	Formaline	Mesh 20	5,69
		Mesh 30	5,09

Table 1 shown that loss on drying of banana peel was 4,46% and banana stem 8,43%. This result was suitable with Li et.al (2010) that banana peel has lower water content than stem. Corn's tree trunck has lower loss on drying value than skin covering the corn's and this is suitable with the research done by Tangendjaja and Elizabeth (2008).

Biosorbent Activity Assay

Copper content in textile industry wastewater was determined using linear regression curve. The results was shown below :

Table 3 Copper Content in textile industry wasterwater

	No of measurement	Content (ppm)	mean (ppm)
wastewater	1	0,8748	0,8852
	2	0,8956	

This assay was done by variating biosorbent type, activator, particle size, adsorption pH, and contact time. Banana peel and stem, skin covering the corn and corn's tree trunk was use as a material for biosorbent. Those material contained cellulose, based on Vanessa et.al 2010 cellulose can be used as copper adsorbent.

After the initial copper content in wastewater was measured by using AAS, biosorbent then applied into the wastewater using 4 different parameter. After application of biosorbent into wastewater of textile industry, the colour change darker. The wastewater then filtered using filter paper and the filtrate was measured with AAS for copper content to determine adsorption capacity. Adsorption capacity was measured using equation :

$$\text{capacity (mg/g)} = (C_2 - C_1) \times \left(\frac{V}{W}\right) \qquad (3.1)$$

Note :

C_1 : Initial copper concentration (ppm)
C_2 : final copper concentration (ppm)
V : wastewater volume (ml)
W : biosorbent weight (g)

Adsorption capacity of the biosorbent toward copper content can be seen at Table 4.

Table 4. Adsorption capacity and adsorption percentage of biosorbent from banana

Biosorbent	Activator	Particle Size	pH	Contact time (hour)	Adsorption Capacity (mg/g)	Adsorption Percentage (%)
Banana Stem	NaOH	Mesh 20	4	12	11,375	51,401
				24	8,323	37,607
			5	12	5,093	23,012
				24	8,480	38,319
		Mesh 30	4	12	11,015	49,774
				24	11,453	51,751
			5	12	6,540	29,553
				24	10,573	47,775
	Formaline	Mesh 20	4	12	14,788	66,821
				24	16,220	73,294
			5	12	10,095	45,617
				24	13,163	59,478
		Mesh 30	4	12	19,700	89,019

				24	14,968	67,634
			5	12	18,205	82,264
				24	10,320	46,634
Banana Peel	NaOH	Mesh 20	4	12	8,430	38,093
				24	11,555	52,214
			5	12	8,763	39,596
				24	13,235	59,806
		Mesh 30	4	12	7,033	31,778
				24	0,052	0,237
			5	12	9,358	42,284
				24	5,610	25,350
	Formaline	Mesh 20	4	12	8,993	40,635
				24	11,625	52,531
			5	12	11,080	50,068
				24	12,858	58,100
		Mesh 30	4	12	11,168	50,463
				24	7,580	34,252
			5	12	6,255	28,265
				24	8,050	36,376

Table 5. Adsorption capacity and adsorption percentage of biosorbent from Corn's

Biosorbent	Activator	Particle Size	pH	Contact time (hour(s))	Adsorption Percentage (%)	Adsorption Capacity (mg/g)
Skin Covering the Corns	NaOH	Mesh 20	5	12	24,065	5,325
				24	54,439	12,044
			4	12	38,986	8,627
				24	39,427	8,725
		Mesh 30	5	12	30,958	6,85
				24	57,009	12,614
			4	12	17,64	3,902
				24	41,939	9,279
	Formalin	Mesh 20	5	12	41,099	9,095
				24	37,427	8,282
			4	12	51,729	11,447
				24	32,976	7,297
		Mesh 30	5	12	46,295	10,244
				24	67,036	14,834
			4	12	51,074	11,302
				24	51,537	11,404
Corns tree trunck	NaOH	Mesh 20	5	12	39,603	8,762
				24	16,238	3,592
			4	12	24,883	5,506
				24	31,308	6,928
		Mesh 30	5	12	28,621	6,332
				24	12,383	2,74
			4	12	35,981	7,962
				24	37,149	8,219
	Formalin	Mesh 20	5	12	51,6161	11,422
				24	47,1878	10,442
			4	12	48,9613	10,834
				24	28,4804	6,303
		Mesh 30	5	12	14,19	3,14
				24	39,201	8,674
			4	12	46,6229	10,317
				24	48,7693	10,791

Table 4 was shown that optimum adsorption capacity for banana stem activated with formaline pH 4, particle size 30 mesh and contact time 12 hours was 19,7 mg/g. This result happened because the surface structure of biosorbent becaming weak acid after activated with formaline and increase negative side of the biosorbent causing easier interaction between copper and biosorbent (Vanessa *et al.*, 2010). Optimum adsorption capacity was got from banana peel having smallest particle size, this result suitable with Patil e.al that smaller particle size will have increasing adsorption percentage.

Optimum adsorption percentage for banana peel was got from biosorbent activated with NaOH pH 5, particle size 20 and contact time 24 hour. This result maybe due to the reaction between NaOH with silica in biosorbent to form sodium silicate (Na_2SiO_3). Sodium silicate was dissolved in water and can be loss after cleaning process with water and leaving a hole outside epidermis and the metals was trapped in (Daffala *et al.*, 2010).

The optimum adsorption percentage for skin covering the corns obtained at pH 5. Research by Francis et al., (2007) showed that for copper biosorpsi place at an optimal pH around 5. At acidic pH below 4 biosorben total surface charge becomes positive, resulting in a repulsive force between the surface of the cation. While at pH above 6, biosorpsi be ineffective because the solubilityof copper decreased (Francis etal., 2007).

Optimum adsorption percentage for corn tree trunck at is 51.61%, obtained in biosorben which are activated with formalin at pH 5 particle size 20 mesh and the contact time of 12 hours. Like the skin covering the corn, the optimum adsorption capacity for corntree trunks are also very good at activating formaline solution and at pH of 5.

CONCLUSION

Banana Peel and Stem, skin covering the corn and corn's tree trunk can be used as a biosorbent of copper content in textile industry wastewater.

ACKNOWLEDGEMENT

Universitas Padjadjaran for supporting the research through Penelitian Peneliti Muda (LITMUD) grant.

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Effects of Chitosan/Montmorillonite Nanocomposites Films on the Growth of Bacteria in Laboratory Media

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Abstract

Chitosan-monmorilonite (chitosan-MMT) nanocomposite, such as those prepared in this study, may exhibit potential antibacterial activity as their unique character. The purpose of this study was to evaluate the in vitro antibacterial activity of chitosan. monmorilonite and chitosan MMT nanocomposite against various microorganisms. Chitosan MMT were prepared based on pilarisation of chitosan with montmorilonit. The physicochemical properties of the chitosan MMT were determined by FTIR analysis, and XRD pattern, tensile strength (TS) and elongation-at-break (E) and permeability to gases. The antibacterial activity of chitosan, montmorilonite, and chitosan MMT against E. coli, and S. aureus was evaluated by calculation of minimum inhibitory concentration (MIC) and relative inhibition time (RIT)**.** Results show that chitosan, montmorilonite and chitosan could inhibit the growth of various bacteria tested. Their MIC values were less than 0.1 µg/mL, and the RITs values of chitosan MMT reached 120 h.

Key word : chitosan,montmorilonite, film, bacteria

1. Introduction

Food packaging technologies due to increased food-borne microbial outbreaks caused by minimally processed fresh products and refrigerated products [1]. Traditionally, antimicrobial additives are mixed into initial food formulations to control microbial growth and extend shelf-life; however, this strategy is not always effective since the protective ability of the antimicrobial agent ceases once it is neutralized in reactions and/or interactions in the complex food system. In addition, the antimicrobial compound directly added into the food cannot selectively target the food surface where spoilage reactions occur more intensively. Antimicrobial packaging is an alternative method to overcome these limitations since it enables controlled release of antimicrobial agent at an appropriate rate during storage, hence, maintains its critical concentration necessary for inhibiting the microbial growth [2]

In the last years polymer/clay composites have received much attention, because of their extraordinary possibility to improve the barrier properties of thin films. These composites are a class of hybrid materials composed of organic polymer matrices and micro/nanoscale organophilic clay fillers [3] and due to their high aspect ratios and high surface area, if clay particles are properly dispersed in the polymer matrix at a loading level of 1–5% (w/w) unique combinations of physical and chemical properties will be obtained, that turn these composites attractive for making films and coatings for a variety of industrial applications [4]

Chitosan, a linear β-1,4-D-glucosamine, has attracted much attention in recent years for its inherent antimicrobial properties. This biopolymer is a natural polysaccharide obtained from the deacetylation of chitin. Moreover, it is a non-toxic compound, and another fascinating advantage of this compoundis thefilm-forming capacity that it presents, which allows its application directly as a coating of the packaging material without the necessity of a carrier matrix. Several works have been carried out on the antimicrobial activity of chitosan [5]. Tsai et al. [6] studied the application on fish preservation obtaining an increase shelf-life of the product from 5 to 9 days. Other research performed by Sagoo et al[7] evaluated the

effect of treatments with chitosan solutions and reached an increase shelf-life of raw sausages stored at chill temperatures from 7 to 15 days. A more recent work on the effect of chitosan–gelatin blends as a coating for fish patties showed a difference of around 2 log cycles between the control and the coated batches for total bacterial counts, Pseudomonas and enterobacteria at 8– 11 days of storage [8]

However, its properties, such as thermal stability, hardness and gas barrier properties are frequently not good enough to meet those wide ranges of applications. Up to now, there is only a limited number of reports about the enhancement of properties of chitosan using PLSNs technology. Asira[9] had a preliminary study about chitosane clay nanocomposites and reported a markedly improved tensile property but inferior thermal property of composites to that of pure chitosan [8]. Ruiz-Hitzky[10] and his coworkers synthesized functional chitosan/ montmorillonite nanocomposites, which can effectively act as active phase for an electrochemical sensor in the detection of different anions . All of them prepared nanocomposites by exfoliatione adsorption method, which used diluted acetic acid solvent for dispersing and dissolving clay and chitosan. However, there are few reports about the effect of acetic acid residue on the effect of chitosan nanocomposites, and the effect of hydrogen bond between chitosan and MMT, which may be the key driving force to make the MMT layers assemble to form flocculated structure in chitosan matrix. Taking these antecedents into account, the aim of the present study is to prepare high-performance chitosan byincorporating montmorillonite at the nanometre scale. The effects of acetic acid residue, the hydrogen bonding force between chitosan and MMT and clay loading on the morphology, thermal stability and mechanical properties of the nanocomposites have been investigated.

2. Experimental procedure

Material

Chitosan of low molecular weight with a deacetylation degree (DD) of ca. 82.5% was supplied by Badan Tenaga Atom Jakarta. The commercial sodium montmorillonite (Na+-MMT), supplied by Laboratori of Geology Trisakti Universiti of Jakarta., had a cation exchange capacity (CEC) of 1 meq/g. It was used without further purification

2.1. Preparation of chitosan MMT nanocomposite

Chitosan solution was prepared by dissolving 4 g of chitosan in 196 mL of 2% v/v acetic acid. The pH of chitosan solution was adjusted to 4.9 with 1 M NaOH in order to avoid any structural change of the MMT. An amount of 2.5 g Na^+-MMT was dispersed in 100 mL of distilled water and then treated in ultrasonic bath for 1 h. The as-prepared chitosan solution was slowly added into the Na^{+-}MMT suspension using peristaltic pump at the rate of about 50 mL/h with vigorous stirring. The mixture was treated at 60 °C for 24 h. The chitosan intercalated Na^+ MMT (Chi-MMT) was washed with distilled water until the pH of the washed water became neutral. Chitosan -MMT was separated from water by centrifugation at 3500 rpm for 10 min and then dried at 60 °C for 48 h.The Chitosan -MMT powder was characterized by XRF, XRD, FTIR.

Wide-angle X-ray diffraction (XRD) patterns of the sheet samples were recorded using a Bruker GADDS diffractometer with area detector operating at a voltagemof 40 kV and a current of 40 mA using Cu Ka radiationm (lZ0.15418 nm). Fourier transform infrared (FTIR) spectra (transmission) were measured on a Perkin-Elmer FTIR spectrophotometer 2000 in the range of 4000e 400 cm^{-1} at a resolution of 4 cm^{-1}

Ultrathin films (with thickness of about 80 nm) for transmission electron microscopy (TEM) observation were prepared by cutting from the epoxy block with the embedded

nanocomposite sheet at room temperature using a Leica ultramicrotome with a diamond knife. The TEM micrographs were taken using a Philips CM300-FEG transmission electron microscope under an accelerating voltage of 150 kV. Sections were observed with 200-mesh carbon coated copper grids without any further modification or coating.

2.2. Mechanical properties: tensile strength (TS) and elongation-at-break (E)

TS and E were measured with an Instron Universal Testing Machine (Model 4500, Instron Corporation) following the guidelines of ASTM Standard Method D 882-91. The initial grip separation was set at 30 mmand the crosshead speed was set at 5 mm/min. TS was expressed in MPa and calculated by dividing the maximum load (N) by the initial cross-sectional area (m2) of the specimen. E was calculated as the ratio of the increased length to the initial length of a specimen (30 mm) and expressed as a percentage. TS and E tests were replicated five times for each type of film.

2.3. Water Vapour permeability

The water vapor permeability (WVP) of the films was determined gravimetrically based on ASTM E96-92 method . The test film was sealed on the top of a permeation cell containing distilled water (100% RH; 2.337 -103 Pa vapor pressure at 20^0 C), placed in a desiccator which was maintained at 20^0 C and 0% RH (0 Pa water vapor pressure) with silica gel. The water transferred through the film and adsorbed by the desiccant was determined from weight loss of the permeation cell. The cups were weighed at intervals of 2 h during 10 h. Steady-state and uniform water pressure conditions were assumed by keeping the air circulation constant outside the test cup by using a fan inside the desiccator[11] The slope of weight loss versus time was obtained by linear regression. The measurement (WVP) of the films was determined as follows:

$$WVP = (WVTR-L)/ \Delta P$$

where WVTR is the measured water vapor transmission rate (g/ m^2 s^1) through the film, L is the mean film thickness (m), and DP is the partial water vapor pressure difference (Pa) across the two sides of the film. For each type of film, WVP measurements were replicated three times.

2.4. Antibacterial activity

Gram-positive bacteria Staphylococcus aureus, Bacillus subtilis and Gram-negative bacteria Escherichia coli, were provided by Laboratory of Microbiology State University of Jakarta and incubated on nutrient agar (peptone 1%, beef extract 0.5%, NaCl 0.5%, agar 2%, pH ¼ 7.2). The antibacterial activity of the nanocomposites was evaluated by finding the minimum inhibition concentration (MIC) and the relative inhibition time (RIT) as follows: the microorganism suspension was adjusted by sterile distilled water to10^5-10^6 cell/ml. The nanocomposites chitosan MMT, chitosan, montmorilonite solutions were prepared in acetate buffer (pH ¼ 5.4) at a concentration of 1% (w/v). The resulting solutions and the nutrient agar were autoclaved at 121^0 C for 20 min. The two fold serial dilutions (1 ml) of each sample were added to sterile petri-dishes together with 9 ml nutrient agar to the final concentrations of 0.1% (w/v), 0.05% (w/v), 0.025% (w/v), 0.0125% (w/v), 0.00625% (w/v), 0.00313% (w/v), and 0.00156% (w/v). A loop of each microorganism suspension was inoculated on cooled nutrient medium by means of drawing a stripe. The bacteria were cultured at 37^0 C. MIC values were read after a 24 h culture and RITs were obtained after 5 days. The minimum inhibition concentration (MIC) was defined as the lowest concentration required to inhibit the growth of the bacteria, i.e. the concentration at which no microorganism colony or less than 5 colonies were visible within 19-38 h. Relative inhibition time (RIT) was determined by the time when the bacterial colonies were deterred to grow, i.e.

the difference between the time when colonies in the experiments were visible on agar plates and the corresponding time in

The positive control was given with doxycycline, and the blank control tubes were only contained Muller–Hinton broth and 0.25% acetic acid. After mixing, the tubes were incubated at 37^0 C for 24h. The tubes were then studied for the visible signs of growth or turbidity.The lowest concentration of chitosan and nanoparticles that inhibited the growth of bacteria was considered as the minimum inhibitory concentration or MIC.

3. Results and discussion

The chemical composition of Na^+-MMT and Chitosan -MMT powder was measured by XRF (Table 1). The molar ratio of Na/Al in Na+-MMT was 0.23. After interaction of chitosan, Na could not be detected by XRF. Thus, the Na^+ ions were completely exchanged by the $–NH_3^+$ groups of chitosan.

Table 1 Chemical composition of Na+-MMT and Chi-MMT measured by XRF

sample	Na_2O	K_2O	MgO	CaO	Al_2O_3	SiO_2	Fe_2O_3	other
Na^+MMT	2.56	0.92	2.74	1.85	16.3	73.1	1.67	0.87
Chitosan MMT	-	0.68	2.89	0.47	17.6	73.1	2.69	1.21

3.1. Characterization of chitosan MMT

XRD Analysis

Fig. 1 shows the XRD patterns of starting Na+-MMT and Chi-MMT powder. The starting Na+-MMTshowed a basal spacing d001 of 1.23 nm. After intercalation with chitosan, d-values of ~1.36 nm and ~2.25 nm were observed. The broad reflection around 2θ~6.6° (d001~1.36 nm) was assumed to be due to monolayers of chitosanin MMT whereas d001~2.25 nm was related to the intercalation of chitosan bilayers.

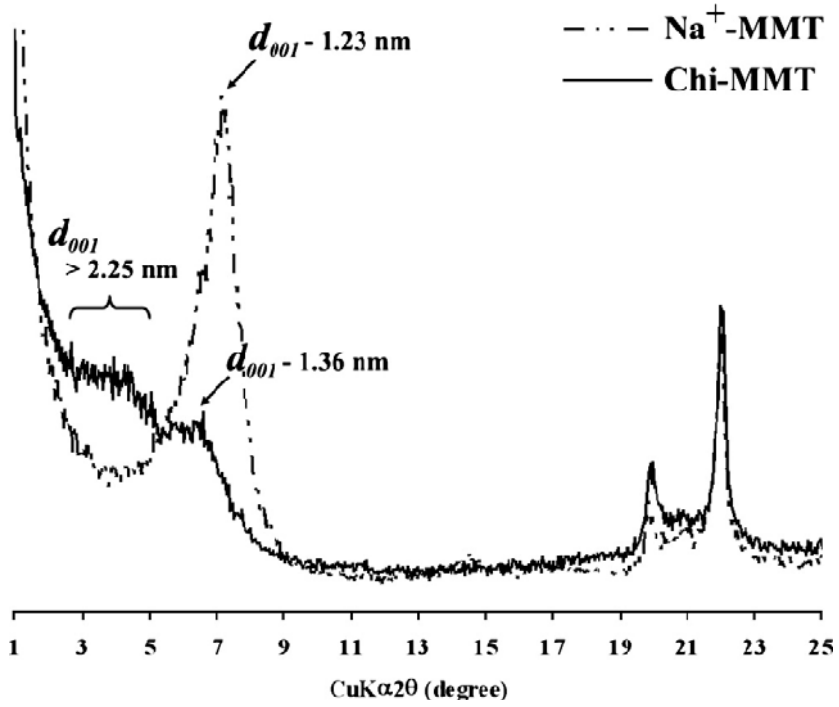

Fig. 1. XRD patterns of (a) Na+-MMT and (b) the chitosan intercalated MMT, Chi-MMT.(Pathavuth and Punnama, 2009)

FTIR analysis

The FT-IR spectrumof Na+-MMT(Fig. 2) shows the vibration bands at 3630 cm^{-1} for O–H stretching, 3442 cm^{-1} due to interlayer and intralayer H-bonded O–H stretching, 1638 cm^{-1} for H–O–H bending,1094 and 1038 cm^{-1} for Si–O stretching, 916 and 626 cm^{-1} for Al–OH, 843 and 795 cm^{-1} due to (Al, Mg)–OH vibration modes and 520 and 467 cm^{-1} for Si–O bending. While the spectrum of chitosan showed peaks at 3436 cm^{-1} due to the overlapping of O–H and N–H stretching bands, 2921 cm^{-1} for aliphatic C–H stretching, 1651 and 1594 cm−1 for N–H bending,1421 and 1382 cm^{-1} for C–H bending,1151 and 1087 cm^{-1} for C–O stretching. The spectrum of the Chitosan-MMT shows the combination of characteristic absorptions due to the chitosan and MMT groups.

The peak at 1594 cm^{-1} of the $–NH_2$ group in the starting chitosan was shifted to 1520 cm^{-1} in the Chitosan-MMT spectrum, corresponding to the deformation vibration of the protonated amine group ($–NH_3^+$) of chitosan. This $–NH_3^+$ group interactswith the negatively charged sites ofMMT. This result was in agreement with the results from XRD and XRF, revealing the intercalation of chitosan in the MMT structure.

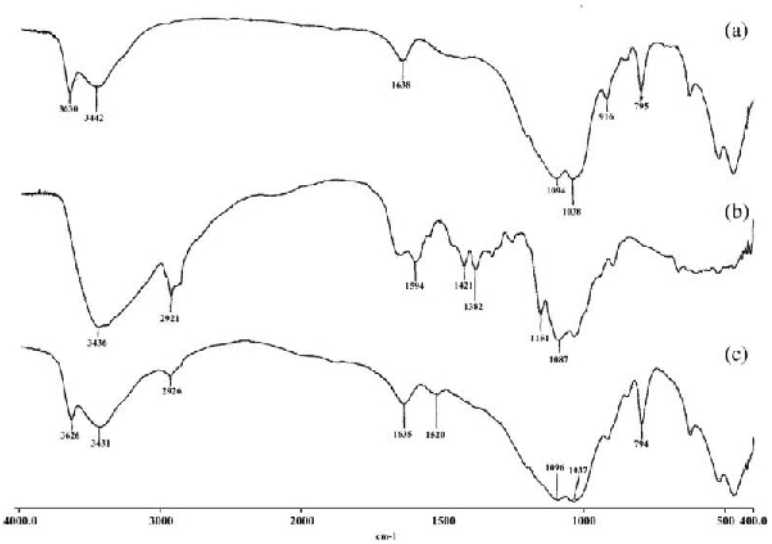

Fig. 2. FT-IR spectra of (a) Na^+-MMT, (b) chitosan and (c) Chitosan-MMT.

BET Analysis

Fig. 3 shows the nitrogen adsorption–desorption isotherms measured on Na^+-MMTand Chi-MMTsamples. The specific BETsurface areas of Na^+-MMT and Chitosan-MMT were 46m2/g and 23m2/g, respectively. The lower surface area of Chitosan -MMTwas attributed to the compact packing of the chitosan molecules in the interlayer space, resulting in pore blocking that inhibited the passage of nitrogen molecules. The average pore diameter of Chitosan -MMT was 16.9 nm compared to 14.2 nm of Na^+-MMT.

3.2. Mechanical properties: tensile strength (TS) and elongation-at-break (E)

As can be observed in Table 2, the TS of chitosan/MNC particles' films increased significantly ($p < 0.05$) with increasing chitosan concentration; while the values of E (Table 2) decreased for high values of chitosan concentration. These results are in good agreement with previously reported values for chitosan films[4]

Previous works have reported that TS values of chitosan/clay films increased significantly with increasing clay concentration due to a possible strain-induced alignment of the clay particle layers in the polymer matrix [11] and the strong interaction between the polymer matrix and silicate layers via the formation of hydrogen bonds[12] . However, in this

work there was no significant influence of the clay concentration on the mechanical properties of the chitosan/MNC films. This behavior may be due to the lower values of clay concentration used, when compared to those of Giannelis[12]. The values of TS and E of the films can be described by the following polynomial equations, respectively

Table 2 TS (MPa) and E(%) of chitosan MMT

No	Sample	TS(Mpa)	E(%)
1	Chitosan	52	4
2	Chitosan MMT 1%	55	4
3	Chitosan MMT 2%	71	6

3.3. Water vapor permeability

The WVP values of the chitosan and chitosan-MMT are shown in Table 3. The water vapor barrier property of the films was significantly improved by incorporation of chitosan-MMT in the film matrix ($p < 0.05$). The greatest values of WVP were obtained for those films with the lowest concentration of chitosan and a significant influence of both chitosan and MMT concentrations ($p < 0.05$) on WVP was found. The WVP of the composite films decreased significantly ($p < 0.05$) by 9–32% depending on the chitosan and clay concentrations.

Table 3 WVP of chitosan MMT

No	Sample	WVP (10^{-12}) kgm(m^2sPa)$^{-1}$
1	Chitosan	3.0 x10^{-12}
2	Chitosan MMT 1%	1.7 x10^{-12}
3	Chitosan MMT 2%	2.2 x 10^{-12}

Rhim et al.[4] reported that the WVP of composite films decreased significantly ($p < 0.05$) by 25–30% depending on the nanoparticles used; these values compare well with the values obtained in the present work. The decrease in WVP of nanocomposite films is believed to be due to the presence of ordered dispersed nanoparticle layers with large aspect ratios in the polymer matrix[13] . This forces water vapor traveling through the film to follow a tortuous path through the polymer matrix surrounding the particles, thereby increasing the effective path length for diffusion.

3. 4. Antimicrobial activity

As to investigate whether Chitosan -MMT themselves have antibacterial properties, chitosan –MMT were tested when dissolved in acetate buffer. As seen in Table4, chitosan MMT only shows a slight antibacterial activity. And all the nanocompositesshow better inhibitory effect than pure Chitosan, Na^+ MMT and MMT. As shown in Tables 1 and 2, all the nanocomposites show excellent inhibition effect on Gram-positive bacteria. The MIC values of the nanocomposites are 4-30 times lower than that of Chitosan, and the RITs of the nanocomposites are more than 3 times longer than that of Chitosan.

Table 4 MIC (%) (w/v) of nanocomposites comparing with chitosan, MMT, Na^+MMT and chitosan-MMT against different microorganisms

No	sample	MIC Bacteria	
		S,Aureus	*E.Coli*
1	Chitosan	0.025	0.05
2	MMT	0.1	0.1
3	Na^+ MMT	0.05	0.1
4	Chitosan-MMT	0.0125	0.0125

Table 5 RITs (h) of chitosan and different nanocomposites against different microorganisms

No	Chitosan-MMT	Concentration	RITs(h) Bacteria	
			S,Aureus	*E.Coli*
1	2 : 1	0.05	>120	>48
2	6 : 1	0.05	>120	>48
3	12 : 1	0.05	>120	>48
4	20 : 1	0.05	>120	>72

It was suggested that a main factor for the antibacterial activity could be due to the positively charged amino groups at C-2 in the chitosan molecule in solution below its pKa 6.3, they could interact with the predominantly anionic molecules at the cell surface. This interaction could change the permeability of the cell membrane of the microorganisms, resulting in a leakage of intercellular components, and then caused the death of the cell [16]. On the other hand, it was reported that clay with large specific surface area could adsorb the bacteria from the solution and immobilize the bacteria with the help of its excellent adsorption capacity, although the natural clay doesn't have inhibitory effect on bacteria. In this system, the antibacterial process of chitosan MMT nanocomposites may be divided into two stages. The first stage was the adsorption of the bacteria from the solution and immobilization on the surface of the clay. The second stage was related to the accumulation of chitosan on the surface of the clay; chitosan chains were in order and aggregated when confined in the interlayer of the silicates, positive charge (amino groups) density in unit volume may increase, accordingly, the stronger interaction between amino groups and bacteria may occur. Hence, the nanocomposites have better inhibitory effect on the growth of bacteria as compared to pure chitosan. In addition, it is seen that the chitosan MMT nanocomposites show much stronger antibacterial activity than the chitosan MMT nanocomposites. The result indicates that the cooperation effect of chitosan, MMT, and CTAB may induce excellent inhibition properties on the Grampositive bacteria for the chitosan MMT nanocomposites.

In addition, it can be observed that the higher amount and the larger dL value of the layered silicates resulted in the stronger inhibitory activity against Gram-positive bacteria. And the 2:1 chitosan MMT nanocomposite with the highest amount of Na^{+}MMT and the 12:1 chitosan-MMT nanocomposite with the largest interlayer distance show the strongest antibacterial activity. As the amount of clay increased, the effective layers in unit weight may increase because of good dispersion, thereupon larger specific surface area was obtained, and more bacteria were adsorbed and immobilized on the surface of clay, then chitosan inhibited them. In the same way, with the increase of the interlayer distance, specific surface area of the layers also magnified; besides more chitosan chains were inserted into the interlayer and positive charge density in unit volume further increased; in this way, chitosan may have more chance to inhibit the growth of the bacteria. Therefore, alternatively, the increase of the amount or the interlayer distance may result in the improvement of the inhibitory activity against Gram-positive bacteria.

However, the inhibitory activity against Gram-negative bacteria for the nanocomposites is not as good as the inhibitory activity against Gram-positive bacteria. The MIC values of the nanocomposites are only 2-4 times lower than that of chitosan, and the RITs of nanocomposites are just 2-3 times longer than that of chitosan. Moreover, as the amount and the interlayer distance of clay increase, the nanocomposites show decreasing inhibitory effect on Gram-negative bacteria. The fact may be related to the cell structure of the bacteria: Gram-positive bacteria have thick cell wall and no outer membrane, whereas

Gram-negative bacteria have thin cell wall and its most layers are outer membranes [5]. Hence, pure chitosan may have different action mechanisms against Gram-negative bacteria and Gram-positive bacteria. Furthermore, the adsorption action of the layered silicates against two species of bacteria may also be different. Further studies should be done to explain the fact.

4. Conclusions

A series of chitosan MMT nanocomposites were successfully synthesized which indicated a good intercalation of the polymeric phase into clay interlayer galleries. FT-IR and XRD results showed the interaction between chitosan matrix and MMT. It was the interaction that caused the enhanced thermal stability and antimicrobial activity in comparison with pure chitosan orMMT, and they were proportional to the amount and the interlayer distance of the layered silicates. With the increase of the amount and the interlayer distance of the layered MMT, the nanocomposites showed stronger antibacterial effect on Gram positive bacteria, while the nanocomposites showed weaker antibacterial effect on Gram-negative bacteria. There were several possibilities to explain the results about Gram-positive bacteria, but for Gram-negative bacteria, consideration was only taken from its different cell structure according to the present work.

The WVP of the films was reduced ($p < 0.05$) by incorporation of such particles, while the water solubility decreased as the MMT concentration increased (for a constant chitosan concentration). The values of TS strength of chitosan MMT films increased significantly ($p < 0.05$) with increasing chitosan concentration, while the values of E decreased for high values of chitosan concentration. The presence of MMT does not seem to influence the values of those two properties. The values of TS increased with the increase of chitosan concentration, but no significant changes in Tm were detected with the addition of MMT.

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Effect of Growth Substances on *in vitro* Callus Induction and Shoots Elongation of Cashew Nut (*Anacardium occidentale* L.)

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ABSTRACT

Growth substances are important factors for plant tissues differentiation whereby their types and concentrations determine the success of *in vitro* plant propagation very much. This research tried to assay the potential of three growth substances which are auxin, cytokinin and gibberellin. These three substances were expected to affect the callus induction and shoot elongation.

The purposes of this research are: 1) To know the effect of types and concentrations of auxin on callus induction and; 2) To know the most effective cytokinin and gibberellin combination in promoting the *in vitro* elongation of cashew nut shoots.

Method used was experiment with the auxin types as the independent variables which were picloram; 2,4 D and 2,4,5 T with concentrations used 25 and 50 mL/L each whereas the dependent variables were length, width, weight, and color of the callus. For experiment on shoot elongation, cytokinin combinations used were zeatin and BAP with gibberellin. Cytokinin concentrations used were 1.3 and 5 mL/L whereas the concentrations of gibberellin used were 0.3, 0.5 and 0.7 mL/L. Quantitative data parameters were length of shoot, number of shoot and leaves increases.

The results for callus induction experiment based on descriptive analysis showed that the most effective auxin type in inducing callus was picloram with concentration 50 mL/L. Callus produced looked green with compact structure and were classified as nonembryogenic. The results for shoot elongation experiment showed that zeatin 5 mg/l was the most effective treatment for shoot elongation.

The conclusions were that types of growth substance affected the callus induction and shoot elongation whilst growth substance combinations did not.

Keywords: *Anacardium occidentale* L., callus induction, shoot elongation, growth substance.

INTRODUCTION

Cashew nut (*Anacardium occidentale* L.) has high economic value. The propagation of this plant could be done in conventional ways, whether generatively or vegetatively. Generative propagation uses grains of spindle. However, the grains of spindle contain different genetic materials compared to the parent plant (Zaubin and Suryadi, 2003). Vegetative propagation could use grafting. However the drawback of this technique is that the growth would be more active at the apex, making it dense quickly. Half-sphere canopy would be formed with relatively low branches, causing low productivity (Hadad, Daras and Wahyudi, 2007).

The other alternative which could be used is through tissue culture technique. It has many advantages over conventional techniques which are: small explant size, able to propagate plants with low reproductive capability and weather-independent (George, Hall and Klerk, 2008). With this technique, it is expected that the cashew nut propagation results in high quality and quantity seeds. The determining factors in tissue culture propagation are the type and concentration of growth substances used.

To improve the plant quality, plant propagation could be done through three ways: adventive shoot formation, lateral shoot formation and somatic embryogenesis (Sukmadjaja,

2005). This research was intended to know the most effective growth substance type and concentration in inducing callus and promoting shoot elongation.

MATERIALS AND METHOD

The research was done at Cellular and Tissue Biology and Center for Biotechnology and Agriculture Genetic Resources Research and Development Laboratory (BB-BIOGEN), Bogor. The research was begun on June and completed on November 2010.

Method used was experiment. The dependent variables were the week the induction takes place, length, width, weight, color and structure of callus for experiment on callus induction and lenght of cashew nut shoot for experiment on shoot elongation. Both experiment used various types and concentrations of growth substances as the independent variables. The experiment's design complied to fully randomized design and all treatments were grown on Murashige Skoog (MS) growth medium. Induction of callus data analysis was done descriptively while shoot elongation data was analyzed statistically with two-way anova.

To induce the callus, there were three auxin types used: picloram; 2,4-D and 2,4,5-T with concentration 25 mg/l and 50 mg/l each (refer to Table 1 for the experiment design). For experiment on shoot elongation, cytokinin combinations which were zeatin and BAP with gibberellin were used (Refer to Table 2 for the experiment design).

Table 1. Design for experiment on callus induction

auxin type	**concentrations**	
	25mg/l (B_1)	**50mg/l** (B_2)
2,4-D (A_1)	A_1B_1	A_1B_2
2,4,5-T (A_2)	A_2B_1	A_2B_2
picloram (A_3)	A_3B_1	A_3B_2
control	A_0	

Table 2. Design for experiment on shoot elongation

		concentration ratios (mg/l)		
gibberellin / **cytokinin**		0.3	0.5	0.7
BAP	1	0.3 : 1	0.5 : 1	0.7 : 1
	3	0.3 : 3	0.5 : 3	0.7 : 3
	5	0.3 : 5	0.5 : 5	0.7 : 5
zeatin	1	0.3 : 1	0.5 : 1	0.7 : 1
	3	0.3 : 3	0.5 : 3	0.7 : 3
	5	0.3 : 5	0.5 : 5	0.7 : 5

The following were the instruments used: laminar air flow, hot plate, pH meter, magnetic stirrer, motor plate, autoclave model HL 36, Wtb binder type oven, analytic balance (4 digits), analytic balance (3 digits), planting tools and Nikon D3000 digital camera.

The materials used were as follows: explants of cashew nut (*Anacardium occidentale* L.) shoot's meristematic tissue which were obtained from in vitro propagations, Balakrisnan-type (B-02) cashew nut shoot measuring approximately 1 cm, growth medium Murashige-Skoog (MS), growth substances picloram; 2,4-D; 2,4,5-T; zeatin; BAP and gibberellin (GA_3).

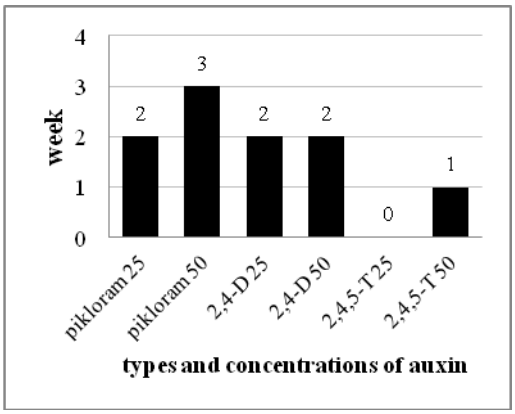

Fig. 1. Weeks of callus formation

The first step of the whole procedures was to sterilise all the equipments and apparatus, followed by the making of MS medium with growth substances added according to the treatments. After all equipments and growth substances had been ready, all prepared explants were planted. Observations and data analysis were done afterwards. The parameters for experiment on callus induction were time in which the induction occured, lenght, width, weight, color and structure of callus. The parameters for experiment on shoot elongation were the shoot elongation and the shoots and leaves number growth.

RESULTS AND DISCUSSION

The results showed that the highest percentage of callus induction was shown by picloram treatment concentration 50 mg/l and 2,4-D concentration 25 mg/l, which was 60%. No induction (0%) was shown by 2,4,5-T concentration 25 mg/l. The percentage of explant with callus is shown in Table 3.

Table 3. Percentage of callus-forming explants

auxin types & concentrations	total	mean (%)
Pik 25 mg/l	200	40
Pik 50 mg/l	300	60
2,4-D-25 mg/l	300	60
2,4-D-50 mg/l	200	40
2,4,5-T-25 mg/l	0	0
2,4,5-T-50 mg/l	100	20
total	1100	
mean		

Notes: 100 = callus induced
0 = callus not induced

Based on the data shown in Fig. 1. the fastest callus induction was shown by the administration of auxin 2,4,5-T with concentration 50 mg/l in which the callus was formed on the first week (4th day).

Parameter observation of calus length in Fig. 2 had showed that the administration of picloram concentration 50 mg/l yielded the highest length average which was 0.88 cm while the lowest length average was shown by 2,4,5-T concentration 50 mg/l which was 0.3 cm.

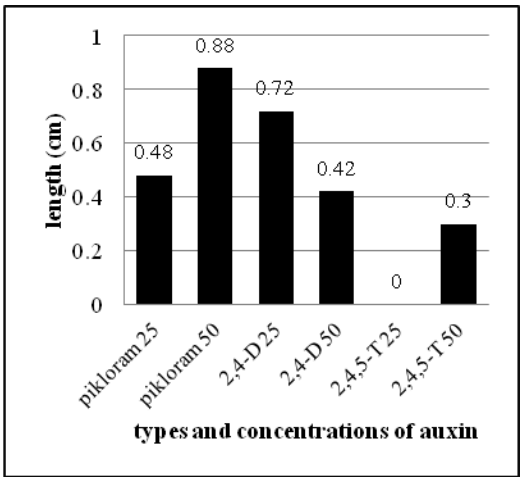

Fig. 2. Callus' length average

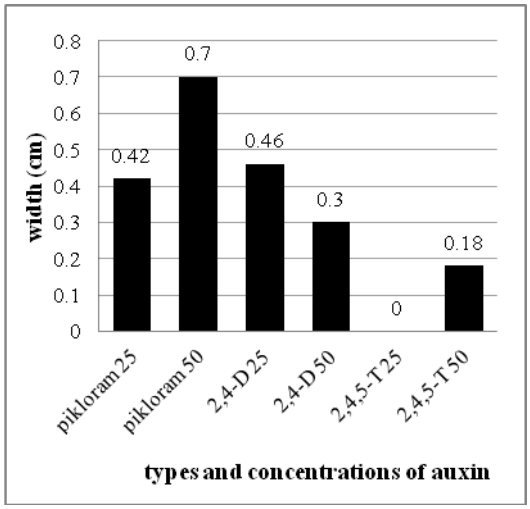

Fig. 3. Callus' width average

Callus' width observation results could be seen in Fig. 3. The callus' most width (0.7 cm) was shown by the treatment picloram concentration 50 mg/l. The callus' least width (0.18 cm) was shown by the treatment 2,4,5-T concentration 50 mg/l.

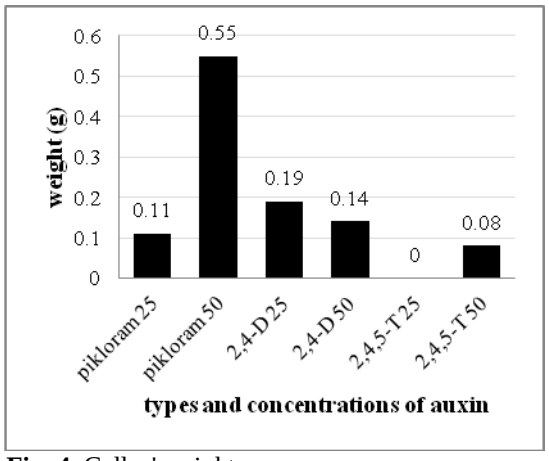

Fig. 4. Callus' weight average

Fig. 4 shows the observation results of callus' weight. Picloram concentration 50 mg/l yielded callus 0.55 g which was the highest among other callus, while the lowest weight of 0.08 g of callus was yielded by treatment 2,4,5-T concentration 50 mg/l.

Based on the data about length, width and weight of callus, it was shown that synthetic auxin picloram was the most effective growth substance to induce callus' growth. The use of picloram as the best growth substance in inducing callus was also confirmed in a previous similar research of *Ficus deltoidea* Jack explant. According to Pick Kiong, Then Chia and Shobri (2007), the use of picloram resulted in better embryogenic callus of *F. Deltoidea* Jack than 2,4-D. George, Hall and Clerk (2008) had stated that picloram is the best auxin to induce callus, especially the embyogenic callus and it is more effective than auxin 2,4-D. Auxin triggers cells differentiation which also triggers morphogenesis or embryogenesis (Lo Schiave *et al*., 1989 *in* George, Hall and Klerk, 2008). The other effect of auxin during embryogensis is acidification of cell wall (Kutschera, 1994 *in* George, Hall and Klerk, 2008). The acidification results in the elastic cell wall and causes cell wall to stretch whereby the cell's permeability increases.

Along with the callus development, there are size and appearance changes. As shown in Fig. 5 and 6, from the first to seventh week the callus's size had risen. The most considerable growth took place in 6th and 7th week. From sixth and seventh week onwards, callus' size and condition varied. According to George, Hall and Klerk (2008), callus' growth has several development stages as follows: cell division activation, active and rapid cell

division, specific cell differentiation and cell division and differentiation decline which ends up in the death of callus' cells.

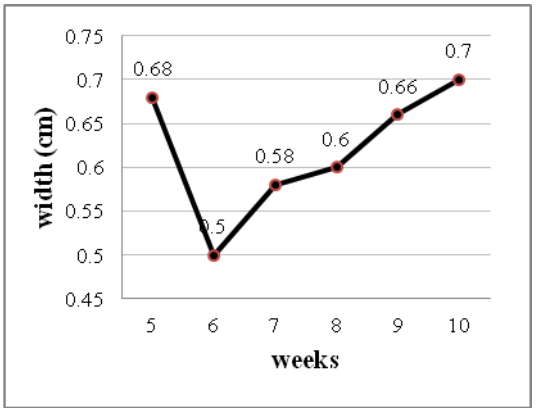

Fig. 5. Callus' width changes from week to week

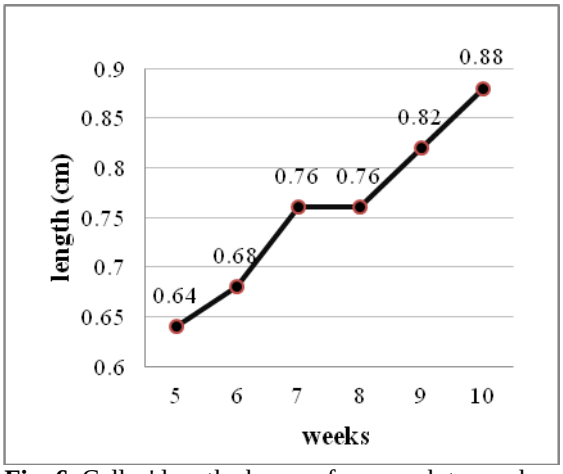

Fig. 6. Callus' length changes from week to week

Callus produced with picloram administration appeared green with long term callus' durability and showed increasing number of soft granules. All callus formed generally were nonembryogenic which were characterized by compact structure, moist and green to brownish color. The possible reasons why no embryonic callus formed in this research are due to low explant's merismatic potential which is the characteristic of woody plant and high callus phenol content which inhibits the work of growth substances.

Whereas in experiment of shoot elongation, it was shown that various combinations between cytokinin and gibberellin with their respective concentrations induced various degrees of elongation. The average length of shoots for each treatment could be seen in Table 4.

Table 4. Average elongation of cashew nut shoots

	shoots elongation		
GA_3 (mg/l) / cytokinin (mg/l)	0.3	0.5	0.7
BAP 1	1.36 ± 0.11	1.44 ± 0.09	1.34 ± 0.19
BAP 3	1.32 ± 0.24	1.46 ± 0.17	1.36 ± 0.29
BAP 5	1.44 ± 0.09	1.46 ± 0.19	1.66 ± 0.25
zeatin 1	1.52 ± 0.19	1.50 ± 0.10	1.46 ± 0.22
zeatin 3	1.34 ± 0.17	1.62 ± 0.22	1.62 ± 0.18
zeatin 5	1.62 ± 0.45	1.42 ± 0.16	1.80 ± 0.25
control		1.30 ± 0.30	

The highest average of shoots elongation in the sixth week was shown by treatment combination zeatin concentration 5 mg/l and GA_3 concentration 0.7 mg/l which was 1.80 $\pm$ 0.25 cm.

Anova test results showed that cytokinin had significant effect by having significant value (0.029) less than 0.05 which means that zeatin and BAP provided significant effect on the

elongation of cashew nut elongation whereas GA_3 had significant value greater than 0.05 which is 0.13. This means GA_3 had no effect on the elongation. It was perhaps due to the inappropriate GA_3 concentrations used. However, Fig. 11a and 11b showed that leaves gained width which indicates the potential of GA_3 in widening the leaves of various plant species (Wattimena, 1988).

Fig. 11a. First week explant with treatment combination zeatin 1 mg/l and GA_3 0.5 mg/l

Fig. 11b. Fifth week explant with treatment combination zeatin 1 mg/l and GA_3 0.5 mg/l

Duncan posthoc test showed that among the various cytokinins used, Zeatin concentration 5 mg/l had the most effect (average score 0.6133).

It was known also from this research that if BAP and zeatin were used on their own instead being combined with GA_3, the yield would be the best. This was confirmed by a similar experiment of *Wrightia tinctoria* plant (Purohit and Kukda, 2004) in which BAP performed well without being combined with other growth substances.

Zeatin gave better results than BAP since it is a natural cytokinin whilst BAP is a synthetic one. George and Sherrington (1984) stated that *in vitro* growth and morphogenesis were affected by the interaction and balance between the supply of growth substances in the growth medium and natural growth substances which are present inside the cultured cells.

Other than to regulate the cellular mechanisms, many synthetic growth substances apparently modify endogenic growth substance concentration. Since zeatin is a natural growth substance, it does not alter the concentration of naturally occuring growth substances. Ellis *et al*. (1991) also came into similar conclusion, stated that zeatin was more effective than BAP on *Picea glauca* shoot growth.

On the observation of cell cycle of *Nicotiana tabacum* cv. Yellow-2 (BY-2), the most obvious cytokinin was zeatin with its highest concentration seen at the initial stage of G1 phase, transition between G1 phase and S phase, middle or late S phase and transition between G2 phase or M (Davies, 2004). George (1993) stated that G1 phase is when organelles' quantity is increasing and cytoplasm's volume is expanding. Right after G1 phase, cell enters S phase. S phase is when DNA is synthesized prior to replication. S phase is followed by G2 phase where cell is preparing itself for mitotic division, whereas M phase is the mitosis phase in which nuclear division and cytoplasmic separation occur.

Various combination and concentra-tion treatments of growth substances resulted in various increases of number of shoots.

Table 5. Average increase of number of shoots at sixth week

	increase of number of shoots		
GA_3 / cytokinin	0.3 mg/l	0.5 mg/l	0.7 mg/l
BAP 1 mg/l	0.2 ± 0.45	0.4 ± 0.55	0.0 ± 0.00
BAP 3 mg/l	0.0 ± 0.00	0.4 ± 0.55	0.4 ± 0.55
BAP 5 mg/l	0.4 ± 0.55	0.2 ± 0.45	0.0 ± 0.00
zeatin 1 mg/l	0.4 ± 0.55	0.2 ± 0.45	0.8 ± 0.45
zeatin 3 mg/l	0.4 ± 0.55	1.0 ±0.71	0.6 ± 0.89
zeatin 5 mg/l	0.6 ± 0.55	0.0 ± 0.00	0.0 ± 0.00
control		0.00 ± 0.00	

The highest increase average of number of shoots was shown by combination of zeatin 3 mg/l and GA_3 0.5 mg/l which was 1.00 $\pm$ 0.71. The lowest increase average of number of shoots was shown by combination of BAP 1 mg/l and GA_3 0.7 mg/l, MS and BAP 3 mg/l and GA_3 0.3 mg/l, BAP 5 mg/l and GA_3 0.7 mg/l, zeatin 5 mg/l and GA_3 0.5 mg/l, and zeatin 5 mg/l and GA_3 0.7 mg/l which was 0.00 $\pm$ 0.00. Fig. 13 shows the best result of the treatment combination of BAP and GA_3 from week to week, while Fig. 12 shows the best result of the treatment combination between zeatin and GA_3.

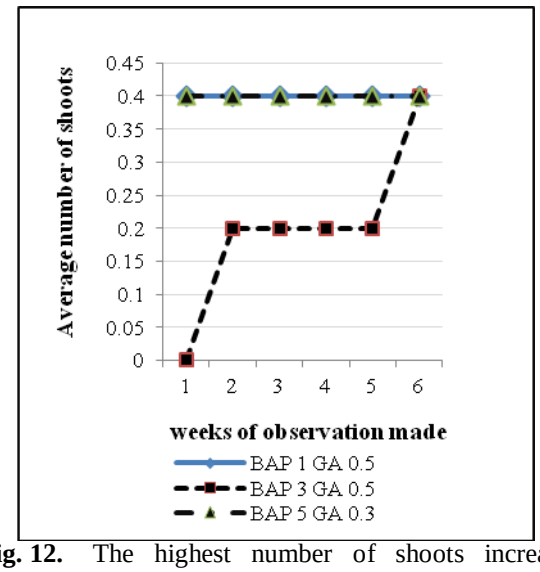

Fig. 12. The highest number of shoots increase induced by various combinations of BAP and GA_3 on six week old cashew nut shoots

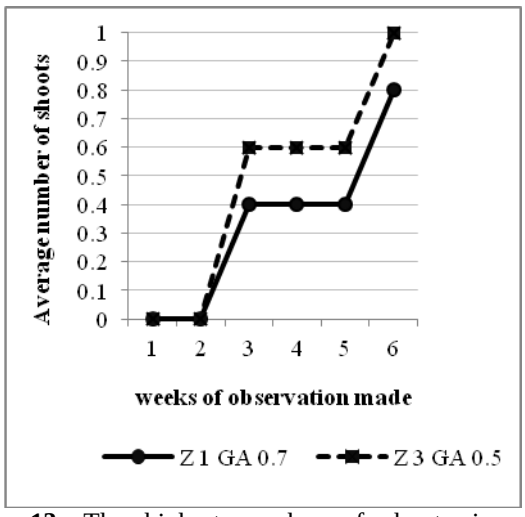

Fig. 13. The highest number of shoots increase induced by two combinations of zeatin and GA_3 on six week old cashew nut shoots

As shown in Fig. 12 and 13, combinations of BAP and GA_3 induced the fastest shoots formation. The possible explanation for this result is that the explants had adapted very fast so even in the first week, they were able to absorb nutrients from the growth medium optimally. The explants which did not respond to treatment possibly had low meristematic potential and high level of phenol oxidation (Mariska and Purnamaningsih, 1997).

Kruskal Wallis test showed that zeatin and BAP had significant value less than 0.05 which was 0.021 which means they had effect on the test subjects, while GA_3 had significant value more than 0.05 which was 0.407 which means no effect had been shown on the test subjects.

Cytokinin increased the number of shoots by stimulating cell division, inhibiting roots formation. It also plays a role during shoot proliferation (George and Sherrington, 1984). This was verified by Akbas *et al.* (2009) who studied almond (*Amygdalus communis* L. Cv. Yaltsinki). They found out that BAP was required to induce multiplications of seven-year-old apex almond shoots. GA_3, on the other hand, did not affect the shoot multiplication since its functions are to induce morphogenesis and inhibit root formations. It also has role during embryo development and cell differentiation (George, Hall and Klerk, 2008).

The effect of combinations and concentrations of growth substances on number increase of leaves could be seen in Table 6.

Table 6. Average of increase of number of six-week-old cashew nut leaves

	increase of number of leaves		
GA_3 (mg/l) / cytokinin (mg/l)	0.3	0.5	0.7
BAP 1	0.80 ± 0.84	0.40 ± 0.55	0.20 ± 0.45
BAP 3	0.20 ± 0.45	0.40 ± 0.55	0.00 ± 0.00
BAP 5	0.40 ± 0.55	0.40 ± 0.90	0.00 ± 0.00
zeatin 1	0.80 ± 0.84	0.20 ± 0.45	0.60 ± 0.55
zeatin 3	0.40 ± 0.55	1.20 ± 0.84	0.40 ± 0.55
zeatin 5	0.40 ± 0.55	0.00 ± 0.00	0.40 ± 0.90
control		0.20 ± 0.45	

The highest average of increase of number of six-week-old shoot's leaves was produced by the combination of zeatin 3 mg/l and GA_3 0.5 mg/l which was 1.20 $\pm$ 0.84 cm, while the lowest was shown by the combination of BAP 3 mg/l and GA_3 0.7 mg/l, BAP 5 mg/l and GA_3 0.7 mg/l, and zeatin 5 mg/l and GA_3 0.5 mg/l which was 0.00 $\pm$ 0.00 cm. The line graph depicting the best result of treatment combinations of BAP + GA_3 and zeatin + GA_3 could be seen in Fig. 6.

To know whether the effect of combination of cytokinin and gibberellin was present or not, data were analyzed by using Kruskal Wallis nonparametric test. The resulting significant value obtained was 0.24 which was greater than 0.05. That means the combination treatment did not have any effect on the *in vitro* leaves' number increase. This absence of effect was suspected due to undifferentiated shoots. Shoots would undergo several growth phases which were initiated by cell division, elongation, differentiation and maturation. It was possible that most of the shoots were not yet entering differentiation phase, but still in elongation phase (Kozlowski, 1971).

CONCLUSIONS

1. The most effective auxin to induce cashew nut shoots was picloram concentration 50 mg/l. Resulting callus had green color, compact structure and were classified as nonembryogenic.
2. Zeatin 5 mg/l had the best effect on elongation.

ACKNOWLEDGEMENT

We thank the head of BB-BIOGEN and all the staffs for all the assistance they provided during our research in the laboratory.

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SCREENING AND IDENTIFICATION OF *p,p'*-DDT DEGRADING BACTERIA FROM AGRICULTURAL SOIL

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1,1,1-Trichloro-2,2-bis(p-chlorophenyl)ethane or p,p'-DDT is one of the most persistent pesticides known to date due to its chemical composition and properties. Microbial degradation is a potential mean of remediation to remove recalcitrant and toxic compounds such as DDT from the environment. This study was conducted to screen and investigate the DDT degradation ability of indigenous soil bacteria in agricultural soil samples from Songkhla province, Thailand with known DDT levels between 0.19 - 9.84 ng/g soil dry weigh (ppb). Twelve bacterial consortia with 53 isolates were screened from agricultural soil samples of Songkhla province inoculated in mineral salts yeast extract medium (MSYM) supplemented with 100 ppm of p,p'-DDT (DDT_{100}). Bacterial consortium PD7, which exhibited a combination of high p,p'-DDT degradation of 94.72% and maximum growth within 72 hrs, was chosen for further biodegradation studies. From the single isolate degradation studies, it was found that DDT degradation level were obtained to be 30.12, 27.80, 39.40, 49.60 and 50.63% for isolates PD7-1 PD7-2 PD7-3 PD7-4 and PD7-5, respectively. Based on 16S rRNA sequences analysis, all five bacterial isolates were identified to be Curtobacterium citreum (PD7-1), Rhodococcus pyridinivorans (PD7-2), Niabella sp. (PD7-3), Bacillus anthracis (PD7-4) and Shinella zoogloeoides (PD7-5).

Keywords: Biodegradation, *p,p'*-DDT, bacteria, pesticides

Introduction

1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane or dichlorodiphenyl-trichloroethane (DDT), discovered as a pesticide in 1939, is the most widely known pesticide of the 20th century. It is also one of the most persistent environmental pollutants because of its toxicity and hydrophobicity, resulting in its bioaccumulation (Kamanavalli and Ninnekar, 2004). Although the use of DDT has been banned by several developed countries in the 1970s, the developing countries continue to use this pesticide in their agriculture and public health program. These have resulted in DDT contamination of various areas around the world, including soil and sediment in Thailand (Kumblad *et al.*, 2001; Thapinta and Hudak, 2000).

Due to the extremely slow Biodegradation of DDT in natural environments, enhancement of the Biodegradation or mineralization process of DDT by microorganisms has been gaining popularity in the last decades. Biodegradation is the important processes involved in the remediation of DDT contaminated areas. In some cases, biodegradation of DDT using single isolate usually yields low Biodegradation result compared with those of bacterial consortium under the same conditions (Bidlan and Manonmani, 2002). Therefore, it is important to study and understand the biodegradation capability and relationships of each bacterial species within a microcosm in order to predict the degradation process of various contaminants in the environment. Thus, in this study we aim to screen and identify for indigenous soil bacteria with DDT biodegradation abilities.

Research Methodology

Materials and Equipments

The chemicals used in this research are analytical standardized Aldrin [1,1,1 Trichloro 2,2 bis(4-chlorophenyl)ethane] or *p,p'*-DDT, Ethyl Acetate and Acetone. All 12 soil samples used in this study are sample stocks collected and prepared by Sonkong (2007) from soil in various agriculture area of Songkhla Province with history of continued farming activities for more than 30 years.

Mineral salt yeast extract medium (MSYM) used for screening and degradation studies consisted of (per 1 liter of distilled water) 0.20 g $MgSO_4.7H_2O$, 0.25 g NH_4NO_3, 0.675 g K_2HPO_4, 0.10 g $Ca(NO_3)_2$ and 0.01% yeast extract (modified from Nadeau, 1995). The medium pH was adjusted to 7.0 and sterilized by autoclaving at 121^oC for 15 min. Supplemented *p,p'*-DDT was filter-sterilized and added to the medium at a concentration of 20, 40, 60, 80 or 100 ppm**.**

Methods

Analytical methods

Two mililiters of sample from cultivation broth was extracted three times with 6 ml of ethyl acetate. Organic phase was collected and then evaporated overnight to dryness. The residue was redissolved in 2 ml of acetone *(modified from Bidlan and Manonmani, 2002)*. A known quantity of the sample was injected into gas chromatography with electron capture detector (GC-ECD) for analysis.

p,p'-DDT concentration was determined by gas chromatography-^{63}Ni micro electron capture detector (GC-ECD Hewlettt-Packard 6890) using the following conditions: HP-35 capillary column (35% crosslinked methyl phenyl siloxane; 30 m x 0.25 μm ID); injector and detector temperatures of 250^oC and 320^oC, respectively; oven temperature programmed at 150^oC for 1 min and increasing at 20^oC/min to 250^oC then held for 4 min. The carrier and make up gasses were helium (2 ml/min) and nitrogen (60 ml/min), respectively. Injection was performed using the split injection technique with 50:1 injection ratio. Standard curve was constructed by injecting 1 μl of *p,p'*-DDT at 1, 5, 10, 50 and 100 ppm. This standard curve was used to quantify *p,p'*-DDT concentration from both soil and culture broth samples.

Experimental methods

- *Screening of bacterial consortium and isolates with the ability to grow in the presence of p,p'-DDT*

Mineral salt yeast extract medium (MSYM) was used to screen for *p,p'*-DDT degrading bacteria. Ten gram of soil sample was added to 100 ml MSYM supplemented with 20 ppm of *p,p'*-DDT (DDT_{20}). Culture was incubated at room temperature, 150 rpm for 2 days or until medium turbidity was observed. Sub-culturing was then performed with 10% inoculum into MSYM supplemented with 40, 60, 80 and 100 ppm of *p,p'*-DDT respectively. Culture was again incubated at the conditions previously described. To screen for single bacterial isolate, 100 μl of bacterial consortium grown at 100 ppm of *p,p'*-DDT was spread on MSYM+DDT_{100} agar and incubated at room temperature until bacterial colony was observed. All bacterial consortia and single isolates were stored as stock cultures in MSYM+DDT_{20} containing 20% glycerol at -20^oC.

- *Screening of bacterial consortium and isolates with the ability to degrade p,p'-DDT*

For starter culture, 100 μl of each stock culture was added to 10 ml MSYM+DDT_{20} and incubated at room temperature, 150 rpm until turbidity was observed (approximately OD_{600} = 0.5). Ten mililiter of sample was added to 100 ml MSYM+DDT_{100} and incubated at room

temperature, 150 rpm until exponential phase of growth was reached. Samples were collected every 12 hours for the determination of growth and *p,p′*-DDT degradation (see Analytical methods). Bacterial consortium showing the highest ability of *p,p′*-DDT Biodegradation was selected for further studies.

Results and Discussion

Screening of bacterial consortium and isolates with the ability to grow on *p,p′*-DDT supplemented medium

Soil samples from agricultural area in Songkhla province with history of continuous farming activities for many decades were used for bacterial screening. Based on previous studies by Sonkong (2007) and Jeenon (2008), these 12 agricultural areas were reported to contain both *p,p′*-DDT and γ-HCH in varying amount of 0.19 - 9.84 and 0.03 - 0.45 ng/g soil dry weigh (ppb), respectively. Since there have been reports showing effective *p,p′*-DDT degradation under aerobic condition (Nadeau *et al*., 1993; Bidlan and Manonmani, 2002), soil were collected from the first 15 cm of the surface. This collecting strategy was chosen in order to obtain high diversities of aerobic/facultative anaerobic bacterial communities, which may be accustomed to the prolong exposure of pesticides, particularly *p,p′*-DDT.

To further increase the probability of isolating *p,p′*-DDT degrading soil bacteria, selective enrichment and acclimatization to the increasing concentration of *p,p′*-DDT from 20 to 100 ppm in the cultivation medium (MSYM) were performed. These technique was proven to be able to increase the ability of growth as well as degradation of *p,p′*-DDT in the cultivation medium. The observation have been made in studies with DDT (Bidlan and Manonmani, 2002) and HCH (Manonmani *et al.*, 2000), where acclimatization improved the degradation ability of DDT/HCH degrading consortium.

From these procedures, all soil samples were shown to contain bacterial consortium tolerant to the supplemented *p,p′*-DDT. Selective enrichment and acclimatization process ultimately resulted in the survival of 12 bacterial consortia with 53 morphologically distinct isolates (data not shown).

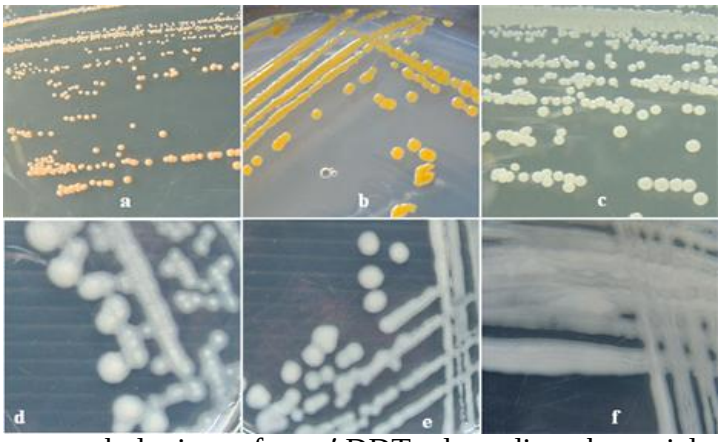

Figure 1. Colony morphologies of *p,p′*-DDT degrading bacterial isolates grown on MSYM+DDT_{100} agar. (a = PD7-2; b = PD7-3, c = PD7-5; d = PD3-4; e = PD5-6; f = PD6-4)

Study of *p,p′*-DDT degradation by bacterial consortium

Bacterial consortia screened from agricultural soils were tested for their *p,p′*-DDT degradation ability by cultivating in MSYM medium supplemented with 100 ppm *p,p′*-DDT at room temperature, 150 rpm for 10 days. *p,p′*-DDT degradation profiles of the bacterial consortia displayed significantly different trends. From these biodegradation activities, we can define the bacterial consortium into 3 groups, i.e., low, moderate and high DDT

degraders. Low DDT degraders, which had *p,p′*-DDT biodegradation less than 50%, while moderate and high DDT degraders have *p,p′*-DDT biodegradation between 50 - 75% and more than 75%, respectively (Fig. 2).

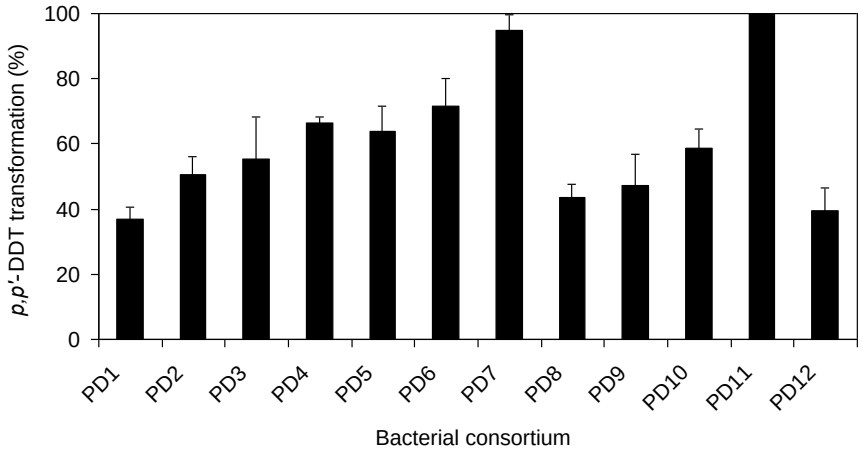

Figure 2. *p,p′*-DDT biodegradation levels by individual bacterial consortium after 10 days of incubation in MSYM+DDT_{100} at room temperature and 150 rpm. Bars represent the standard deviation from three determinations. (Negative control = No bacteria; Level of *p,p′*-DDT biodegradation = 3.47%)

Comparison of the two highest *p,p′*-DDT degraders, bacterial consortium PD7 and PD11, showed approximately the same level of *p,p′*-DDT degradation. However, the rate of degradation, amount and rate of growth were significantly different. Bacterial consortium PD7 displayed high rate of *p,p′*-DDT degradation from day 1 of cultivation, while that of PD11 were very slow and required over 5 days before rapid *p,p′*-DDT biodegradation was observed (data not shown). The growth profile of both consortia was also different. Although maximum growth by bacterial consortium PD7 took longer to reach than PD11 (5 days compared to 2 days), but bacterial consortium PD7 was able to generate higher growth of up to 193.83 μg/ml total cell protein within 5 days of incubation while PD11 generated only 75.35 μg/ml in day 2 of incubation. This phenomenon suggested that PD11 did not utilized *p,p′*-DDT as the sole source of carbon. The reduction of DDT concentration could be attributed to other mechanism such as bioaccumulation/biomagnifications into either cellular body or adsorption on the cell surface. Based on this reasons, bacterial consortium PD7, which consisted of 5 isolates (Fig. 3), was selected for further DDT biodegradation studies and investigated for its single and mixed bacterial cultures ability to degrade *p,p′*-DDT.

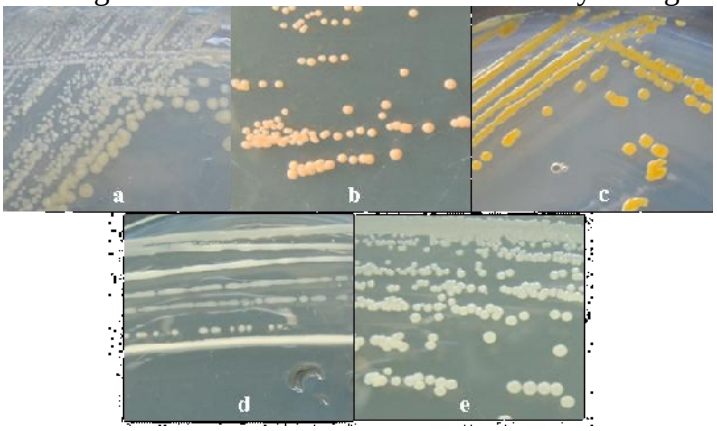

Figure 3. Five bacterial isolates from consortium PD7. (a = PD7-1; b = PD7-2, c = PD7-3; d = PD7-4; e = PD7-5)

Study of *p,p′*-DDT degradation by single isolate

The selected bacterial consortium PD7 composed of five isolates, i.e. isolate PD7-1, PD7-2, PD7-3, PD7-4 and PD7-5. These isolates were used to investigate *p,p′*-DDT biodegradation by single and mixed bacterial culture.

The profiles DDT biodegradation of each bacterial isolates after 10 days of incubation displayed similar trend of increasing within the first five days and exhibited slower biodegradation rate from day 5 to day 10 (Fig. 4 and 5). The exception was for isolate PD7-5, which still showed the increase of growth after day 5 of incubation with no significant increase in *p,p′*-DDT biodegradation. In this stage, isolate PD7-5 may be encountering a substrate selection situation where more than one substrate is available, since some metabolites product such as DDE, which was less stable and toxic, were generated in the medium. This was a possible reason of just slightly increase of *p,p′*-DDT degradation was achieved by PD7-5 from day 5 to day 10, while growth showed significant increase at the same period (Fig. 4 and 5). However, the *p,p′*-DDT biodegradation profiles of bacterial consortium PD7 showed continuous increase from inoculation until day 10 of incubation with growth entering stationary phase by day 3 of cultivation (Fig. 4).

All bacterial isolates displayed *p,p′*-DDT biodegradation of 27.80% to 50.63% from the initial 100 ppm *p,p′*-DDT after 10 days of incubation in MYSM+DDT_{100} medium at room temperature and 150 rpm (Fig. 4). Bacterial isolate PD7-1, PD7-2, PD7-3, PD7-4 and PD7-5 showed *p,p′*-DDT biodegradation levels of 30.12, 27.80, 39.40, 49.60 and 50.63%, respectively (Fig. 5). The biodegradation levels of all five bacterial isolates were quite lower than that of bacterial consortium PD7 (more than 90% *p,p′*-DDT degradation). The possible reason was a synchronous (symbiotic) interaction between the isolates within the bacterial community, which worked together to increase the overall level of *p,p′*-DDT biodegradation.

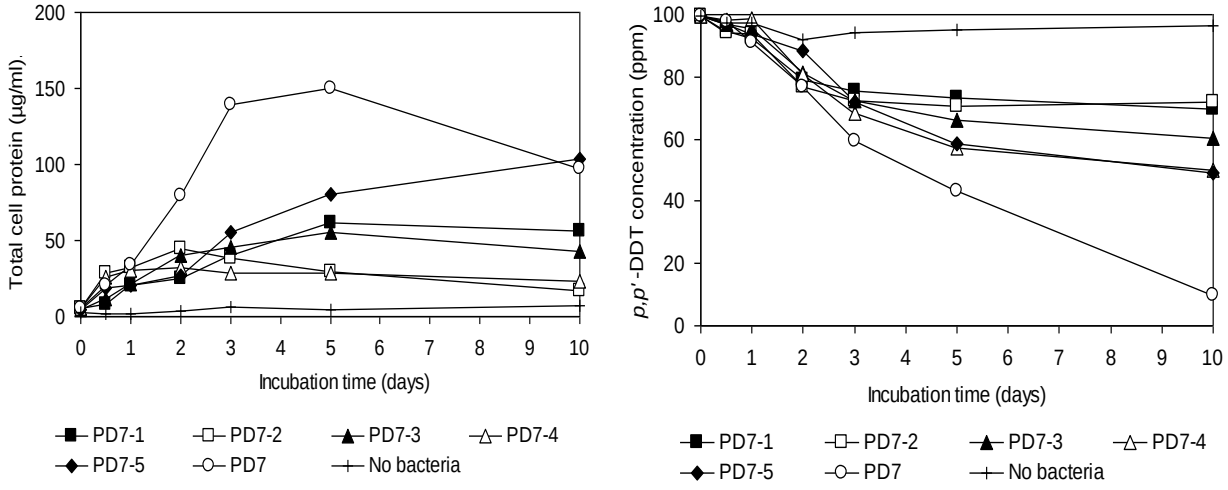

Figure 4. Bacterial growth and *p,p′*-DDT biodegradation profile of bacterial consortium PD7 and its isolates (PD7-1 to PD7-5) grown in MYSM+DDT_{100} at room temperature, 150 rpm for 10 days.

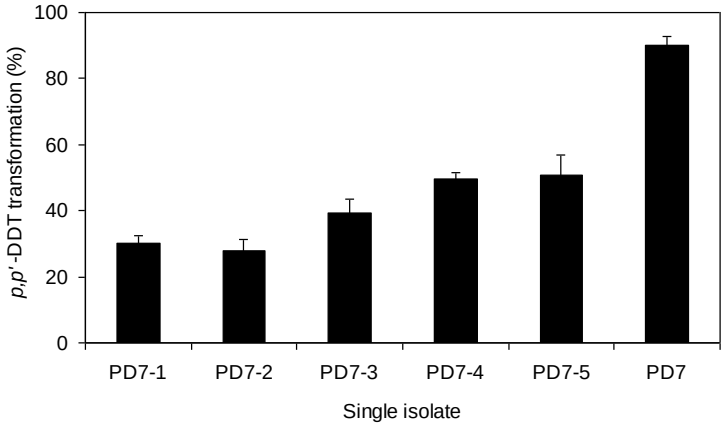

Figure 5. *p,p′*-DDT biodegradation levels by individual bacterial isolates grown in MSYM+DDT_{100} at room temperature, 150 rpm for 10 days. Bars represent the standard deviation from three determinations. (Negative control = No bacteria; Level of *p,p′*-DDT biodegradation = 2.81%)

Similar results were observed in the studies of *p,p′*-DDT biodegradation by soil consortium and single *Serratia marcescens* DT-1P culture (Bidlan and Manonmani, 2002), immobilized mixed bacterial cultures (Beunink and Rehm, 1988), and the biodegradation of blended crude oil by marine sediment Hii *et al.* (2008). Bacterial consortium generally showed higher *p,p′*-DDT degradation ability in comparison with that of bacterial isolate. For example, soil consortium were shown to completely degrade 25 ppm *p,p′*-DDT within 144 hours under aerobic condition at room temperature, while only 80 % was achieved by single isolate under the same condition (Bidlan and Manonmani, 2002).

Identification of bacterial isolates by biochemical characteristics and 16S rRNA sequences analysis

Based on their colony/cell morphologies and biochemical properties (data not shown), there were 12 bacterial consortia with 53 distinc bacterial isolates were identified to be able grow on *p,p′*-DDT supplemented medium.

For the five bacterial isolates from consortium PD7, i.e. PD7-1, PD7-2, PD7-3, PD7-4 and PD7-5 further molecular identifications were performed by 16S rRNA sequences analysis. Full length sequences from each isolate (more than 1300 bases) were BLAST to GenBank database with the returned result between 95 - 99% identities. Based on 16S rRNA sequences analysis data, all five bacterial isolates were identified to be closely related to *Curtobacterium citreum* (PD7-1), *Rhodococcus pyridinivorans* (PD7-2), *Niabella* sp. (PD7-3), *Bacillus anthracis* (PD7-4) and *Shinella zoogloeoides* (PD7-5) (Table 1).

Table 1. 16S rRNA gene sequences analysis of *p,p′*-DDT degrading bacterial isolates from consortium PD7.

Isolates	Predicted genus/specie	Identity	Sequences similarity
PD7-1	*Curtobacterium citreum*	99%	1482/1483
PD7-2	*Rhodococcus pyridinivorans*	99%	1461/1463
PD7-3	*Niabella* sp.	95%	1325/1390
PD7-4	*Bacillus anthracis*	99%	1449/1451
PD7-5	*Shinella zoogloeoides*	98%	1426/1446

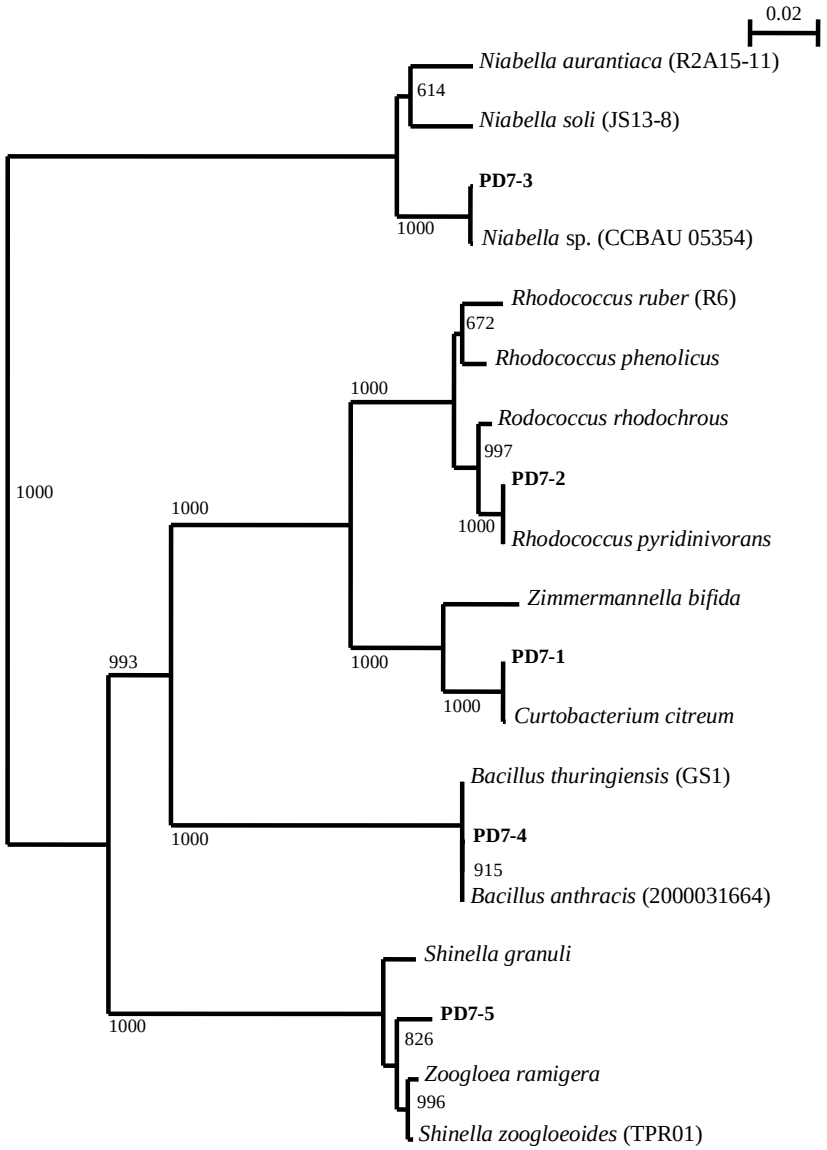

Figure 6. Phylogenetic analysis of the 16S rRNA sequence analyses of *p,p'*-DDT degrading bacterial isolates PD7-1, PD7-2, PD73, PD7-4 and PD7-5.

Conclussions

Twelve bacterial consortia with 53 distinct bacterial isolates were obtained from 12 soil samples collected from various agricultural areas in Songkhla Province, Thailand, through selective enrichment and acclimatization processes in mineral salt yeast extract medium supplemented with 100 ppm of *p,p'*-DDT (MSYM+DDT_{100}). All twelve bacterial consortia were shown to have the ability of 37.03 to 100% biodegradation from initial 100 ppm of *p,p'*-DDT within 10 days of incubation. Bacterial consortium PD7 which consisted of 5 single isolates was chosen for further study since it exhibited high *p,p'*-DDT biodegradation ability (94.72%) and generate high total cell protein (193.83 μg/ml). From the single isolate degradation studies, it was found that DDT degradation level were obtained to be 30.12, 27.80, 39.40, 49.60 and 50.63% for isolates PD7-1 PD7-2 PD7-3 PD7-4 and PD7-5, respectively. Based on 16S rRNA sequences analysis, all five bacterial isolates were identified to be *Curtobacterium citreum* (PD7-1), Rhodococcus *pyridinivorans* (PD7-2), *Niabella sp.* (PD7-3), *Bacillus anthracis* (PD7-4) and *Shinella zoogloeoides (PD7-5).*

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Antibacterial Activity Test and Phytochemical Screening of *Smilax celebica* Tuber

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ABSTRACT

Development of science and technology has brought many advances in various areas of life, one of which is the exploration of plant as medical herb. One of the plants that have potential as a medical herb is *Smilax celebica*. *S.celebica* is a type of shrub that growth wild, has spreading growth form, stems have sharp spines, bulbous and the root is tough and fibers.

This study aims to investigate antibacterial activity of ethanol extract obtained from *S.celebica* tuber against *Staphylococcus aureus* ATCC 35923 and *Shigella flexneri* ATCC 12022 and to screen the phytochemical components of *S.celebica*.

The result showed that ethanol extract of *S.celebica* tuber have potential as an antibacterial against *S.aureus* ATCC 35923 and *S.flexneri* ATCC 12022. Minimum Inhibitor Concentration (MIC) value of their ethanol extract against *S.aureus* ATCC 35923 was 3,13% and Minimum Bactericidal Concentration (MBC) value was 12,5%.
Whereas, MIC value of their ethanol extract against *S.flexneri* ATCC 12022 was 12,5% and its MBC value was 50%. Phytochemical screening of the plant was showed the presence of alkaloids, flavonoids and saponins.

Key word: *Smilax celebica*, antibacterial, phytochemical screening.

A. INTRODUCTION

The use of plants as a medical herb has currently become the focus of many studies. Plants are important source of potentially useful for development medical herb (Mahesh, 2008). The use of medical herb may offer a new source of antibacterial and antifungal agents with significant activity against infective microorganisms (Mun, 2003). Organization (WHO) has listed more than 20.000 species of medical plants (Devi, 2009). One of the plants that have potential as medical herb is *Smilax sp*. Ginting (2008) reported that tuber of Raru Gadong (*Smilax sp*) contains flavonoids and tannins and therefore has potential as antibacterial agent. Ivanova has reported that *Smilax excelsa* contain saponin (Ivanova *et al*, 2009). Saponin is bioactive compound has potential as antibacteria. *S.aureus* is a common bacteria found everywhere, including in many place in your body, normal microflora, negative Gram, anaerob fakultatif, non-motil, causes gastroenteritris at people (Ambarwati, 2007). Whereas, *S.flexneri* is negative Gram bacteria, non-motil, anerob fakultatif, causes shigellosis (Tian-Yi *et al*, 2005).

Smilax celebica is the member of Genus *Smilax* that grows in the RPH Mangunan forest, Imogiri, Bantul Yogyakarta. *S.celebica* is a type of shrub with single elliptical leaves, sharp edges, curved bone leaves, sharp thorns on green stems and hard and fiber roots. *S.celebica* at RPH Mangunan forest has presence abundant. *S.celebica* usually grows wild and is not widely known by local people. In addition, research on *S.celebica* from Imogiri as a potential agent of antimicrobial activity has not been conducted. Therefore, this study aims to determine the potential *S.celebica* primary as an antibacterial agent against *Staphylococcus aureus* ATCC 35923 and *Shigella flexneri* ATCC 12022 as well as to screen the phytochemical component of *S.celebica* tuber.

B. MATERIAL AND METHOD

Peparation of extract

S.flexneri celebica tuber was collected from RPH Mangunan forest, Imogiri, Bantul Yogyakarta. The plant was identified at the center for Plant Conservation Botanical Gardens LIPI Bogor. A total of 308 g of *S.celebica* tuber was macerated with 900 mL ethanol 70% in a closed container and incubated for 24 hours at the room temperature, while mixed repeatedly. The separated extracted were then filtered through filter paper and the ethanol filtrate were separately concentrated to dryness in vacuo using a rotary evaporator to remove the ethanol. Afterwards, stock solution of crude ethanol extract of were ready to use for dilution test. *S.celebica* tuber was made into concentration of 50%, 25%, 12,5%, 6,25% and 3,13% (b/v) with a total volume was 1mL.

Antibacterial activity

1. Determination of MIC
   The diluton method was performed to determine minimum inhibitory concentration (MIC). Determination of MIC was conducted by adding 1 mL suspension of bacteria into concentration was made, so the final concentration of extract become 25%, 12,5%, 6,25%, 3,13% and 1,56% (v/v). 4 tubes preparated for control, control of suspension (Cs, contain of 1 mL of bacteria + 1 mL of BHI media), control of media (Cm, contain of BHI media), control of extract (Ce, contain of 1 mL ethanol extract of *S.celebica* + 1 mL of BHI media). All tube were incubated for 24 hours, clarity and turbidity observed. The smallest concentration with a revealing clarity on the tube is MIC value of extract.
2. Determination of MBC (Minimum Bactericidal Concentration)
   MIC test result of ethanol extract of *S.celebica* then streaking on the BHI agar media to known value of MBC and incubated for 24 hours at 37ºC. Coloni of bacteria observed. The smallest concentration that is not overgrown with bacteria is a value MBC of extract.
3. Analysis of chemical compound and Thin Layer Chromatography (TLC)
   This analysis to know the content of chemical compounds of the tuber *S.celebica* alkaloids, flavonoids and saponins. The solvent used is ethanol, methanol, chloroform, n-Heksana and etil asetat. Mixed solvent system by trying different variations of the comparison, ethyl acetat : n-Hexane, ethyl acetat : ethanol, ethyl acetat : methanol, n-Hexane : methanol, methanol : chloroform, ethyl acetat : n-Hexane : methanol, and n-hexane : methanol : chloroform. Silica gel plates inserted into the vessel developers to eluted until the specified limits. It was observed by UV 245 nm. Phytochemical screening to determine the content of alkaloids are sprayed with dragendorf produce orange or pink, flavonoids sprayed with $AlCl_3$ produce brown and sprayed with a saponin anisaldehid produce purple.

C. RESULT

Antibacterial activity test of ethanol extract of *Smilax celebica* was repeatedly. The result of Minimum Inhibitor Concentration (MIC) and Minimum Baktericidal Concentration (MBC) has showed that ethanol extract of *S.celebica* has better antibacteri potential against *S.aureus* ATCC 35923 than *S.flexneri* ATCC 12022. It was showed in the table 1 and 2.

Table 1. The Result of Minimum Inhibitor Concentration (MIC)

No	Concentration	*Staphylococcus aureus* ATCC 35923			*Shigella flexneri* ATCC 12022		
		Repetition I	Repetition II	Repetition III	Repetition I	Repetition II	Repetition III
1	1,5 %	+	+	+	+	+	+
2	3,13 %	-	-	-	+	+	+
3	6,25 %	-	-	-	+	+	+
4	12.5 %	-	-	-	-	-	-
5	25 %	-	-	-	-	-	-
6	50 %	*	*	*	-	-	-
7	60 %	*	*	*	-	-	-
8	CM	-			-		
9	CE	-			-		
10	CSSa	+					
11	CSSf				+		

Description: (+) : turbid
(-) : clear
(*) : not done
CM : Control of media
CE : Control of extract
CSSa : Control of suspension of *S.aureus* ATCC 35923
CSSf : Control of suspension of *S.flexneri* ATCC 12022

Table 2. The Result of Minimum Bactericidal Concentration (MIC)

No	Concentrationi	*Staphylococcus aureus* ATCC 35923			*Shigella flexneri* ATCC 12022		
		Repetition I	Repetition II	Repetition III	Repetition I	Repetition II	Repetition III
1	1,5 %	+	+	+	+	+	+
2	3,13 %	+	+	+	+	+	+
3	6,25 %	+	+	+	+	+	+
4	12.5 %	-	-	-	+	+	+
5	25 %	-	-	-	+	+	+
6	50 %	*	*	*	-	-	-
7	60 %	*	*	*	-	-	-
8	CM	-			-		
9	CE	-			-		
10	CSSa	+					
11	CSSf				+		

Description: (+) : grow
(-) : not grow
(*) : not done
CM : Control of media
CE : Control of extract
CSSa : Control of suspension of *S.aureus* ATCC 35923
CSSf : Control of suspension of *S.flexneri* ATCC 12022

The result of TLC showed that the value of Rf were various, it is indicated that *S.celebica* have various compounds. The result of Phytochemical screening has showed that *S.celebica* contain compound of alkaloids, flavonoids and saponin. Complete of result showed in the table 3.

Table 3. The result of phytochemical screening

No	Compound	Sprayed (reagen)	Result	Value of Rf	Color
1	Alkaloid	Dragendorf	+	0,90	Orange
2	Flavonoid	AlCl3	+	0,88	Kuning
3	Saponin	Anisaldehid	+	0,80	Ungu

Description: (+) : yes (-): no

D. DISCUSSION

The Minimum Inhibitory Concentration (MIC) of ethanol extract of *Smilax celebica* has different result for *Staphylococcus aureus* ATCC 35923 and *Shigella flexneri* ATCC 12022. The MIC of extract *Smilax celebica* against *Staphylococcus aureus* ATCC 35923 was at a concentration 3,13% as shown by clearity as opposed on the medium contained 1,56% of extract. Whereas, at the concentration of extract was 3,13%, *S.flexneri* ATCC 12022 could be grow. The MIC of extract *S.celebica* against of bacteria *Shigella flexneri* ATCC 12022 was 12,5% (table.1). This concentration of ethanol extract *S.celebica* was able to inhibit growth of *S.flexneri* ATCC 12022 as indicated by clearity of medium. May be it cause, *S.aureus* ATCC 35923 was a Gram-positive bacteria has only one a cytoplasmic membrane and *S.flexneri* ATCC 12022 was a Gram-negative bacteria with 2 membrane of cell, so that it was causes the different inhibitory concentration(Atlas, 1996).

Cell membrane has an important role in the cell, namely as a chanel selective permeability, active transporting and controlling the composition of cell. Cell membrane is a continuous bilayer formed by lipids that are oriented with the polar lipid heads toward the outside and the non polar heads toward the center of membrane (Talaro, 2005). The extracted compounds are mostly obtained polar. This compound can deal directly with the outside of the cell membrane, interfere with its performance and cause of death.

The minimum bactericidal concentration (MBC) of extract *S.celebica* against *S.aureus* ATCC 35923 has antibacterial activity potential more a good than *S.flexneri* ATCC 12022. At a concentration of extract 12,5%, *S.aureus* ATCC 35923 can not grow. Whereas, *S.flexneri* ATCC 12022 at a predetermined concentration was up to the limit of 25%, *S.flexneri* could still grow although not as much at a concentration of 12,5 %, 6,25 %, 3,13 % and 1,56%. Therefore, the extract concentration increased to 50 % by 60%. At a concentration of 50 %, *S.flexneri* ATCC 12022 can not grow or die, so it can be determined that the KBM *S.flexneri* ATCC 12022 is 50% (table.2).

The result antibacterial activity of ethanol extract of *S.celebica* tuber compared with result studies Ambarwati (2007) about extract mimba seed with same method against *S.aureus,* the ethanol extract *S.celebica* has a better potential as antibacterial than extract mimba seed. Ethanol extract of *S.celebica* tuber compared with ethanol extract of Guajava leaves against *S.flexneri* has conducted by Ketut *et al* (2004), then the ethanol extract of *S.celebica* tuber have lower potential than extract Guajava leaves. Generally, the ethanol extract of *S.celebica* tuber has the high activity against *S.aureus* ATCC 12022 than *S.flexneri* ATCC 12022, which makes the plant have a potential as bioprospecting for antibiotic.

The test of compound content of secondary metabolites the spots on the TLC plate were sprayed with dragendorf a test reagent for alkaloids, $AlCl_3$ sprayed reagent to test

flavonoids and sprayed with a reagent anisaldehid to test saponin. The result phytochemical screening of ethanol extract of *S.celebica* tuber presence of alkaloid, flavonoid and saponin. Result showed that ethanol extract of tuber *S.celebica* contain alkaloids at Rf 0,9, flavonoids at Rf 0,88 and saponins at Rf 0,80.

E. CONCLUSION

Overall, ethanol extract of *Smilax celebica* tuber have a potential as antibacterial against *Staphylococcus aureus* ATCC 35923 and *Shigella flexneri* ATCC 12022. *S.celebica* tuber contains compounds alkaloid, flavonoids and saponins.

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Antimicrobial Activity of Leaves, Stems, and Barks of Palasu (*Mangiferra Caesia* Jack) Against Microorganisms Associated with Fish Spoilage

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ABSTRACT

Fish is a good protein source, but very perishable than the other foods. Several chemical preservatives are used to inhibit fish spoilage or to prevent the growth microorganisms. Plant extracts from palasu **(***Mangiferra caesia* Jack) may offer a new source of antimicrobial agent to against microorganisms associated with fish spoilage. Preliminary phytochemical analysis proved that flavonoid, phenolic, and tannin were in ethyl acetate and methanol fractions of leaves, stems, and barks of palasu. Antimicrobial activity from crude methanol extract, ethyl acetate, methanol and n-hexane fractions of palasu plant were tested to fourteen microorganisms using agar diffusion well. This results showed that ethyl acetate fraction from the leaves, stems and barks of palasu have the best antimicrobial activity followed by methanol fraction, crude methanol extract and n-hexane fraction, respectively. The Furthermore of examine revealed that ethyl acetate fraction from leaves at concentrations 1000μg exhibited antimicrobial activities against all test microorganisms. The order of sensitivity of microorganisms to this fractions are as follows *Klebsiella pneumonia* > *Bacillus subtilis* > *Aeromonas hydrophila* > *Bacillus cereus* > *Bacillus sp.* > *Eschericia coli* > *Candida albican* > *Enterobacteria* > *pseudomonas aeuroginosa* > *Salmonella sp* > *Citrobacter freundii* > *Staphylococcus aureus* > *Listeria monocytogene* and *Vibrio cholera.* Based on this finding, ethyl acetate fraction of palasu plant may be an alternative to chemical preservatives and used as natural antimicrobial preservatives to reclaim the shelf-life of fish.

Keywords: *Antimicrobial, microorganism of fish spoilage, palasu Mangifera caesia Jack*

I. Introduction

Fish is an important protein source and one of the main foods for human consumption. During storage and processing, the quality of fish may decline due to a complex reactions in Chemical, enzymatic or microbial activity (Ghaly *et al*., 2010). Bacterial change are considered the most important cause of fish spoilage (Gram and Dalgaard, 2002). Microbial spoilage and chemical deterioration cause losses of gross primary fish and fishery products.

Fish spoilaged causes fish to be unacceptable for consumption due to change in sensory characteristic and nutritional value. Microbial growth and metabolisms cause of fish spoilage which produce amines, biogenic amines such as cadaverin, histamine, putrescine, suphides, organic acids, aldehydes and ketons with unpleasant or off flavors (Gram and Dalgaard, 2002; Olafsdottir,*et al*., 2005; Dalgaard, *et al.,* 2006).

Improvement in shelf life of fish can have an important economic impact by reducing lost of attributed to spoilage. There is an increasing concern of the consumers for use food free of chemical preservative and then more interest for use of natural compound. Some of the synthetic preservatives are not safe, these are related with the usage of formaldehyde for fish preservation recently. Therefore, the exploration of antimicrobial and antioxidant compound from plant that potential as a natural preservative still continue.

Palasu (*Mangifera caesia* Jack) fruit empirically has a preservative activity in fresh fish. Dayak People in Sanggau West Kalimantan, use this fruit which is growth in wild forest for preservation their fresh fish, but until today the preservative characteristic of palasu is not

been studied. Previous research we had done indicated that extract of palasu fruit have antimicrobial activity on some bacteria.

In the present study we have been made to screen crude extract of palasu leaves, stems, and barks for antimicrobial activity on various Gram positive and Gram negative of spoiling fish and or pathogen food related bacteria .

II. Materials And Methods

2.1.Materials

The leaves, stems, and barks of palasu (*Mangifera caesia* Jack) were collected from Sanggau West Kalimantan. Chemicals such as normal hexane, methanol, ethyl acetate were purchased from Merck, *Listeria monocytogene* ATCC 7644, *Candida albicans, Vibrio cholerae, Enterobacteria, Bacillus cereus* ATCC 11944, *Bacillus sp, Salmonella sp, Staphylococcus aureus, Aeromonas hydrophila, Klebsiella pneumoniae, Bacillus substilis, Pseudomonas aeruginosa, Citrobacter freundii,* and *Eschericia coli* were purchased from laboratory of Microbiology Bogor Agriculture Institute (IPB), Nutrient Agar (NA) and Nutrient broth (BH) Oxoid, brain heart infusion agar, amoxycilin, cefadroxyl, and aquadest.

2.2. Procedure

Preparation of extract

About 400g of air dried powder of leaves, stems and barks of palasu were macerated with methanol for 3×24 hours. After that the methanolic extract was filtered. The solvent of supernatant was removed under vacuum using rotary evaporator. The dry residue was partitied with n-hexane, and then ethyl acetate. The organic solvent was evaporated. The dried crude fractions then dissolved in dimethyl sulphoxide (DMSO) for antimicrobial bioassay.

Preliminary phytochemical investigations

All the fractions were screened for detection of alkaloid, flavonoid, phenolic, saponin, and tannin according standard procedure by Harbone 1998.

Antimicrobial assay

The screening and evaluation of antimicrobial activities were used agar diffusion as well as described by Valgas *et al*., 2007.

III. Results And Discussion

3.1. Preliminary Phytochemical Analysis

Phytochemical analysis of the crude fractions of palasu (*Mangifera caesia* Jack) revealed that the presence of non nutrient phytochemical compound like alkaloid, flavonoid, phenolic, saponin and tannin (Table 1). These bioactive compounds which are synthesized as secondary metabolites to protect the plant from microbial attacks or animals predator. These compounds are generally associated with antimicrobial and antioxidant activity.

Table 1. The phytochemical screening of palasu (*Mangifera caesia* Jack) plant

Part used	Metabolite secondary compound				
(Fractions)	Alkaloid	Flavonoid	Phenolic	Saponin	Tannin
Leaves					
(n-Hexane)	-	-	-	-	-
(EtOAc)	+	+	+	-	+
(MeOH)	+	+	+	+	+
Stems					
(n-Hexane)	-	-	-	-	-

(EtOAc)	-	+	+	-	-
(MeOH)	-	+	+	+	+
Barks					
(n-Hexane)	-	-	-	-	-
(EtOAc)	-	+	+	-	-
(MeOH)	-	+	+	+	+

(+) detected
(-) not detected

In this result, the antimicrobial activities shown by fractions which have contain one or more of phytochemical constituents like in Table 1. Flavonoid is a special class of phytochemical which have antimicrobial characteristics to against both Gram-positive and Gram-negative bacteria. The antibacterial mechanisms of action of selected flavonoid like as quercetin has been attributed to inhibition of DNA gyrase. It has also been proposed that sophoraflavone G and (−)-epigallocatechin gallate inhibit cytoplasmic membrane function, and that licochalcones A and C inhibit energy metabolism (Cushnie and Andrew, 2005).

The research of genus Mangifera showed that the ethanolic extract of Thai mango (*Mangifera indica* L) seed kernel (MSKE) contains phenolic pentagalloylglucopyranose and methyl gallate (Nithitanakool *et al*.,2009). The alcoholic extract from MSKE and its phenolic like gallic acid, methyl gallate and pentagalloylglucopyranose actively against *S. aureus* and 19 MRSA strains. The inhibitory mechanism of this extract may be due to the damage of the bacterial membrane. This extract causes in change permeability of outer or inner membrane bacterial cell and disruption of membrane, so the small celluler molecul go out from the cell (Jiamboonsri *et al.,* 2011). The ethyl acetate and methanol fractions of palasu leaves, stems, and barks contained flavonoid, phenolic, saponins, tannins and moreover alkaloids which are known to have antimicrobial activity.

3.2. Agar Well Diffusion Assay

Based on the result, the methanolic extract was considered to be actively inhibit microorganism and it was further partitioned with normal hexane (n hexane) and ethyl acetate (EtOAc) to separate polar, semipolar and non polar compounds. The n hexane, EtOAc and MeOH fractions of leaves, stems and barks of palasu were screened for antimicrobial activities for fourteen microorganisms. The antibacterial activity of the two different concentrations (500 and 1000ug/well) of palasu plant tested are presented in Tables 2 to 4. All plant extracts showed antimicrobial activity against a number of microorganisms test. The data showed that EtOAc fraction of palasu plant got higher antimicrobial activities for all test microorganisms as compared to MeOH fraction, MeOH extract and n hexane fraction. The EtOAc of palasu leaves have the best antimicrobial activity followed by part of stems and barks, respectively. This means that the part of plant and the type of solvent used might have effect in it activity. The higher antimicrobial activities of EtOAc of palasu leaves might be due to type of chemical constituent and amount of chemical compound in EtOAc fraction, and also the different sensitivity of the test microorganism.

Table 2. Antimicrobial activity of methanolic extracts, ethyl acetate, n hexane and methanol fractions of leaves palasu (*Mangifera caesia* Jack)

	Concentration 500 μg				Concentration 1000 μg			
Microrganisms	MeOH Extract	EtOAc Fraction	Hexane Fraction	MeOH Fraction	MeOH Extract	EtOAc Fraction	Hexane Fraction	MeOH Fraction
Listeria monocytogene	-	+	-	-	-	+	-	-
Candida albicans	-	+	+	-	+	+	+	-

Vibrio cholerae	-	-	-	-	-	+	-	-
Enterobacteria	-	-	-	-	+	+	-	-
Bacillus cereus	+	+	+	+	+	+	+	+
Salmonella sp	-	+	-	-	-	+	-	-
Bacillus sp.	+	+	-	+	+	+	+	+
Staphylococcus aureus	-	-	-	-	-	+	-	-
Aeromonas hydrophila	+	+	+	+	+	+	+	+
Klebsiella pneumoniae	+	+	+	+	+	++	+	+
Bacillus substilis	+	+	+	+	+	+	+	+
Pseudomonas aeruginosa	-	+	-	-	-	+	-	-
Citrobacter freundii	-	+	-	-	-	+	-	-
Eschericia coli	-	-	-	-	-	+	+	+

Description:
(+) Actively inhibit microbial growth test
(-) Does not actively inhibit the growth of test microbes

Table 3. Antimicrobial activity of methanolic extracts, ethyl acetate, n hexane and methanol fractions of stems palasu (*Mangifera caesia* Jack)

Microrganisms	Concentration 500 µg				Concentration 1000 µg			
	MeOH Extract	EtOAc Fraction	Hexane Fraction	MeOH Fraction	MeOH Extract	EtOAc Fraction	Hexane Fraction	MeOH Fraction
Listeria monocytogene	-	-	-	-	-	-	-	-
Candida albicans	-	+	-	-	+	+	+	+
Vibrio cholerae	-	-	-	-	-	-	-	-
Enterobacteria	-	-	-	-	-	-	-	-
Bacillus cereus	+	+	-	+	+	+	-	+
Salmonella sp	-	+	-	+	-	+	-	+
Bacillus sp.	+	-	-	-	+	-	-	-
Staphylococcus aureus	-	-	-	-	-	-	-	-
Aeromonas hydrophila	+	+	+	+	+	+	+	+
Klebsiella pneumoniae	+	+	-	+	+	+	-	+
Bacillus substilis	+	+	+	+	+	+	+	+
Pseudomonas aeruginosa	-	+	-	-	-	+	-	-
Citrobacter freundii	-	-	-	-	-	-	-	-
Eschericia coli	-	+	-	-	-	+	-	+

Table 4. Antimicrobial activity of methanol extracts, ethyl acetate, n hexane and methanol fractions of barks palasu (*Mangifera caesia* Jack)

Organisms	Concentration 500 µg				Concentration 1000 µg			
	MeOH Extract	EtOAc Fraction	Hexane Fraction	MeOH Fraction	MeOH Extract	EtOAc Fraction	Hexane Fraction	MeOH Fraction
Listeria monocytogene	-	-	-	-	-	-	-	-
Candida albicans	-	+	-	-	+	+	+	+

Vibrio cholerae	-	-	-	-	-	-	-	-
Enterobacteria	-	-	-	-	-	-	-	-
Bacillus cereus	+	+	-	+	+	+	-	+
Salmonella sp	-	+	-	+	-	+	-	+
Bacillus sp.	+	-	-	-	+	-	-	-
Staphylococcus aureus	-	-	-	-	-	-	-	-
Aeromonas hydrophila	+	+	+	+	+	+	+	+
Klebsiella pneumoniae	+	+	-	+	+	+	-	+
Bacillus substilis	+	+	+	+	+	+	+	+
Pseudomonas aeruginosa	-	+	-	-	-	+	-	-
Citrobacter freundii	-	-	-	-	-	-	-	-
Eschericia coli	-	+	-	-	-	+	-	+

Antimicrobial activity of EtOAc fraction of palasu leaves were evaluated by measuring the diameters of clearing zone on microorganism test. The highest diameters of clearing zona was shown by EtOAc fraction (500ug) against at Gram-negative bacteria like as *K.pneumoniae* (12.6 mm), *A.hydrophila* (6.5 mm), *salmonella sp* (4.9 mm), *Pseudomonas aeruginosa* (3.4 mm), *Citrobacter freundii* (1.9 mm), *E.coli* (0 mm), *Enterobacteria* (0 mm), and *V. cholerae* (0 mm). The Value clearing zonas of Gram positive bacteria were *Bacillus sp* (9.8 mm), *B. cereus* (8.9 mm), *B subtilis* (6,8 mm), *Staphylococcus aureus* (4.2 mm), and *L. Monocytogene* (1.8 mm) (Table 5). These results show that not all the Gram-negative bacteria are susceptible to action of tested fractions at 500ug concentration like as *E.coli*, *Enterobacteria*, and *V. cholerae*. The differential sensitivity of Gram-positive and Gram negative bacteria to this extracts may be due to the morphological differences between these bacteria. But the EtOAc fraction from leaves at 1000ug concentration was active inhibit all the test microorganisms and n hexane fraction of barks of palasu plant was active inhibit only three microorganisms. All the test the test microorganisms were susceptible to palasu fractions though to varying degrees. In this study, increasing the concentration of plant extract resulted in higher antimicrobial activity against specific microorganism.

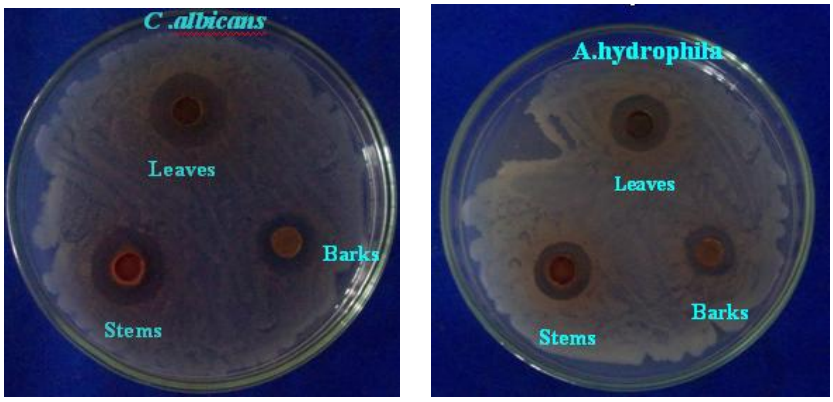

Figure 1. Zones of inhibition of bacterial growth around the well containing palasu (*Mangifera caesia* Jack) extract

The antimicrobial activity profile showed that palasu (*Mangifera caesia* Jack) fractions were active against fungi and both of Gram-positive or Gram-negative bacteria. In our present study, it is deduced that EtOAc fraction of palasu leaves showed the broadest spectrum of action. The antimicrobial activity against all the types of microorganisms test

may be due to the presence of metabolite secondary compounds from palasu plant which have antimicrobial activity like a broad spectrum antibiotic.

Gupta *et al.*, 2008 reported that the antimicrobial activity of the amchur (dried pulp of unripe *Mangifera indica* extract on the selected food borne bacteria that cause food spoilage. The antibacterial activity of amchur extract (50% ethanol) was tested against ten bacterial strains (7 Gram-positive and 3 Gram-negative) by agar well diffusion method. The crude extract showed a broad spectrum of antibacterial activity be inhibiting both the groups of bacteria. However the ethanolic extract was most effective against *Staphylococcus aureus* and was least effective against *Micrococcus luteus.*

3.3. Minimum Inhibitory Concentration (MIC)

The EtOAc fraction from palasu leaves were further tested for Minimum inhibitory concentration determination. The determination of MIC at a concentration 0.1mg/mL showed a growing of a number of bacteria and no growing all of the bacteria tested at concentrations over 0.7mg/mL. MIC values obtained by using the agar diffusion method revealed that EtOAc fraction of palasu leaves on *K.pneumoniae* as 0.10mg/mL and *Bacillus substilis* as 0.15mg/mL. However, the EtOAc fraction gave low inhibitory effect against *V. cholerae* and *Enterobacteriae* with the MIC value 700 µg/mL and the results are presented as shown in Picture 2.

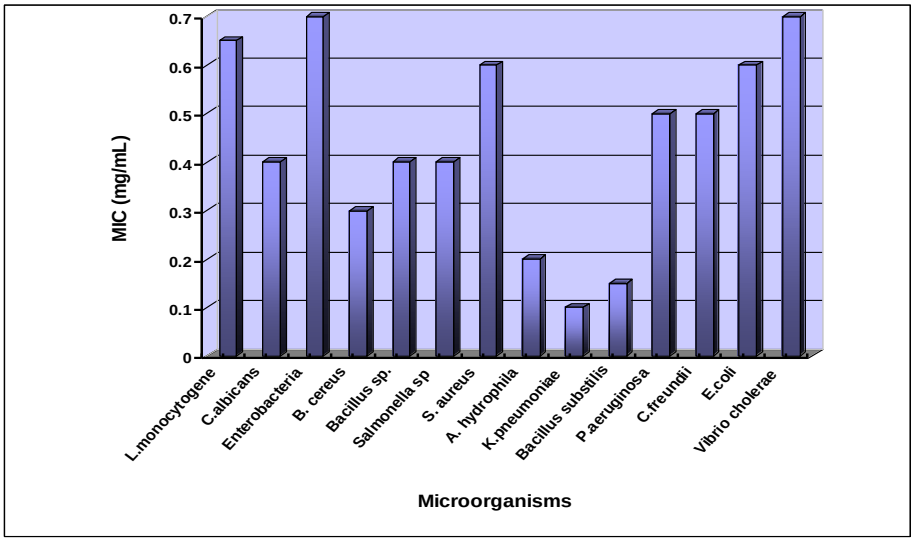

Figure 2: Minimum inhibitory concentration (MIC) of ethyl acetate leaves the plant *Mangifera cesia* Jack against some microorganisms

The EtOAc fractions of palasu leaves were able to againt *K.pneumoniae* with highest activity among the others test microorganisms. The EtOAc of fractions exhibited antimicrobial activities against the test microorganisms in the order of sensitivity as *Klebsiella pneumonia* > *Bacillus subtilis* > *Aeromonas hydrophila* > *Bacillus cereus* > *Bacillus sp.* > *Eschericia coli* > *Candida albican* > *Enterobacteria* > *pseudomonas aeuroginosa* > *Salmonella sp* > *Citrobacter freundii* > *Staphylococcus aureus* > *Listeria monocytogene* and *Vibrio cholera.*

IV. Conclusion

The results of the present study indicated that palasu plant, successfully displayed antimicrobial activities and contained alkaloid, flavonoid, saponin, phenolics, and tannin. The leaves of palasu especially EtOAc fractions have great potential as antimicrobial compound against fish spoilage causing microorganisms and or pathogen food related bacteria This

investigation provides preliminary information for future development of this plant for application as natural fish preservatives. Further investigation is needed in order to identify the chemical structure of compound presence in the active fractions.

V. Acknowledgements

The authors acknowledge our gratitude to Ministry of Research and Technology Indonesian, for fully supporting this research activity.

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ISSN: 2088-9771

Table 5.The mean inhibitory zone diameter (mm) of methanol extract, ethyl acetate, n-hexane and methanol fractions at a concentration of 500 µg against some microorganisms

Microorganism	leaves				stems				Barks			
	MeOH Extract	EtOAC Fraction	Hexane Fraction	MeOH Fraction	MeOH Extract	EtOAC Fraction	Hexane Fraction	MeOH Fraction	MeOH Extract	EtOAC Fraction	Hexane Fraction	MeOH Fraction
Listeria monocytogene	0.0 ± 0.0	1.8 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Candida albicans	3.5 ± 0.3	6.3 ± 0.2	2.3 ± 0.7	0.0 ± 0.0	0.0 ± 0.0	2.9 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3.5 ± 0.4	0.0 ± 0.0	0.0 ± 0.0
Vibrio cholerae	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Enterobacteria	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.9 ± 050	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
Bacillus cereus	4.6 ± 0.1	8.9 ± 0.1	5.6 ± 0.2	6.1 ± 0.4	4.4 ± 0.5	6.1 ± 0.0	2.9 ± 0.4	5.0 ± 0.0	4.40 ± 0.0	4.6 ± 0.0	0.0 ± 0.0	1.5 ± 0.1
Bacillus sp.	4.4 ± 0.2	9.8 ± 0.2	0.0 ± 0.0	4.0 ± 0.5	0.0 ± 0.0	5.5 ± 0.0	0.0 ± 0.0	4.0 ± 1.1	0.0 ± 0.0	4.4 ± 0.0	0.0 ± 0.0	1.5 ± 02
Salmonella sp	0.0 ± 0.0	4.9 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	7.0 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	7.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Staphylococcus aureus	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Aeromonas hydrophila	3.3 ± 0.2	6.5 ± 0.0	5.7 ± 0.2	2.2 ± 0.3	5.9 ± 0.3	2.7 ± 0.2	3.3 ± 0.2	2.3 ± 0.5	5.9 ± 0.4	3.3 ± 0.2	3.3 ± 1.0	0.0 ± 0.0
Klebsiella pneumoniae	4.3 ± 0.1	12.6 ± 0.4	5.7 ± 0.4	4.3 ± 0.1	8.9 ± 0.1	4.6 ± 1.1	0.0 ± 0.0	3.1 ± 0.2	8.9 ± 0.2	4.3± 1.0	0.0 ± 0.0	4.1 ± 0.2
Bacillus substilis	4.6 ± 0.2	6.8 ± 0.4	7.5 ± 0.1	4.0 ± 0.3	9.3 ± 0.5	6.2 ± 0.0	3.2 ± 0.4	7.0 ± 0.0	9.3 ± 0.1	4.6 ± 0.1	3.70± 0.0	1.8 ± 05
Pseudomonas aeruginosa	3.4 ± 0.2	3.4 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3.2 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3.4 ± 0.7	0.0 ± 0.0	0.0 ± 0.0
Citrobacter freundii	0.0 ± 0.0	1.9 ± 02	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Eschericia coli	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.7 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

ISSN: 2088-9771

Table 6.The mean inhibitory zone diameter (mm) of methanol extract, ethyl acetate, nhexane and Methanol fractions at a concentration of 1000 µg against some microorganisms

Microorganism	Leaves				Stems				Barks			
	MeOH Extract	EtOAc Fraction	n Hexane Fraction	MeOH Fraction	MeOH Extract	EtOAc Fraction	n Hexane Fraction	MeOH Fraction	MeOH Extract	EtOAc Fraction	Hexane Fraction	MeOH Fraction
Listeria monocytogene	0. 0± 0.0	4.0 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Candida albicans	8.7 ± 0.0	7.1 ± 0.1	3.7 ± 0.2	0.0 ± 0.0	8.1 ± 0.3	6.7 ± 0.1	2.1± 0.2	3.4± 0.3	5.0 ± 0.2	4.5 ± 0.5	1.9± 0.3	0.0 ± 0.0
Vibrio cholerae	0.0 ± 0.0	1.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Enterobacteria	4.1 ± 0.4	6.8 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.0 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.7 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Bacillus cereus	6.0 ± 0.2	8.8 ± 0.2	2.7 ± 0.4	4.7 ± 0.7	7.3 ± 0.5	7.1 ± 0.1	0.0 ± 0.0	3.5± 0.1	5.0± 0.3	4.7± 0.1	2.0± 0.2	1.7 ± 0.2
Bacillus sp.	0.0 ± 0.0	8.6 ± 0.0	2.3 ± 0.1	5.5 ± 0.4	0.0 ± 0.0	6.5 ± 0.2	0.0 ± 0.0	4.6± 0.1	0.0 ± 0.0	4.4 ± 0.0	1.6± 0.1	1.5 ± 0.1
Salmonella sp	6.5 ± 0.2	5.9 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	4.9 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3.5± 0.2	0.0 ± 0..2	0.0 ± 0.0	0.0 ± 0.0
Staphylococcus aureus	0.0 ± 0.0	4.2 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Aeromonas hydrophila	6.5 ± 0.0	9.0 ± 0.2	4.4 ± 1.0	4.4 ± 0.2	5.5± 0.1	8.0 ± 0.2	2.3± 0.1	5.4± 0.3	3.9± 0.4	6.1± 0.2	2.7 ± 0.1	2.8 ± 0.1
Klebsiella pneumoniae	7.7 ± 0.5	14.9± 0.1	5.5 ± 0.1	5.0 ± 0.2	6.9 ± 0.2	6.7 ± 0.1	0.0± 0.0	4.9± 0.2	5.6± 0.1	5.7 ±0.4	4.0 ± 1.0	0.0 ± 0.0
Bacillus substilis	6.6 ± 0.3	9.3 ± 1.5	2.1 ± 0.2	4.0 ± 0.1	6.2 ± 0.1	4.8 ± 0.0	2.0± 0.2	3.7± 0.1	4.4±0.2	4.7 ± 0.7	2.5 ± 0.2	0.0 ± 0.0
Pseudomonas aeruginosa	0.0 ± 0.0	6.7 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	4.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3.8 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
Citrobacter freundii	0.0 ± 0.0	4.5 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Eschericia coli	0.0 ± 0.0	8.1 ± 1.2	5.3 ± 0.2	5.6 ± 0.1	0.0 ± 0.0	6.8 ±0.2	0.0 ± 0.0	4.5± 0.1	0.0 ± 0.0	4.4 ±0.5	0.0 ± 0.0	0.0 ± 0.0

(Mean diameter values (mm) ± S.D of three replicates results)

Effort to Build Environmental Care Attitude with Environmental Education through Integrated Biology Learning Based on Science Process Skills to Support Character Education Development in Indonesia

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ABSTRACT

Rescue efforts and the preservation of natural resources of Indonesia are known for having very high biodiversity, especially through Environmental Education (PLH), which has long conducted since 1988. However, it seems still not effective because the results have not been thoroughly positive impact on environmental conditions. Classrooms are dirty, barren school grounds, litter, etc., indicates that the attitude of students tend to be less concerned about the environment. Not optimal due to the handling of these efforts only scratched the cognitive aspects (acquisition of knowledge about the environment) and have not touched on the affective aspects (cultivation of values) that impact on behavior change among environmentally conscious students. While, even though the behavior change requires a process, but it can be grown through training and habituation. Development of environmental care attitude is closely related with effort to character development that lately being promoted government, so it needs to support its implementation.

The nature of biology learning that refers to the process, product and scientific attitudes and characteristics of the biology materials are relating with the environment of life and the universe, it is suitable to develop a caring environment among learners. Through the integration of Environmental Education on the subjects of biology in the design of biology learning based on process skills using a variety of relevant approaches and methods such as: approach to science, technology, and society (STS), contextual teaching and learning (CTL), science, environmental, technology, and society (SETS), etc., be the effective way in the environmental care attitude development. Departing from this, how the design techniques of learning biology through integration of Environmental Education with biology subjects based on science process skills and its assessment instrument will be the focus of explanation in this paper.

Keywords: *Environmental Education (PLH), the design of integrated biology learning based on science process skills, assessment of integrated biology learning based on science process skills.*

A. INTRODUCTION

For Indonesia, the advancement of science and technology and the dynamics of life as a developing country that is actively building impact on the necessities of life are ever increasing and complex. Demands the fulfillment of basic needs, has led to an increase in human activities that tend to lead to the exploitation of natural resources excessively. Exploitation is not only against a variety of biological resources as forest products such as: wood, rattan, etc.., but also the exploitation of other natural resources forms such as: iron, asphalt, coal, petroleum, etc.. To acomodate other human needs such as housing, for the people of Indonesia that amount from time to time continue to increase has been accelerating environmental change towards environmental degradation. Infrastructure development also led to rapid environmental changes such as changes in forest ecosystems into a factory, office building, etc.. While the use of fuel oil, the impact of air pollution is one of the factors

driving global warming. The influence of global warming we can feel them is the phenomenon of environmental changes such as: increased temperatures, rising sea levels, etc.., will have an impact on the occurrence of environmental degradation. The decline in environmental quality will disrupt the ecosystem of life, which means a threat to the preservation of natural resources including biodiversity is in it.

Is the fact that the wealth of Indonesia's natural resources, especially biological resources its have very high diversity. Of all biodiversity on earth, almost 15% owned by Indonesia even its biodiversity richness was ranked fifth of the world. Indonesia also was one of eight countries to become as a center of biodiversity of the world and earned the nickname of the mega biodiversity countries. However, biodiversity will be extinct if not properly managed and conserved. Therefore needs to be done a variety of efforts to rescue and environmental conservation, because if not then the wealth of Indonesia's biological resources as the pride of the world would be destroyed and become extinct.

In Indonesia, efforts to rescue and environmental conservation have been done through a variety of sectors, including through education. Government of Indonesia through the Ministry of Environment since 1988 has launched the implementation of environmental education (PLH) through formal education in various types and levels of schooling. Through the integration of PLH in a variety of subjects, aimed at developing knowledge, skills, attitudes, motivations and concerns of students to solve and anticipate environmental problems (Rochyadi, 2009:24). But it seems the implementation of PLH in the school has not yielded significant changes in student behavior, especially in developing environmentally conscious attitude. Class condition, dirty, barren school yard, school environment are less well organized, is an indicator that the school community care for the environment is low.

The results of the analysis to the design of learning show that the low effectiveness of the implementation of PLH in school, because the integration of PLH especially in biology learning, only develop cognitive aspect while the aspect of its psychomotor and affective less developed. That's because the teachers are generally difficulties when having to integrate the PLH in the design of biology learning (Suciati, 2009). Departure from these problems, then focus on the exposure of this paper is directed at the PLH design development through the integration of biology learning based on science process skills which include: review of psychological aspects of the process of behavior change in individuals, especially concern for the environment, techniques for designing PLH through the integration of biology learning based on science process skills with using various approaches and methods relevant to the material characteristics and the nature of biology as a science learning.

B. CONTRIBUTION OF ENVIRONMENTAL EDUCATION IN EFFORTS TO RESCUE AND CONSERVATION OF THE ENVIRONMENT AND BIODIVERSITY RESOURCES IN INDONESIA

Indonesia is one country that has a rich diversity of biological resources of the world's largest wealth even forests described as the lungs of the world. Indonesia is also one of eight countries that earned one of the mega biodiversity countries in the world as the center of the world's biodiversity. The wealth of biodiversity that includes various levels of both the ecosystem level, species level, as well as genetic level. The level biodiversity ecosystem includes about 47 species, the biological richness of species that live naturally in Indonesia include various types include: approximately 28,000 species of plants, 350,000 species of animals, and 10,000 types of microbes. While the level of biological richness of genetic diversity include different types of bacteria (about 1000 genes), diversity of fungi (10,000 genes), diversity of mammals (100,000 genes), and diversity of flowering plants (400,000 genes). Rich diversity of biological resources is a natural wealth that can provide the benefits,

vital and is the basic capital for national development and is a "lung" of the world that mankind is absolutely necessary both now and in the future.

But as a developing country, Indonesia in addition to developing various sectors of life are also being confronted with increasingly complex environmental problems. Indonesia recorded in the Guinness Book of Records as the country's fastest forest destruction. That's because the increasing needs of the community encourage high activity toward the exploration of natural resources excessively. As an impact of the rate of environmental degradation are getting faster, so the safety and preservation of natural resources increasingly threatened. The data show that the quality of the environment in Indonesia, including soil, water, and air from time to time tend to decrease. Even the level of environmental degradation and extinction of biodiversity that occurs in almost all regions in Indonesia today signed in the severe category. Narrowing of mangrove land in the area of the Northeast Wildlife Langkat Ivory Coral North Sumatra. Land area of 3,000 hectares of mangroves in the region has been converted into residential areas, ponds and rice fields by the browser (Kompas, 20 April 2009). Narrowing of the primary catchment is happening in Sleman. About 40% of the area now covered by buildings which could potentially lead to decreased quality of ground water, while according to the rules only by 20% the area that may be closed.

In the 1990's Indonesia imposed a motto: "building without damaging" as as a basis for development based on environment of various sectors. But apparently it was not able to inhibit the rate of environmental degradation, environmental problems appear even more complex. The increasing number of motor vehicle and other technology products, if viewed from the economic aspect of it indicates the increasing the purchasing power of community as well as describing the economic improvement. On the other hand, these conditions will encourage the increased use of fuel, especially fuel which can not be updated (such as: gasoline, diesel fuel and the like). As a result, an increasing number of gas emissions in the atmosphere (such as CO2, SO2, NO, NO2, CH4, CFCs), to exceed the ability of plants and oceans to mengabsorbsinya. Composition imbalance can lead to an increase in temperature in the atmosphere, ocean, and land which we are familiar with the greenhouse effect. Based on data from IPCC (Intergovermental Panel on Climate Change) shows that during the last century the global average temperature at Earth's surface has risen by 0.74 which is about 0.18 degrees Celsius (in Rochyadi, 2009:36). The impact of global warming in addition to an increase in temperature also result in reduced water availability in tropical countries (a decline of about 10-30%) and the melting of icebergs (gleser) in the polar regions. According to calculations, if the polar ice caps melt, it is predicted by 2012 sea levels would rise to 7 meters. The phenomenon of global warming these days we feel them more real: the long summer, the increase in extreme weather, an increase in the height of the waves on the beach, and occurrence of floods everywhere. The above conditions indicate that there has been a decline in environmental quality and is a threat to the preservation of natural resources including biodiversity contained there in. Environmental Education (PLH) is a form of education active role in solving environmental problems in Indonesia. PLH applied in the school aims to develop knowledge, skills, attitudes, motivations and concerns of learners in solving and anticipating environmental problems. PLH concept is not given as an independent subject, but integrated in various subjects at every level of education. PLH implementation through integration in various subjects in schools aims to develop knowledge, skills, attitudes, motivations particular concern to solve and anticipate environmental problems (Rochyadi, 2009:24). Although factually contribution PLH in solving environmental problems have not shown significant results, but because it can be done in stages and power a wide range, believed to PLH may be one effective way to instill the values of a caring attitude to the environment since the early learners, that is expected to

cultivate positive character of the individual to its environment. Thus, PLH should be viewed as a long term investment that will contribute positively to the efforts to rescue and conservation of biodiversity in Indonesia today and in the future.

C. PSYCHOLOGICAL ASPECTS AND THE NATURE OF BIOLOGY LEARNING IN THE ENVIRONMENTAL CARE ATTITUDE DEVELOPMENT

Classroom conditions are filthy, barren school yard, garbage strewn everywhere, is an indicator of low awareness of the school community toward their environment. This reflects that the implementation of PLH in the school has not been effective. The results of the analysis to the design of learning PLH PLH showed that integration of PLH in the learning of biology tend to the development of cognitive aspects as a transfer of knowledge the environmental concepts. While aspects of psychomotor and affective aspects have not been developed, so the concept of PLH tend to be delivered verbally and theoretical (Suciati, 2009). As a result of learning have not been able to make an impact on the occurrence of a change in attitude toward the habits to form an environment care attitude as character behavior. Given the existence of environment and biodiversity resources are increasingly threatened due to declining environmental quality, the efforts to rescue and conservation the environment through education, especially PLH to be continuously improved its effectiveness. Therefore necessary to develop a design of biology learning which develop learner's environment care attitude effectively, so it can be an alternative for rescue efforts and conservation of wealth of environment and biodiversity resources of Indonesia.

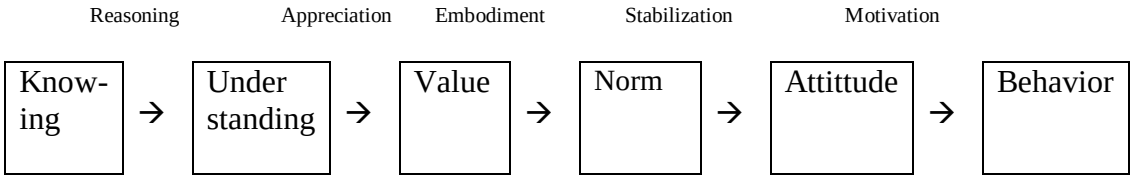
A learning plan will be effective when accordance with the principles of teaching ang learning. Therefore to achieve an effective design learning of PLH, teachers need to understand about the principles of teaching and learning especially the occurrence of the learner's behavioral changes. In the context of learning, Warsita (2008:62) argues that learning is an effort as a process of change someone's behavior as a result of interaction of learners with various learning resources in their environment. Behavioral changes including changes in cognitive, affective and psychomotor the learners themselves, indicate that learners have learned. Therefore, the positive behaviors can be generated as expected the goals of of PLH, learning activities must be designed by considering the theories and principles of learning that will give direction learning priority.

The principles of PLH related with efforts to develop of learner's attitudes from less concerned became caring for the environment as environmental care attittude. Changing individual behavior is not easy, because the attitude change can not be done instantly but necessary process. This attitude stems from feelings (like it or not like) associated with a person's predisposition to respond to things or objects. Attitudes also as an expression of values or outlook on life which is owned by someone. Attitudes can be formed, so it can happen that the desired behavior or action. Meanwhile, according to Harry (2008: 102) attitudes consists of three components including: affective, cognitive, and conative. Affective component is the feeling of belonging to individuals or assessment of an object. Cognitive component, is a trust or a person's beliefs about the object. While the conative component is the tendency to behave or act with respect to the presence of certain way related with attitude object.

In the context of PLH, attitudes related to values and norms about the environmental issues. In this case, the learners themselves need to develop appropriate attitudes towards environmental issues going on around them based on the positive values or negative attitudes in context. For example: positive attitude toward classroom and school environment cleanliness, and so on. In other contexts, the learners themselves need to be developed negative attitudes towards the habit of littering, and so on. Learners who do not have a

positive attitude towards environmental issues, it will tend to ignore things that are taught. Thus, if learners do not have a positive attitude towards environmental issues, then the recording will be less likely to care about the environment.

While in manual Book of Environmental Education (Department of Education, 1997:40) stated that in order to arrive at behavioral change regardless of the environment, required a long process. Psychologically, formal channels are gradually changing one's behavior occurs as follows:



Based on the diagram can be explained that from just knowing until learners have an understanding of necessary reasoning. Understanding will develop into the values if the students do appreciation to what would have understood. The values are developed will form the norm after the manifestation of those values. If the norm has been steadily built up, it will shape attitudes. Attitude that has formed will be the behavior if there is no intention on the individual self

The principle of PLH is to creating an effective biology learning with learning situations that learners can establish themselves positively on environmental issues. For that the teacher is required to select, define and develop learning strategies (approaches, methods) appropriate to the learning outcomes can be achieved. In the context of realizing integration of PLH in the design of effective learning biology, especially in developing environmental care attitude, the teachers are required to understand the characteristics of the material and the nature of learning. Biology its characteristics as a part of science are closely related to natural phenomena are very relevant to serve as learning resources in the PLH. In addition, the nature of biology learning as an integral part of science that refers to the process, product and scientific attitude, fully compatible with the concept of PLH. Biology is seen as a process, meaning that biology learning can be developed through a variety of science process skills both basic science process skills including skills: observing, classifying, communicating the data, interpret data, make predictions, raising questions, draw conclusions or integrated science process skills includes: set variables, define problems, create a hypothesis, design experiments, conduct experiments, draw conclusions is a strategic way to cultivate the values of environmental awareness in learners at the school from an early age. Through the integration of PLH in biology learning based on science process skills is expected to be grown to develop learner's environmental care attitudes.

In the context of learning methods, in the manual Book of Environmental Education stated that the methods suggested in the PLH include: field trips (field trips), playing the role (role playing), simulations, problem solving (problem solving), and clarification of values (values clarification). But along with the development of learning, there are a variety of innovative learning approaches that can be used in design of PLH such as environmental approach, science process skills approach, a contextual teaching and learning approach, science, technology, and society approach, science environmental technology and society approach, etc. as well as a variety of methods such as: experiment, simulation, etc.. Thereby changing the individuals behavior is not enough if only given through the lecture method but must be through the process until building the values of attitude. It is relevant to the statement of Freire (in Zaim, 2007:41) that education not only as a venue for the transfer of knowledge, but how knowledge is used as a means to educate people to be able to read social reality. According to Stapp (1974) the role of teachers in the PLH is not giving lectures about the

environment, but to create learning in which students can obtain information relevant to the concerns of students on the environment. Lecture method is less effective in helping learners gain "belief", attitudes, values and skills that are conductive in order to form and develop environmental awareness.

D. DESIGN OF INTEGRETED ENVIRONMENTAL EDUCATION WITH BIOLOGICAL MATERIAL CONCEPTS THROUGH LEARNING ACTIVITY BASED ON SCIENCE PROCESS SKILLS

Design of teaching and learning activity has understanding by teachers as the lesson plan. To be able develop a good learning design, teachers must understand the constituent components of curriculum which include: objectives, materials, strategies, media, and evaluation of learning. The steps for preparing the design of learning are as follows:

1. Establish learning goals based on: Competency Standards (Standar Kompetensi), Basic Competencies (Kompetensi standar), Indicators (Indikator), and Learning Objectives (Tujuan Pembelajaran). For the components of Competencies Standard, Basic Competencies, teachers can immediately take to the curriculum (syllabus) in accordance with student's degree and subject matter. Based on the basic competencies and then translated into indicators. Learning objectives are aligned with the indicator and the concepts of environmental education which will be integrated.
2. Describe the learning materials. Translation of materials refers to the essential concepts of biological material contained in basic competencies. In this case teachers are required to harmonize with the concept of environmental education will be integrated.
3. Choosing the appropriate learning strategies. Learning strategies include: methods, approaches, or models are selected according to the concept of material learning and is intended to provide learning experiences to learners in accordance with the demands and characteristics of the material and environmental education concepts to be integrated. Teachers can use the method suggested in PLH are like: field trips, role playing, simulations, problem solving, or values clarification. In accordance with the characteristics of the design model, the learning approach used focused on the science process skills approach contains activities such as: observing, data classification, data interpretation, communicating the data, making prediction based on data, designing of experiments, doing the experiments, formulate hypothesis, making conclusions.
4. Determining the media in accordance with the characteristics of the material and concepts that will be integrated environmental education and strategies (methods and approaches) are used.
5. Arranging learning activities including preface activity, core activity, closing activity in accordance with learning material, learning strategy and learning media.
6. Choosing tools, materials and learning resources which needed in teaching and learning process.
7. Setting up the learning evaluation instruments (learning outcomes assessment) includes assessing cognitive, affective and psychomotor.
8. All of components above organized into learning design in accordance with KTSP.

Characteristics of design integration PLH in learning biology based on science process skills is the existence of learning activities that learners enable to proceed scientifically developed range of skills that can process both basic and integrated science. Of course a variety of science process skills only can be developed if the teacher facilitates the provision of special tables to communicate the results of observational data such as: table for observation, data classification, data analysis, and the like on the design of learning.

Generally, the tables are accommodated by the teacher referred to in the form of student worksheet. Without these instruments to facilitate, it is impossible to develop science process skills that are the hallmarks of this design model. In addition, the implementation of this design model can be effective if teachers understand principles such as: characteristics of biological materials, the harmony between the concept of PLH with the concept of biological materials, the nature of biology learning, curriculum development and the principles of lesson plan. The profile of environmental education on biology learning based on science process skills as a whole can be seen in the example lesson plans, worksheets, assessment instrument (attached).

E. CONCLUSION

Based on the above description, it can put forward some conclusions:

1. Design of integrated model of environmental education in biology learning based on science process skills is intended as an alternative solution related with the low effectiveness of environmental education in schools, especially in developing student's environmental care attitudes.
2. Implementation of integrated model of environmental education in biology learning based on science process skills can be effective if teachers understand principles such as: characteristics of biological materials, the harmony between the concept of environmental education with the concept of biological materials, the nature of biology learning, curriculum development, the principles of lesson plan, psychological aspects of behavior change processes at the individual and learning strategies, media, and assessment techniques which appropriate .

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G. APPENDIX

(Example: Profile design of integreted environmental education based science process skills on lesson plan)

LESSON PLAN

Subject : Science (Biology)
Class / Semester: X / 1
Time : 3x45 seconds

Competency Standards:
1.8 Applying science and technology in environmental management

Basic Competence:
1.8.1 Planning the use of science and technology in environmental management

Indicators:
1. Observing the environmental problems that exist in the vicinity
2. Analyzing the causes of environmental problems that exist in the vicinity
3. Suggests an alternative way of handling environmental problems using technology and science

Learning Objectives:
After learning of students can:
1. Observing the environmental problems that exist in the vicinity
2. Analyzing the causes of environmental problems that exist in the vicinity
3. Suggests an alternative way of handling environmental problems using technology and science

Learning Materials:
1. Observation of the environmental problems that exist in the vicinity
2. Analysis of the environmental problems caused that exist in the vicinity
3. An alternative way of handling environmental problems using science and technology

Learning Strategies:
1. Approach: Science Process Skills
2. Method: question-answer, discussion, observation, assignments, practice

Learning Activities:

Steps	Kinds of activities	Teacher's activities	Student's activities	Sciene Process Skill
Preface activity	Motivasi Apersepsi	Providing motivation through questions • Conduct apersepsi owned by linking students with the knowledge that the material will be taught • Divide groups	• Responding to answer the question • Form a group according to the direction of teachers	

Core activity	Exploration Elaboration Confirmation	• Distribute worksheets and provide a brief explanation of procedures for group work • Being a facilitator of students in conducting field observations • Observe and evaluate students in group activities • Being facilitator on class discussion • Being a student facilitator presentation • Provide confirmation	• Listening to teachers' explanations • Conducting observations in groups about the environmental problems that exist in the vicinity of schools based on the work sheet • Collect & classify data in observation table • Analyze data through focus group discussions • put forward the idea of solving problems using science and technolo • Discussion about questions work sheet • Presenting the results of group discussions	• Observing • Classifying • Communicating data • Interpret data • Analyze data • Make predictions based on data • Solving problems • Ask questions • Interesting conclusion • Plan your experiment • Interesting conclusion • Make a hypothesis
Closing activity		• Being a facilitator of students in making conclusions • Giving students the task of planning the use of science and technology activities to address environmental problems	• Make conclusions • Record the assignment of teachers to make use of science and technology planning activities to address environmental problems	

Media/Tools/Materials:
Media : chart, video, white-board.

Learning Resources:
Handbook of Biologi for Senior High School XII grade
Handbook of Environmental Education for Senior High School XII grade

APPENDIX II (Example: Observation table on work sheet student's activities in design of integreted environmental education based science process skills)

WORK SHEET STUDENT'S ACTIVITIES

Table 1: Observation Results Regarding Environmental Problems in School Vicinity

No.	Environmental Problems	Cause	Kinds of Technology & Science Solution
1.	There were puddles of water in front of the school yard every time the rain out	Drainage is too narrow and somewhat stuffy and school position lower than the road	Creating culverts, infiltration wells, biopori.
2.	Offensive odor from the toilet smell down to the classroom	Many students are not disciplined after the urine is not watered because of limited water supply	Replacing the water reservoir
etc.	School yard looks barren and hot	There is no shade plants, because the school yard narrow	Plants with hanging pots, hydroponics.

Table 2: Types of Science and Technology Options Analysis Solutions for Environmental Problems in Schools Vicinitiy

Kinds of Technology & Science Solution	Difficulties	Utilies	Cost Categories
Creating culverts	High, because people need some expert (mason) to make it	Low, only to drain water disposal Must dig up the soil, damaging the environment	High, should make concrete and need to pay workers more
Creating absorption wells	Medium, because just one person skilled (masons) to work	Rather high, because it can accommodate and store water, enough environmental damage	Medium, masons and material needs is quite expensive
Make biopori	Low, because it can be made by students with teacher guidance.	Water can absorb quite a lot, not much damage the environment	Cheap, simple because the material

Comfort Level in the District Sleman

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ABSTRACT

The research be located in a Sleman District. The area of research is 577,56 km2. Expected results of this study can be used in urban spatial planning is more focused, as it can be determined through research that needs to be done at the preventive or mitigating the effects of physical changes in the city, especially the level of comfort in residential areas.

Activities of collecting data and processing data until getting the result of comfort level at settlement area was carried out in digital manner using satellite imagery year 2008 and Geographic Information System (GIS). Before carrying out interpretation data of satellite imagery was carried out various something that will be easier interpretation satellite imagery is mosaic satellite imagery, rectification, and enhancement using software ER Mapper. Activity of collecting data the research was interpretation of mosaic satellite imagery in visual manner by screen digitizing and collecting data of climate element in the area of the research. By interpretation satellite imagery was gotten data of density of settlement, vegetation coverage, distance to trade center, and distance to main street.

A. BACKGROUND OF PROBLEM

Development basically purpose to give prosperity for humans. However there are impacts negative from development are unavoidable, include changes in environmental quality. Changes in environmental quality is certainly have an impact on human and life. One change in environmental quality is decline in air quality.

Effects of reduced air quality also affect on environmental quality of settlements. This is because the human and natural environment can not be separated, both interact and the interplay, including also affect about neighborhood. Changes in environmental components are not aligned and balanced bring negative impacts to the environment.

Su Rito Hardoyo and Risyanto (1994), said that the neighborhood formed due to interaction between humans and ecosystems. Theoretically interrelated neighborhoods with various components. General biophysical components (biotic components and physical components of natural) consists of climate, rocks, soil, water, topography, vegetation, and animals have a reciprocal relationship with the human component. In relations between the two components lead to the formation of the target component, one of which is the human settlement, which is essentially a change in biophysical components as a result of human intervention in order to adapt to the challenges of the natural environment.

In Setijati H. Ediyono, et al (1999) argues that the greenhouse effect is the phenomenon of infrared light absorption by greenhouse gases there are particles in the lower atmosphere. With this phenomenon, the air near the earth's surface becomes warm. Greenhouse gases include carbon dioxide (CO2), NH 4, CFCs, N2O, Ozone and water vapor. Called the greenhouse effect, because it has properties such as greenhouses, which can be penetrated by infrared light so it will withstand the heat emitted by the earth's surface.

Condition of the city with various phenomena such as the greenhouse effect, heat islands and air pollution, of course, affect the comfort of residents in residential areas. One element that affects the climate of comfort of living and the change is closely related to the phenomenon mentioned above is the air temperature. Tuller (1977) in S. Astin (1995) say that rising air temperatures will reduce comfort levels of urban society in conducting all its

activities. Clearer if it is associated with one of the causes of temperature rise is that increased air pollution levels.

The study relied on the ability of satellite imagery to intercept information from land use studies. Satellite imagery is able to present a complete picture of objects and phenomena on earth. Object and symptoms consistent with the actual location on earth. While the way of recognition that is based on the elements of image interpretation in the form of color and the color, shape, size, pattern, tekstu, shadow, site and association. Elements of image interpretation that is used to identify the forms of urban land use based on actual usage by adapting the website[1].

Discomfort in residential areas that are affected by the climatic phenomenon is physiological discomfort. According to Eddy Indarto (1993), physiological discomfort shelter residents consider two Gatra. The first is to build a house, the second is the effect of heat shock on the organ. To test the above two Gatra required information relating to homes, residents and the influence of heat shock in human organs.

In this study, to determine the level of comfort in a residential area is required environmental information related to the settlement and information affecting the changes in climate elements such as air temperature, relative humidity, and wind speed. Remote sensing data used in this study is the satellite image. Satellite imagery is used to obtain data or information such as building density, vegetation coverage, distance to industrial centers, the distance to the center of trade and proximity to main roads. Tapping the data is done using digital methods and obtained Screen Digitizing. Determination of the data or information should be tailored to the factors that affect the comfort level in residential areas as well as the ability of satellite imagery.

Processing the data in this study using geographic information system (GIS). In Hartono (1997) geographic information system (GIS) is very useful for spatial data handling areas primarily for storage, editing, performance, change and modeling. The first three related to the usefulness of the data processing for presentation and presentation of data, while the utility to know the changes that are very useful for monitoring activities, especially the fast-changing variables. Modeling is very important to generate new information for planning and implementation.

Sleman regency is one of regencies in the province of Yogyakarta, which has grown rapidly from year to year. This can be seen from the development of residential areas that tend to Yogyakarta Sleman district in the north and building a more dense in Sleman regency. Sleman Regency is the city of tourism, cultural city, a university town and city industries are pushing the pace of development and urbanization with all the activities of its inhabitants. This causes a high density of buildings, less green areas, industrial and transport activities increase as well. In this condition need to know how they affect comfort in residential areas.

B. FORMULATION OF THE PROBLEM

These studies are needed to answer the following questions questions :

1. What level of accuracy of the information extracted from satellite images that the density of residential buildings, vegetation coverage, the industry is the distance to the center, commercial center and main street, with a level of comfort in a residential area based on the comfort index?
2. What level of comfort in some residential areas based on the parameters of the density of residential buildings Sleman, vegetation coverage, the distance to the center of industrial, commercial center and main street?

C. RESTRICTION PROBLEM

To clarify the objectives to be achieved in this study performed restriction problems, which this study is the Sleman district consisting of 17 subdistricts. Seventeen counties are (1) Moyudan, (2) Minggir, (3) Seyegan, (4) Godean, (5) Dalkeith, (6) Mlati, (7) Depok, (8) Berbah, (9) Prambanan (10) Kalasan, (11) Ngemplak, (12) Ngaglik, (13) Sleman, (14) Tempel, (15) Turi, (16) Pakem, and (17) Cangkringan.

D. SIGNIFICANCE OF THE PROBLEM

Expected results of this study can be used :

1. In urban spatial planning is more focused, as it can be defined directions of research to be carried out preventive measures or to counter the effects of physical changes in the city, especially the level of comfort in residential areas.

E. RESEARCH RESULTS

1. The Selection of Satellite Images

The selection of satellite images is very important because it can affect the image quality of satellite imagery, there are some things to do, namely: satellite imagery is paid or not, are usually paid a very high image resolution and excellent color clarity, while not being paid is not very clear, so for that purpose kevalitan satellite image data are required to pay. More recent satellite imagery is also more expensive, this is because new data can still be accounted for.

Figure 2. Satellite images that are not paid

Figure 3. Satellite image of the paid

2. Mosaic of satellite images

In the process of making a mosaic of satellite images will be obtained a combination of satellite imagery and continuous representation which is the area of research. Mosaic of satellite imagery will be used for mapping the variables considered in this study digital.

To simplify the process of making a mosaic of satellite images, satellite images of the black edges should be eliminated. This is done by planting cut the black edges of the satellite imagery.

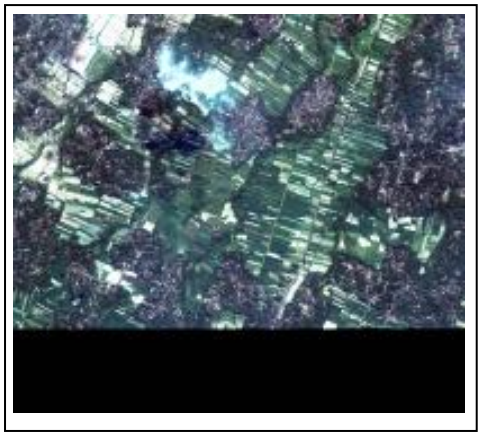

Figure 4. Satellite images before cropping

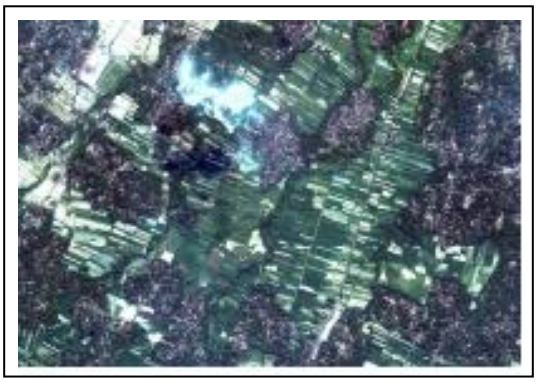

Figure 5. Satellite imagery after the cropping

Mosaic of satellite images can be viewed on the district sleman Figure 7.

3. Land Use Maps and Identification of Research Variables

Object recognition in the activities of this interpretation, holding the key to the interpretation of colors, shapes, patterns, websites and associations. Besides using the key interpretation, interpretation activities are also assisted by local knowledge about areas of research. Land use classification is applied to the interpretation of land use is determined by the purpose of research and satellite imagery capabilities. The result is a map of land use and soil physical characteristics are considered in this study. Map of residential density and land use can be seen in Figure 8 and Figure 9.

4. Map-Class Distance to The Town of Physical Variables

Because in the Sleman District No industrial centers the map class distance to the central industrial district not Sleman. At this stage, the class will be generated map to center of trade and class maps the distance to the main road. Receive such data in a manner that is the buffer in the field of physical quantities Trade Centre and the main street. Map to center of trade and the main road can be seen in Figure 10 and Figure 11.

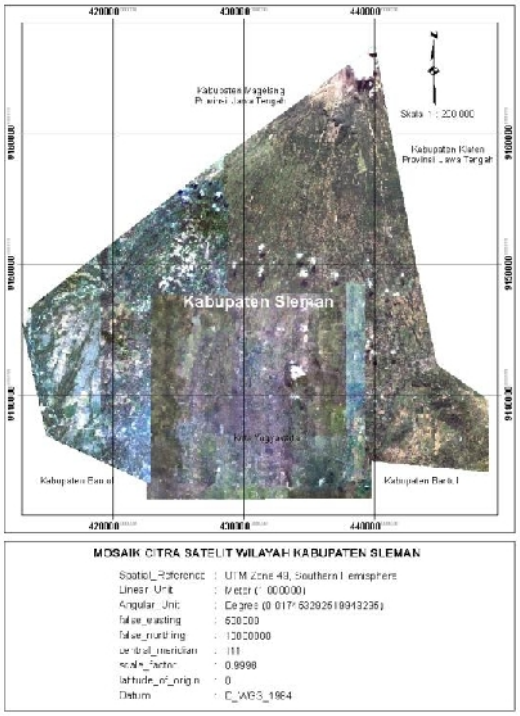

Figure 7. Satellite Image Mosaic District Sleman

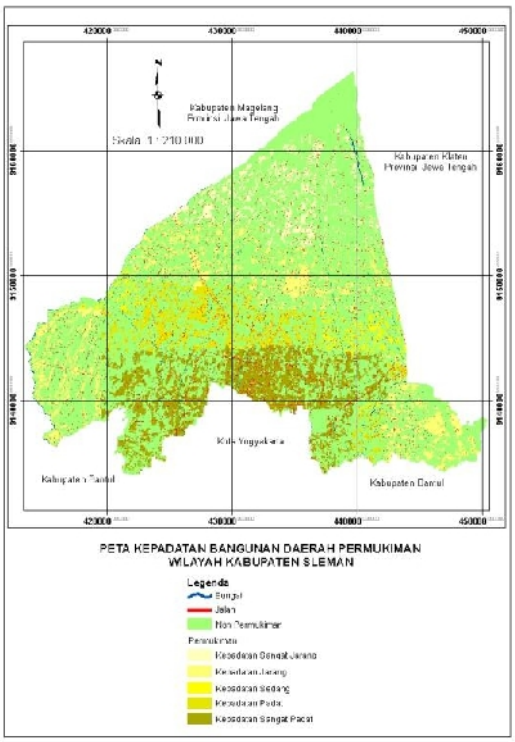

Figure 8. Map The Density of Settlement

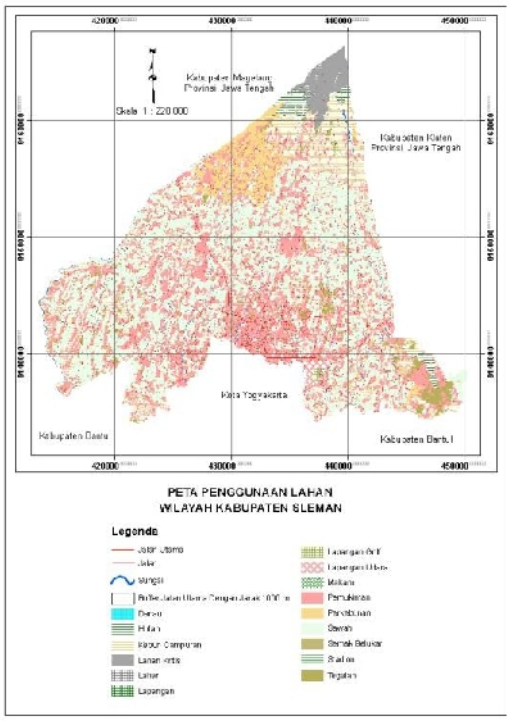

Figure 9. Land Use Maps

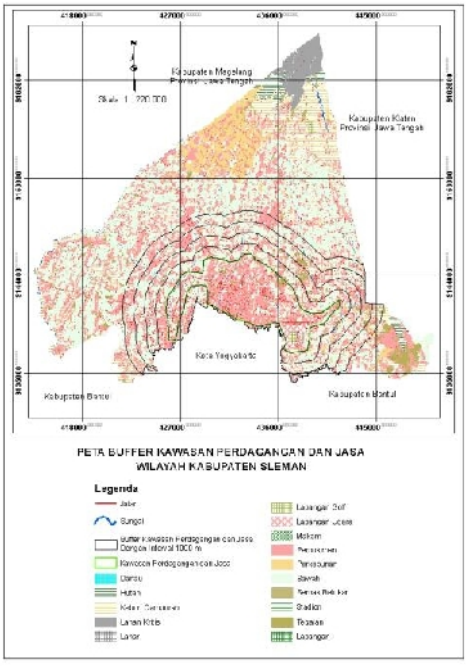

Figure 10. Map distance to the center of trade

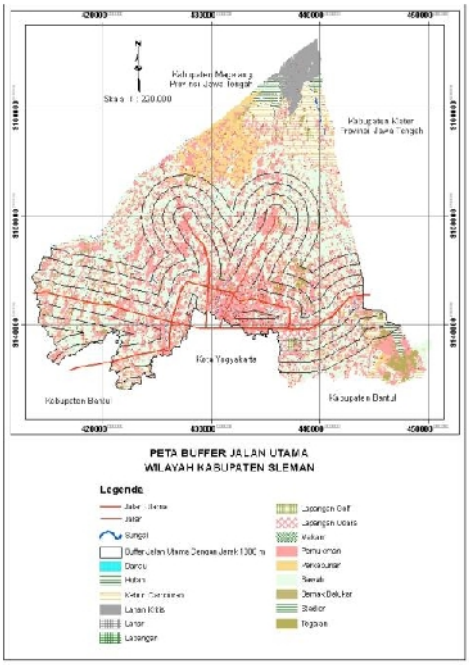

Figure 11. Map Distance to The Main Road

5. Settlement Area Map Comfort Level by Tapping the Data From Satellite Imagery

In this result is clearly seen that the pattern of residential areas are very comfortable in center city and surrounding area, while residential areas are not comfortable standing behind him. A residential area is conveniently situated in the suburbs, which generally have more vegetation coverage compared with the downtown area. This shows that the variation of the physical characteristics of the region affects the level of comfort. Map of area residential comfort level can be seen in Figure 12.

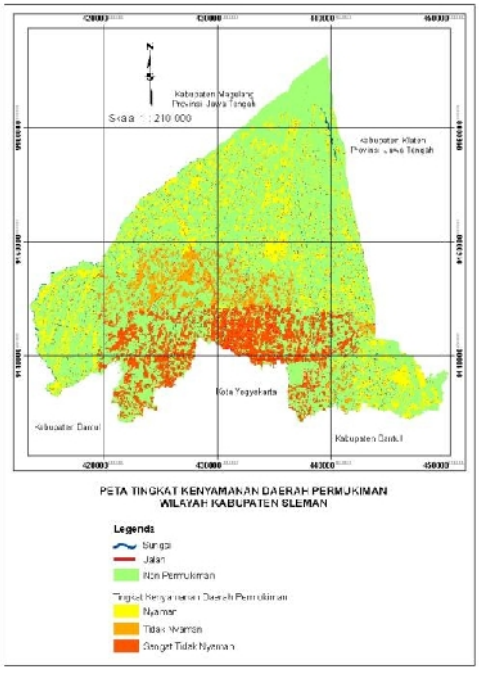

Figure 12. Comfort level map residential area

6. Distribution of Air Temperature and Relative Humidity are Spatially

Based on field measurements of air temperature for three days and then made a Similarly isotherms map is a map of the isotherms of averaging time measurement in the morning at 09.00, in the afternoon 13.00 and evening 16.00.

In this study the relative humidity necessary for both spatial information, relating to the use of relative humidity as a climatic element to be considered for the determination of the level of comfort. Isohumidity map and isotherms map can be seen in Figure 13 and Figure 14.

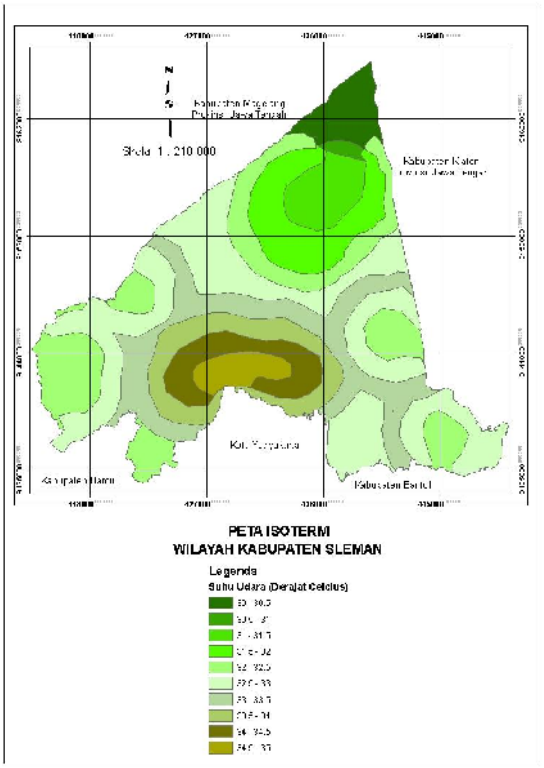

Figure 13. Isotherm Map

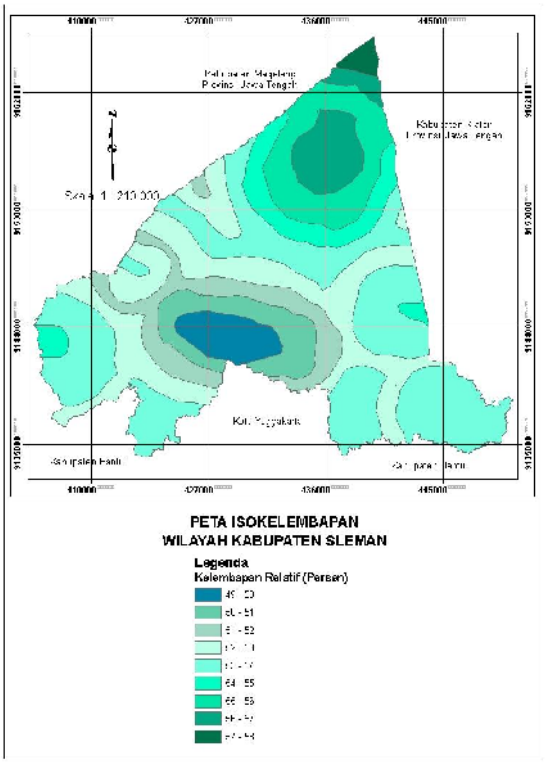

Figure 14. Isohumidity Map

7. Map of Residential Areas Based on The Comfort Level of Temperature Humidity Index (THI)

Map of residential areas based on the comfort level Temperature Humidity Index (THI) was used as a reference to the comfort level of the actual residential area. Map of residential areas based on the comfort level Temperature Humidity Index (THI) is contained in Figure 15.

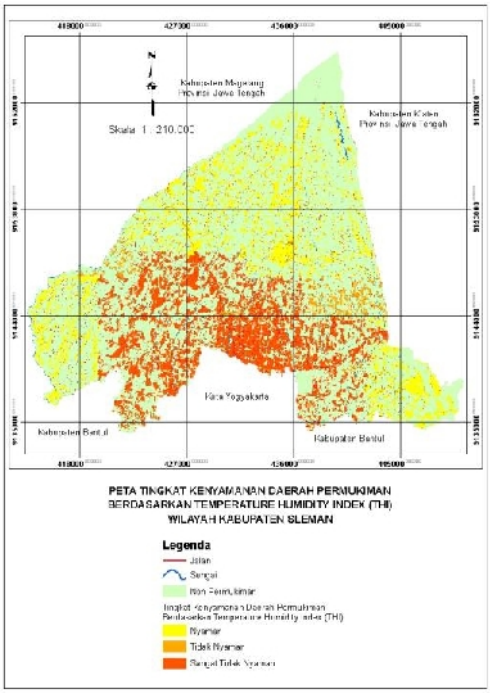

Figure 15. Map of Residential Areas Based on The Comfort Level of Temperature Humidity Index (THI)

F. CONCLUSIONS AND SUGGESTIONS

1. Conclusions

The results show that our comfort level residential area, obtained either by using satellite imagery as well as those based on the THI has the same pattern. The results show that our comfort level residential area, obtained either by using satellite imagery as well as those based on the THI has the same pattern. Even when viewed from the same category have not been based on the results of the analysis indicate that the second map showing the settlements near the city center has a tendency increasingly uncomfortable or conversely more likely to be more comfortable. This was seen when compared between the total value of the dignity of all variables with a value of THI. It can be concluded that satellite imagery and gografis information systems (GIS) can be used to determine the level of comfort in residential areas.

2. Suggestions

Although based on the final results obtained, the hypothesis can be proved, but in some parts of this research can still be developed, particularly methods of research in general. Further research is needed to get a more complete and accurate. Enhancement is the addition can be considered such factors as traffic density. Moreover, it also could be increased comfort index, not only air temperature and relative humidity are used but also other climatic elements such as wind speed, sunshine duration and others. For the accuracy of satellite image mosaic can be improved by measuring fotogrametris. And in the field of remote sensing can be developed on terapannya to measure the level of comfort in a region, using satellite imagery data in addition to satellite imagery.

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The Bacterial Growth and "Crude" β-Galactosidase Characteristics of *Klebsiella pneumonia* and *Lactobacillus bulgaricus*

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Abstract

The β-galactosidase enzyme isolated from bacteria is important for human which have "lactose intolerance" because the enzyme hydrolyzed lactose to be glucose and galactose in which these compounds can be digested by human ulcer. To know the characteristic differences between bacteria species producing β-galactosidase, the bacterial growth and "crude" β-galactosidase characteristics of *Klebsiella pneumonia* and *Lactobacillus bulgaricus* were researched. The bacteria were grown in MRS media, the growth of bacteria was based on the values of absorbances, characteristics (pH, temperature, incubation time, optimum activity) of "crude" β-galaktosidase of the two bacteria were detected and the enzyme activities were measured by modified Marteau *et al.* (1990) method.

The research results show that the highest bacterial growth of *K. pneumonia* was reach at time period for 24 hours, while that of *L. bulgaricus* was at 27 hours. The highest optimum activity of "crude" *K. pneumonia* β-galactosidase was reached at pH: 5.5, temperature 39^{o}C and incubation time for 35 minutes, while that of *L. bulgaricus* was reached at pH 6.5, temperature 45^{o}C and incubation time for 40 minutes. The activities of "crude" *K. pneumonia* β-galactosidase before optimazion was 0.340 U/mL and after optimazion was 0.425 U/mL, while that of *L. bulgaricus* before optimazion was 0.047 U/mL and after optimazion was 0.096 U/mL It can be concluded that there were the differences in bacterial growth and characteristics of "crude" β-galactosidase between the two bacteria of *K. pneumonia* and *L. bulgaricus.*

Keywords: bacterial growth, characteristics, β-galactosidase, *K. pneumonia*, *L. bulgaricus*

Introduction

The β-galactosidase enzyme is enzyme hydrolyzing lactose to be glucose and galactose (Chakraborti *et al*., 2000; Rhimi *et al.,* 2009; Chen *et al*., 2008). This enzyme was found from various types of bacteria (Li *et al*., 2009), included lactic acid bacteria (Salminen and Wright, 1998), and probiotic bacteria (Khusniati *et al*., 20011a; Hsu *et al*., 2005). Beside, this enzyme can be used in production of low/free lactose milk in which this milk products were very good for milk consumers which have "lactosa intolerance" (Haider and Husain, 2009; Rhimi *et al.*, 2010).

Various types and species of bacteria were reported to contain β-galactosidase enzyme (Khusniati *et al*., 20011a,b; Li *et al*., 2009). The difference in type and spesies of bacteria producing β-galactosidase enzyme resulted the difference in character of enzyme produced. The difference in character of β-galactosidase from the different bacteria was estimated to produce the different optimum condition of β-galactosidase (incubation time, temperature and pH) of bacteria as mentioned. It has been reported that there were the different optimum condition of β-galactosidase activites between the different type of microorganism (Gerday *et al.*, 2001; Hsu *et al*., 2005; Li *et al*., 2009;).

The main selected bacteria of *K. pneumonia* and *L.bulgaricus* were estimated to contain β-galactosidase enzyme hydrolyzing lactose to be glucose and galactose. The

characteristic difference of β-galactosidase from various types and spesies of bacteria haven't been known yet. To know the characteristic differences between bacteria species producing β-galactosidase, the bacterial growth and "crude" β-galactosidase characteristics of *Klebsiella pneumonia* and *Lactobacillus bulgaricus* were researched.

Materials and Methods

Subculture of the selected main bacteria, K. pneumonia and L. bulgaricus

The selected main bacteria, *K. pneumonia* and *L. bulgaricus* were sub-cultured by using slant agar media

The growth of K. pneumonia and L. bulgaricus

K. pneumonia and *L. bulgaricus* sub-cultured were grown in MRS media. The growth of bacteria was based on the values of absorbances,

Isolation of β-galactosidase enzyme from K. pneumonia and L. bulgaricus

Isolation β-galactosidase enzyme from *K. pneumonia* and *L. bulgaricus* were conducted by using centrifuge of 10.000 rpm for 15 minutes.

Characteristic of β-galactosidase

Characteristic (incubation time, temperature and pH) of β-galactosidase enzyme were observed its optimum condition. The activities of β-galactosidase in optimum condition were measured by using spectrophotometer

The activity of β-galactosidase enzyme

The activity of β-galaktosidase enzyme was measured by modified method of Marteau *et al*. (1990). The activity of β-galactosidase was detected by ONPG method. The amount of 1000 μl buffer phosphate 0.1 M pH 7 and 50 μl enzyme poured into reaction tube and poured solution was incubated at temperature 37^{o}C for 15 minutes. The solution incubated was then added 200 μl o-nitrophenyl-β-D-galactoside (ONPG) 4 mg/ml and the solution added was incubated at temperature 37^{o}C for 15 minutes. After incubation, the solution was added 1000 μl Na_2CO_3 1M. The solution was then analyzed by Spectrophotometer UV VIS with λ 420 nm. The activity of β-galactosidase was measured in U/mL.

Statistical analysis

The differences in β-galactosidase activities of *K. pneumonia* and *L. bulgaricus* before and after optimum condition were statistically analyzed by using CRD (Complete Randomized Design) with three replications.

Results

Research results show that the highest bacterial growth shown in absorbance of *K. pneumonia* was at times for 24 hours, while that of *L. bulgaricus* was at time for 27 hours. The bacterial growth shown in absorbances of *K. pneumonia* were different to that of *L. bulgaricus*. The bacterial growth shown in absorbances of *K.* pneumonia and *L. bulgaricus* in various hours werre shown in Table 1.

Table 1. The bacterial growth shown in absorbances of *K. pneumonia* and *L. bulgaricus*

Hours	Absorbances	
	K. pneumonia	*L. bulgaricus*
0	0.021	0.004
3	0.121	0.015
6	0.349	0.238
9	0.551	0.473
12	0.841	0.752
15	1.140	0.991
18	1.444	1.258
21	1.754	1.534
24	**2.045**	1.744
27	2.043	**1.974**
30	2.041	1.970
33	2.041	1.969
36	2.042	1.969

The optimum pH of the *K. pneumonia* was 6.5, while that of *L. bulgaricus* was 5.5. The optimum pH of *K. pneumonia* β-galactosidase was different to that of *L. bulgaricus*. The optimum pH of the *K. pneumonia* and *L. bulgaricus* β- galactosidase were shown in Figure 1.

The optimum temperature of *K. pneumonia* β-galactosidase was t 29^{o}C, while that of *L. bulgaricus* was at 45^{o}C. The optimum temperature of *K. pneumonia* β-galactosidase was different to that of *L. bulgaricus*. The optimum temperatures of the *K. pneumonia* and *L. bulgaricus* β- galactosidase were shown in Figure 2.

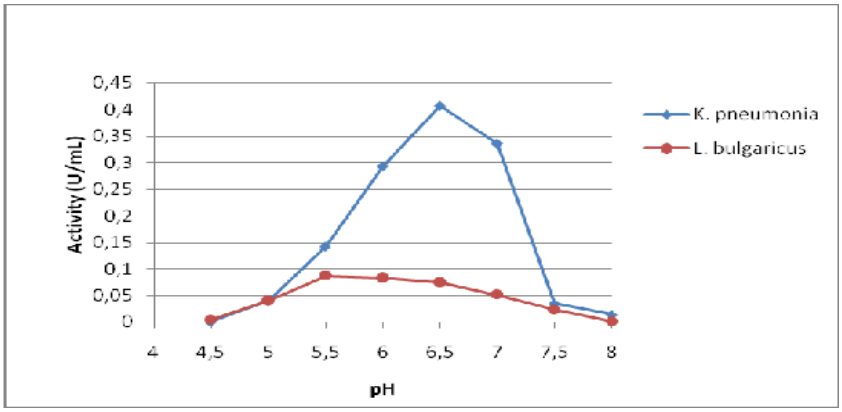

Figure 1. The optimum pH of *K. pneumonia* and *L. bulgaricus* β-galactosidase

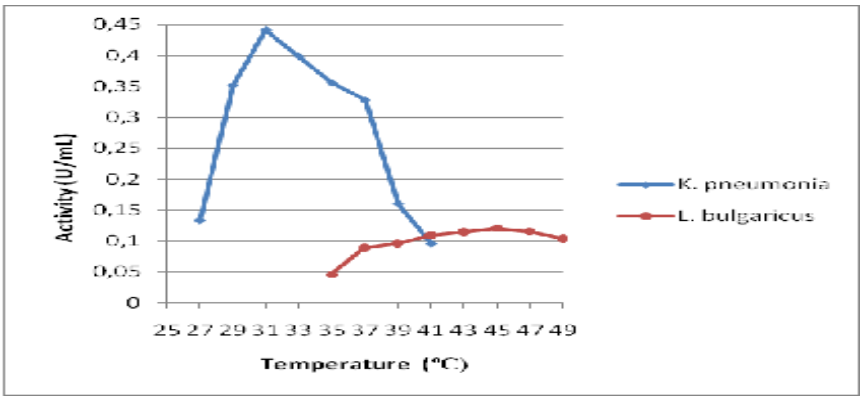

Figure 2. The optimum temperature of the *K. pneumonia* and *L. bulgaricus* β-galactosidase

The optimum incubation time of *K. pneumonia* β- galactosidase was 35 minutes, while that of *L. bulgaricus* was 40 minutes. The optimum incubation time of *K. pneumonia* β-galactosidase was different to that of *L. bulgaricus*. The optimum incubation times of the *K. pneumonia* and *L. bulgaricus* β- galactosidase were shown in Figure 3.

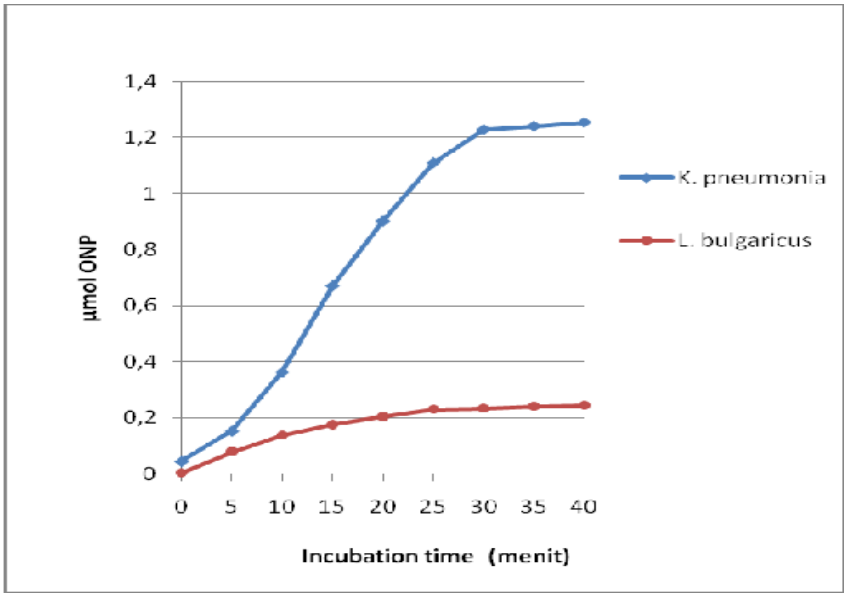

Figure 3. The optimum incubation times of the *K. pneumonia* and *L. bulgaricus* β-galactosidase

The β-galactosidase activities of *K. pneumonia* and *L. bulgaricus* after optimum condition were higher than that before optimum condition.. The β-galactosidase activities of *K. pneumonia* before and after optimum condition were 0.340 U/mL and 0.425 U/ml, respectively, while that of *L. bulgaricus* were 0.047 U/ml and 0.096 U/ml (Figure 4). These results show that the β-galactosidase activities of *K. pneumonia* were significantly higher than that of *L. bulgaricus* in both before and after optimum condition (P<0.05).

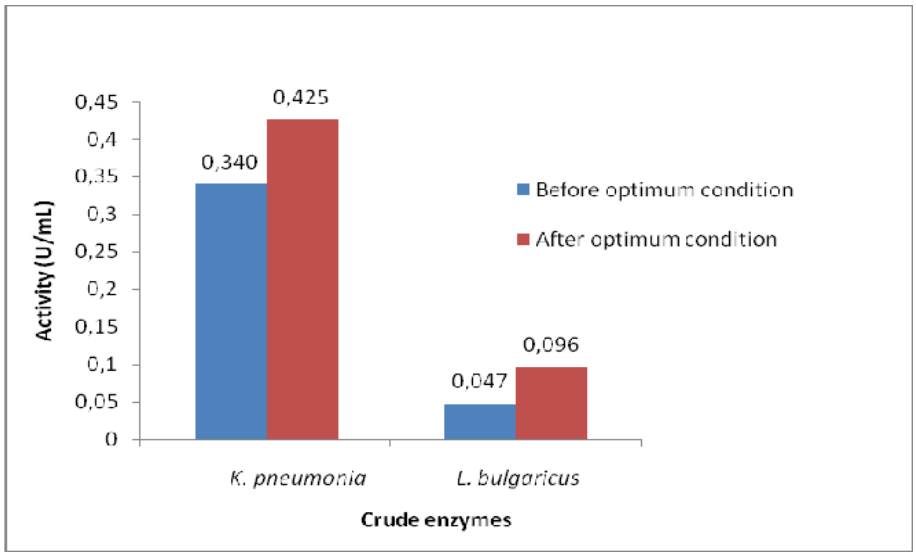

Figure 4. The β- galactosidase activities of *K. pneumonia* and *L. bulgaricus* before and after optimum condition

Discussion

The differences in bacterial growth shown in absorbances between β-galactosidase of *K. pneumonia* and *L. bulgaricus*. may be due to the differences in type and species of those bacteria. It has been reported that the differences in type and species of bacteria may have resulted in the differences in bacterial growth of those two bacteria resulted (Khusniati *et al*., 2011a,b).

The differences in optimum conditions (pH, temperature and incubation time) between β-galactosidase of *K. pneumonia* and *L. bulgaricus*. may be due to the differences in type and species of those bacteria producing β-galactosidase. It has been reported that the differences in type and species of bacteria producing β-galactosidase may have resulted in the differences in β-galactosidase optimum conditions resulted (Chakraborti *et al*., 2000; Chen *et al*., 2008; Rhimi *et al*., 2010; Li *et al*., 2009).

The higher β-galactosidase activities of *K. pneumonia* before and after optimum condition than that of *L. bulgaricus* may be due to the differences in characteristics of β-galactosidase produced between those two bacteria. It has been reported that the differences in characteristics of β-galactosidase produced between the different type and species of bacteria may have resulted in the differences in β-galactosidase activities resulted (Chen *et al*., 2009; Gerday *et al*., 2001; Hsu *et al*., 2005; Li *et al*., 2009).

Conclusion

The highest bacterial growth of *K. pneumonia* was reach at time period for 24 hours, while that of *L. bulgaricus* was at 27 hours. The highest optimum activity of "crude" *K. pneumonia* β-galactosidase was reached at pH: 5.5, temperature 39^{o}C and incubation time for 35 minutes, while that of *L. bulgaricus* was reached at pH 6.5, temperature 45^{o}C and incubation time for 40 minutes. The activities of "crude" *K. pneumonia* β-galactosidase before optimazion was 0.340 U/mL and after optimazion was 0.425 U/mL, while that of *L. bulgaricus* before optimazion was 0.047 U/mL and after optimazion was 0.096 U/mL It can be concluded that there were the differences in bacterial growth and characteristics of "crude" β-galactosidase between the two bacteria of *K. pneumonia* and *L. bulgaricus.*

Acknowledgements

The authors wish to acknowledge to Mrs. Neneng Karimaryati for her technical assistance during the research.

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In Vitro Antagonism Test between *Fusarium solani* Caused *Fusarium Wilt* on Orchid *Phalenopsis* Taida Salu by Using Endophytic Fungi

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Abstract

Endophytic fungi is the fungi that associated with plant organs. Endophytic fungi (Mycorrhizal) is very profitable because its antagonistic to pathogens in cultivation plant. One of the pathogens that against orchids is *Fusarium solani*. The utilization of endophytic fungi as a biological control of *F. solani* is an important alternative without negative effect to enviroment.

The purpose of these research was to observe the ability of endophytic fungi in suppressing the growth of *F. solani* caused *Fusarim wilt* in orchid plant. Antagonism test *in vitro* of endophytic fungi was done by using dual culture method by putting the colony of endophytic and pathogen (which had been grown previously in media) with 5 mm diameters. And then both of this fungi were placed side by side with a distance of 5 cm on 9 cm diameters petri dishes that had been filled with PDA. As a control was pathogen that were cultured on PDA. And then the colony was incubated until the pathogen fungi reached the edge of petri dishes.

Result of this research showed that *Ceratorhiza* sp. and *Epulorhiza* sp. suppressed the growth of *F. solani* caused *Fusarium wilt*. *Ceratorhiza* sp. suppressed the growth of *F. solani* up to 69,52% and *Epulorhiza* sp. suppressed the growth of *F. solani* up to 30%. The type of growth *Epulorhiza* sp. was submerged in the medium whereas the type growth of *F. solani* were above the surface of the medium.

Key words : endophyte, Fusarium, antagonism, Phalaenopsis, in vitro

INTRODUCTION

Orchids are one of favourite ornament plants, and have many kinds of varieties. Orchids are marketable in local markets or international markets. According to Abidin (1997) *cit* Dwiatmini (2002) *Phalaenopsis* hybrid became favourite cut flower in Netherlands. Beside having varieties, the flower of *Phalaenopsis* hybrid bloom in a long time, but when the high humidity and decreased light intensity in rainy season, this orchids are often infected by *Fusarium wilt* . Therefore, it is necessary to control the fungal disease which save to the enviroment such as by utilizing with endophytic fungi.

Several study showed that endophytic fungi (mycorrhizal) can be associated with the host, both in terms of nutrition supply and protected from the disease. Whereas endophytic fungi could get nutrition such as carbohidrate that is the result of photosynthesis of plant (Bonfante & Andrea, 2010)

Based on the result of these studies, it can be used as a guide to overcome the *Fusarium wilt* that caused by *Fusarium* and to investigate the identity of endophytic fungi that reduce the growth of *Fusarium* in hybrid *Phalaenopsis*. The research about *Fusarium wilt* on hybrid *Phalaenopsis* has not been done before. So it is necessary to isolate the endophytic fungi and investigate the potency of endophytic fungi in reducing Fusarium wilt caused by *Fusarium.*

The purpose of this research was to observe the ability of endophytic fungi in suppressing the growth of *F. solani* caused *Fusarim wilt* on orchid plant.

MATERIALS AND METHODS

Isolation and identification of fungal pathogens

Fusarium solani was isolated from *Fusarium wilt* lesions of *Phalaenopsis* taida salu. The diseased areas were cut into small pieces (0,5 cm) and each piece was sterilized in 1% sodium hypoclorite for 1 min and washed in 3 series of sterile water. The washed tissues were then placed separately on potato dextrose agar (PDA) plates and incubated at room temperature (25-30^{o}C). After incubation, the hyphae were transfered into fresh PDA plates. Identification of the fungal isolate was carried out under microscopic observation based on appropriate taxonomic key and description (Lee *et al*., 2002).

Isolation and identification of endophytic fungi

Endophytic fungi that used were *Ceratorhiza* sp. and *Epulorhiza* sp. were isolated from healthy roots of *Phalaenopsis amabilis*. The roots were cut into 1-2 mm and each piece was sterilized in 75% ethanol for 40 sec, 4% sodium hypoclorit for 10 min and washed in 3 series of sterile water for 1 min. The washed tissues were then placed separately on potato dextrose agar (PDA) plates and incubated at room temperature (25-30^{o}C). Chlorampenicol 0.5 g/ was added to the culture media to inhibit the growth of bacteria. Identification of the fungal isolate was carried out under microscopic observation based on appropriate taxonomic key and description (Yuan, 2008)

In vitro antagonism assay

An in vitro antagonism test has been done on 9 cm diameters petridishes filled with PDA. The antagonism test was done by placing side by side of the 5 mm colony of endophytic fungi and pathogenic fungi grown on PDA with 5 cm in space. Control treatment was done by using only pathogenic fungi. The samples were then incubated on 25-30^{o}C for 7 days. Percent of inhibitory zone was calculated by the following formulation :

$$\text{Percentage of inhibitory zone (\%)} = \frac{(A - B)}{A} \times 100$$

Keterangan : A = diameters of pathogen control

B = diameters of pathogen on dual culture

(Nuangmek *et al*., 2008).

RESULT

In vitro effect of endophytic isolates on mycelia growth of pathogens

The in vitro studies showed the endophytes produced inhibition of radial growth of the pathogens. On day 1 to 3 antagonist test, the contact between fungal hyphae endofit with *F. solani* had not occurred. The zone of inhibiton of mycelial growth of *Fusarium solani* ranged from 4.27 to 9.8 mm representing 30 to 69.52% compared to the control at 100%. *Ceratorhiza* sp. showed the high potential in the in vitro assay (16.69 to 69.52%) to in hibit mycelial growth of the pathogens, while *Epulorhiza* sp. the potential at 0.76 to 30% myceliah inhibiton, recpectively (figure. 1)

DISCUSSION

Table 1 showed that on day 1 to 3 antagonist test, the contact between fungal hyphae endofit with *F. solani* had not occurred. On day 4, the inhibitory zone of *Ceratorhiza* sp. (19.69%) was noticeably faster than on *Epulorhiza* sp. (0.76%). This occurred until day 12 of observations, where at the end of the observation showed that the percent inhibition zone of *Ceratorhiza* sp. was 69.52%, which was 43% greater to inhibit *F.solani* than *Epulorhiza* sp. which only showed 30% inhibition at day 12. Based on the results of this study indicated that endophytic fungi had the ability to inhibit *F. solani*, the causal agent of Fusarium wilt in orchids. Differences on the ability between the two endophytic fungi in inhibiting *F. solani* (Fig. 1), were due to different growth rate between *Ceratorhiza* sp. and *Epulorhiza* sp. Research conducted by Schubert *et al.*, (2008) on *Trichoderma* also showed that competitiveness against pathogenic fungi was influenced by the growth rate factors. Fungi that have rapid growth surpassed the competition of space and in turn can suppressed the growth of antagonistic fungi (Golfarb *et al.* (1989) cit Purwantisari and Hastuti (2009). In addition to space, the nutrient competition factor also can influence antagonism test results.

Table 1. Percentage of inhibitry zone to *Fusarium solani* observed for 12 days on PDA.

Days	Pathogen control diameters	*Ceratorhiza* sp.		*Epulorhiza* sp.	
		Pathogen diameters on dual culture	Inhibition (%)	Pathogen diameters on dual culture	Inhibition (%)
1	1,1	1,10	0	1,10	0
2	2,00	2,00	0	2,00	0
3	3,20	3,20	0	3,20	0
4	4,40	3,53	19,69	4,32	0,76
5	5,67	3,53	36,90	5,30	6,46
6	6,87	4,13	39,74	5,33	22,28
7	8,07	4,27	47,48	5,67	29,73
8	9,47	4,27	54,94	6,47	31,69
9	10,67	4,27	59,94	7,53	29,32
10	11,80	4,27	63,82	8,53	27,68
11	13,33	4,27	67,98	9,80	26,49
12	14,00	4,27	69,52	9,80	30,00

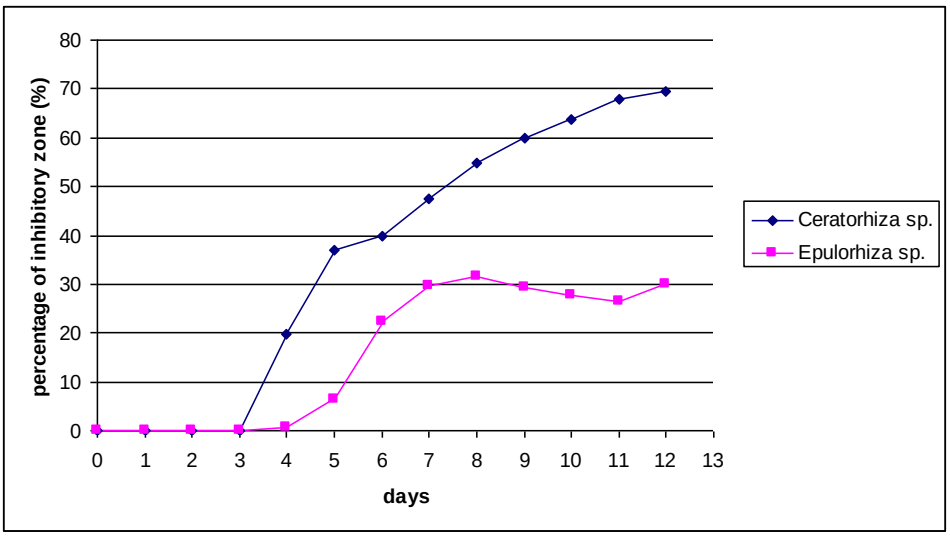

Fig. 1.Percentage of inhibitory zone of *Ceratorhiza* sp. and *Epulorhiza* sp. to *Fusarium solani*

Figure 2 indicated that based on the results of in vitro assays at 7 days after inoculation, there was pigment observed at the edges of the culture of *F.solani* seen through the bottom of petridish. Pigment was produced by *F. solani* associated with reduced nutrients in the area. Culture conditions and reduced nutrients in the media would affect the pH of the culture so it might triggers the excretion of pigment by the pathogen (Leslie and Breet, 2006). The pigment color was more brown produced by *F. solani* in dual culture with *Ceratorhiza* sp., compared with the light brown color on the dual culture of *F. solani* with *Epulorhiza* sp. suggested that nutrient competition in dual cultures between *Ceratorhiza* sp. and *F. solani* was greater than in dual cultures between *Epulorhiza* sp and *F. solani*.

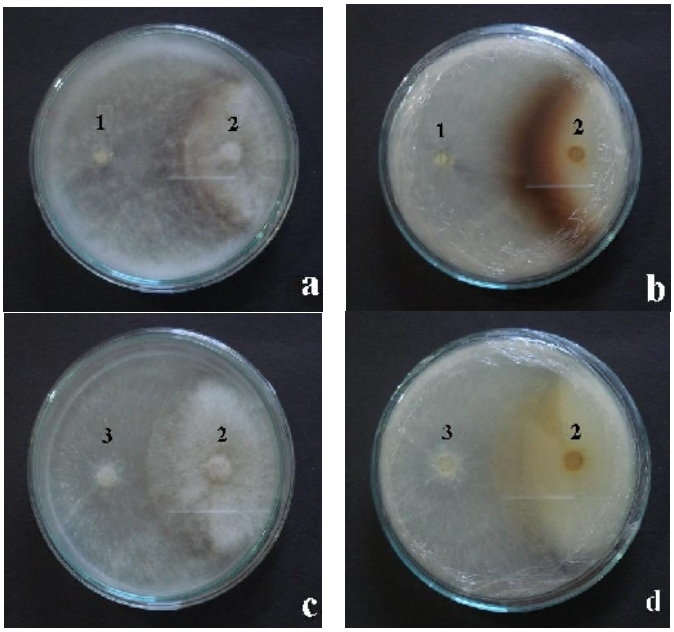

Fig 2. In vitro antagonism test on PDA at day 7 (a) dual culture of *Ceratorhiza* sp. and *F. solani* - surface view (b) dual culture of *Ceratorhiza* sp. and *F. solani* - below view (c) dual culture between *Epulorhiza* sp. and *F. solani* -surface view (d) dual culture between *Epulorhiza* sp and *F. solani* - below view (1) *Ceratorhiza* sp. (2) *F. solani* (3) *Epulorhiza* sp.

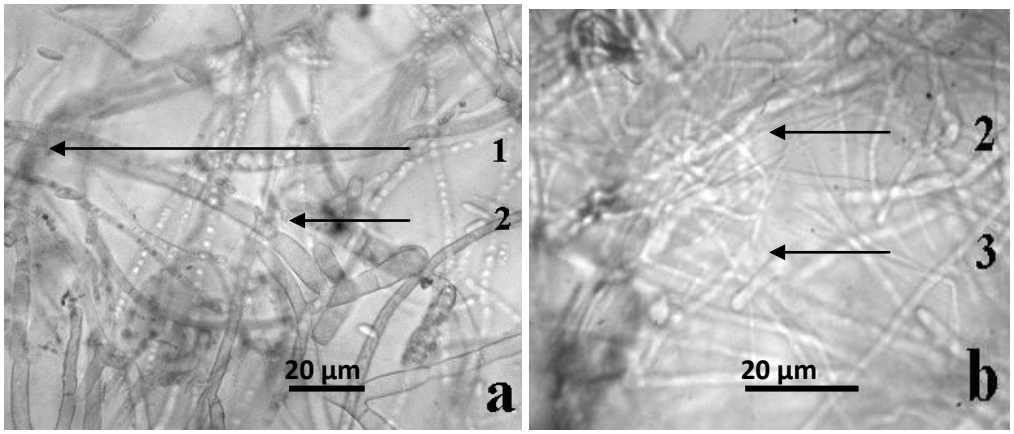

Fig 3. Microscopic observation on an in vitro antagonism test between *Ceratorhiza* sp and *F. solani* (a), and *Epulorhiza* sp. and *F. solani* (b). , 400 X (1) *Ceratorhiza* sp. hyphae (2) *F. solani* hyphae (3) *Epulorhiza* sp. hyphae

Microscopic observation at a contact of the two hyphae of fungi in dual culture between endophytic fung and *F. solani* as presented in figure 3, showed that there was no interaction

occurs in the form of coiling hyphae or the death of hyphae. The hyphae of endophytic fungi appeared to have a larger diameter than the hyphae of *F. solani*. The results showed that the test of antagonism was more influenced by differences in growth rate and growth patterns which in turn affected the competition of nutrients and space. *Epulorhiza* sp. was known to grow submerged in the media, causing contact that occurs with hyphae *F. solani* was fewer than *Ceratorhiza* sp. which had the same growth pattern of hyphae as *F. solani*. Nutrients, types of media and different test methods greatly affected the antifungal activity in dual culture (Prince *et al.*, (2011).

ACKNOWLEDGEMENT

We are thanked to Mekar Lestari nursery for providing the Orchid and taxonomy plant laboratory of Biology department for materials.

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ISSN: 2088-9771

Improving the Environmental Awareness and Creative Thinking Students Through the Application Problem Based Learning Model

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Indonesia is an archipelago country that has the potential of natural resources and abundant biodiversity. The potential of natural resources contributes to economic value and welfare of the community.Current environment and natural resources in Indonesia suffered an alarming crisis. Many of the environmental damage due to excessive exploitation of natural resources and without regard to environmental sustainability.Therefore necessary that proper educational environment so that the potential of existing natural resources maintained the quality and quantity.Education has a very strategic role in shaping the generation of caring and responsibility towards nation building and to overcome various environmental problems. Therefore necessary that proper environmental learning model in order to form the character of self-care for their students and also hone students' creative thinking on a variety of environmental problems that exist around the students. Internalization of character education in the learning environment can be done with problem-based learning model.

Education has a very strategic role in shaping the generation of caring and responsibility towards nation building and to overcome various environmental problems. Therefore necessary that proper environmental learning model in order to form the character of self-care for their students and also hone students' creative thinking on a variety of environmental problems that exist around the students. Internalization of character education in the learning environment can be done with problem-based learning model.

Problem-based learning is one model of learning which is oriented on problem solving. Problem based learning is designed to help teachers convey as much information to students. Problem based learning is designed primarily to assist students in developing thinking skills, problem solving and intellectual skills, learn about the different roles of adults through the involvement of students in real or simulated experiences, and to make autonomous and independent learners.

Key words :environmental awareness, creative thinking, problem-based learning

International Conference on Bioscience and Biotechnology (ICBB) 2011

October 11th-12th, 2011, Yogyakarta, Indonesia

Poster Paper

The Effect of Repeated Exposure of Formalin-Containing Fish Against Liver Cell of Mice Based on the Ratio of SGOT/SGPT

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Abstract

Formalin is often found in foodstuffs, although this chemical is prohibited for use as a food additive. Repeated exposure of formalin-containing foodstuffs suspected to lead the increase of structural damage and functional liver disorders. Therefore, the purpose of this study was to determine the effect of repeated exposure of formalin-containing fish against the ratio of SGOT (serum glutamic oxaloacetic transaminase) / SGPT (serum glutamic pyruvic transaminase) of mice (*Mus musculus*). The levels of SGOT and SGPT in mice serum was determined by *Cobas Mira*® Automatic Analyzer. The data of the ratio of SGOT/SGPT were analyzed using two-way ANOVA with treatment (four categories: negative control, positive control of fish, positive control of formalin, and treatment of formalin-contained fish) and time factor (four categories: 0, 2, 14, and 62-days of repeated exposure) as independent variables. The results of data analysis showed that the formalin treatment either in the form of a single compound (positive control of formalin) or in the mixed form with fish meat (fish treatment formalin) in mice causes an increase in the ratio of SGOT / SGPT. Based on the time factor, the ratio of SGOT / SGPT was increased on 14th day, and relatively stable up to 62nd day of repeated exposure.

Keywords: formalin-containing fish, repeated exposure, the ratio of SGOT/SGPT.

Introduction

Formalin is often found in foodstuffs, although this chemical is prohibited for use as a food additive (Cahyadi, 2006). There have been many reports about the negative effects of formalin in experimental animals. This effect occurs first from the molecular, cellular, tissue, organ, and organism level. This influence will increased if the consumption of foodstuff containing formalin be done repeatedly (Kartikaningsih, 2008). We have previously reported that exposure of formalin can increase the value of SGOT and SGPT of mice (Maramis et al., 2010). The ratio of these two enzymes is also often used as a marker of liver functional disorder (Cohen & Kaplan, 1979). Therefore, the purpose of this study was to determine the effect of repeated exposure of formalin-containing fish against the ratio of SGOT/ SGPT.

Materials and Methods

Design of The Research

This research uses randomized block design, 4 (treatment) x 4 (time) factorial. Four categories of treatment factor consists of: negative control; fish containing formalin; fish containing formalin plus chlorophyllin; and chlorophyllin treatments. While four categories of time factor consists of: repeated exposure over 0; 2; 14; 62 days.

Experimental Animals

Study were carried out using male BALB/c mice (15-25 g), 2,5 months old. The animal were grouped and housed in polyacrylic cages and maintained under standard laboratory conditions (25 ± 2 ^{0}C, 12/12 h photoperiod, and relative humidity of 50 – 60 %). The fish used as foodstuff models was *Oreochromis niloticus*.

Formalin Treatment and Chlorophyllin Supplementation to Mice

The concentration of formalin in fish flesh was 100 ppm (mg/kg) and chlorophyllin was 6.000 ppm. The concentrations of each substances that induces to each mice (using gavage tube) were adjusted to the weight of the animals. The mice were euthanized by cervical dislocation and then dissected to taking the blood samples straight from the heart.

Determining The Levels of SGOT and SGPT

Activities of SGOT and SGPT were determined using *Cobas Mira*® Automatic Analyzer. The ratio was calculated from the value of SGOT and SGPT.

Statistical Analysis

The ratio of SGOT/SGPT were statistically analyzed using two-way analysis of variance, and adjusted with Duncan Multiple Range Test (DMRT), by using SPSS 15.0 software. P value of < 0.05 was considered as statistically significant.

Results and Discussion

The Ratio of SGOT/SGPT Based on Treatment Factor

The mean ratio of SGOT/SGPT in the serum of mice among the four categories of the treatment factor is presented in Table 1 and Figure 1. There were significant differences between the four categories of treatment factor for the ratio of SGOT/SGPT. Formalin, both in the form of a single compound or a mixture with foodstuff (fish meat), causing a significant increase of the ratio of SGOT/SGPT. Increased markers of liver damage by formalin occurs through two mechanisms that formalin acts as a mutagen (DNA-protein crosslink formation) and as free radicals/reactive oxygen species (Wu & Cederbaum, 2003; Barker et al., 2004). Both of these mechanisms ended in structural damage of liver cells (loss of cell membrane integrity) so that the enzymes in cells scattered out and get into the bloodstream.

Table 1. The result of DMRT test of the ratio of SGOT/SGPT based on treatment factor.

Categories of the Treatment Factor	Mean of the Ratio of SGOT/SGPT
Negative control	2.9168^{ab}
Positive control of fish	2.7380^{a}
Positive control of formalin	3.1916^{bc}
Formalin-containing fish	3.3139^{c}

Note: The numbers that are followed by the same letters indicate no significant differences, whereas the numbers that are followed by different letters indicate significant differences between groups of treatment factor. The data of the ratio of SGOT/SGPT are the value of serum glutamic oxaloacetic transaminase (IU/L) per the value of serum glutamic pyruvic transaminase (IU/L) of mice. This note also applies to Table 2.

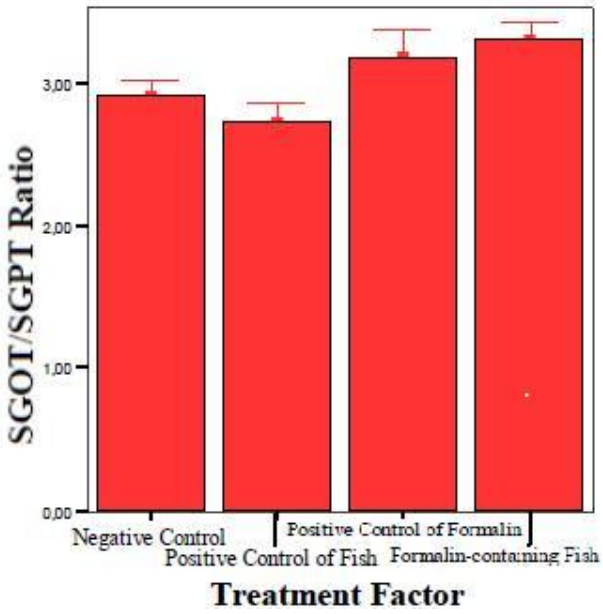

Fig. 1. Bar graph of the mean of the ratio of SGOT/SGPT based on treatment factor.

The ratio of SGOT/SGPT Based on Time Factor

The mean ratio of SGOT/SGPT in the serum of mice among the four categories of the time factor is presented in Table 2 and Figure 2. There were significant difference between the four categories of time factor for the ratio of SGOT/SGPT. Repeated exposure for 14 and 62 days led to an increase the ratio of SGOT/SGPT. Repeated exposure for 14 and 62 days led to an increase in the ratio of SGOT/SGPT. This fact implies that continuously consumption of foodstuffs contaminated with formalin can cause structural damage as well as functional disorders on liver.

Categories of the Time Factor	Mean of the Ratio of SGOT/SGPT
0 day	2.8252[a]
2 days	2.8516[a]
14 days	3.1921[ab]
62 days	3.2892[b]

Table 2. The result of DMRT test of the ratio of SGOT/SGPT based on time factor.

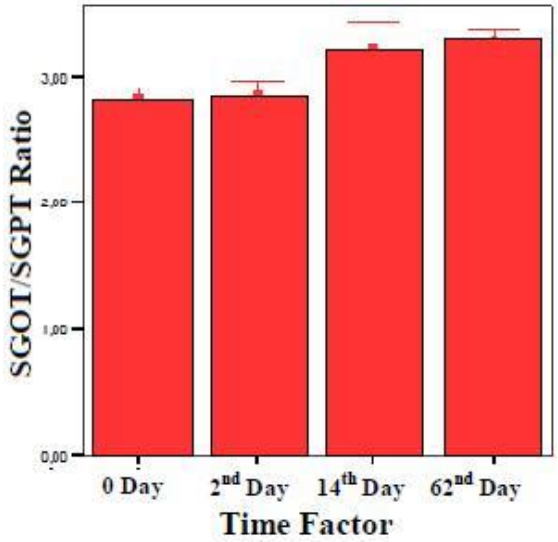

Fig. 2. Bar graph of the mean of the ratio of SGOT/SGPT based on time factor.

Conclusion

Repeated exposure to formalin-containing food (fish meat) causes an increase in the ratio of SGOT/SGPT which is a marker of structural damage and functional disorders of liver tissue.

Acknowledgement

A. A. Maramis would like to thanks to Directorate General of Higher Education, Ministry of National Education, Republic of Indonesia, and Rector of State University of Manado for partially financial support through Dissertation Research Grant and Creativity Research Grant, respectively.

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Determination of Rhodamine B in Cosmetics and Food by Using Spectrophotometry UV Visible and TLC

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Abstract

Rhodamine B is a colouring agent for paint industry, textile and paper. This coloring agent is causing irritation at respiration systems and has a carcinogenic effect. At high concentration rhodamine B causing liver damages. Rhodamine B has been reported being added into cosmetics and food product which are lipstick, eye shadow, blush on, food paste and kerupuk. This research is conducted to determine rhodamine B content in that product by using TLC and spectrophotometry UV Vis. This research was done through sample collection, sample preparation, validation of analytical method, analysis of rhodamine B content by using TLC and spectrophotometry UV Vis. Validation of spectrophotometry UV Vis method showed linierity 0,999, precision test with CV 0,2- 0,46%, accuration test with recovery percentage 99,64- 100,5% and LOD value 0,268 ppm. The research showed four sample of crackers and four sample of food shrimp paste contain rhodamine B by using TLC and spectrophotometry UV Vis but rhodamine B didn't found in cosmetics product.

Key words : Rhodamine B, TLC, Spectrophotometry UV Vis

INTRODUCTION

The using of additive compound in food are increasing lately. This are happen because of discoveries in synthesizing new chemical compound that are more practical, cheaper and easy to have. Additive compound are use in food to increase the quality of the product so can compete with other product in the market.

In Indonesia, additive exploiting in food was happened a lot. For example, textile colouring agent that has been used in terasi and sirup (Sardjimah, 1996). This case can be happen because of the lack of the community knowledge about colouring agent for food and also because textile colouring agent are cheaper than food (Hidayati, 2006). The government through Peraturan Menteri Kesehatan (Permenkes) No.239/Menkes/Per/V/85 has declare 30 dangerous colouring agent. Rhodamin B belong to one of coloring dyes described as dangerous and banned substances used in food products(Syah et al. 2005; Permenkes, 1992).

However, abuse of rhodamine B as the food coloring is still common in the field and is reported in some media. Saleh research in 2003 indicate that from 25 samples of hawker food and drink circulating in the area of Bandung, there are five positive samples contain prohibited colors rhodamine B the product are syrup products snacks, cereals and red shrimp (Soleh, 2003 in Cahyadi, 2005).

The use of this coloring are prohibited in Europe from 1984 due to the strong carcinogenic effect. Other negative effects are causing interference or even liver cancer (Syah et al. 2005). Rhodamin B is known causes liver cell changes from normal to the surrounding tissue, causes necrosis. Damage of the liver tissue is marked with hyperchromatic from the nucleus, fatty degeneration of the cytoplasm and cytolisis (Siswati, 2009).

In order to make sure that the community get health security in the use of cosmetics and food from this colouring agent, this research need to be done. This research was

conducted through materials collection, spesific chemical reagent for rhodamine B, rhodamine identification by using TLC and UV Visible.

RESEARCH METHODS

Materials

Materials used in this research is the Rhodamine B, sample food and cosmetics, ammonium monovanadat (Merck), aquades, concentrated nitric acid (HNO3) (Merck), concentrated sulfuric acid (H2SO4) (Merck), ethanol pa (Merck) , formaldehyde (Brataco), potassium sodium tartrat (Merck), sodium hydroxide (Brataco), copper (II) sulfate (Merck).

Instrument

The instrument used in this research are the glass tools commonly used in Chemical Analysis Laboratory, 90mm diameter No.4 Whatman paper, plate drops, UV spectrophotometer (200-222U170 SEPCORD), analytical balance (Dragon 204), TLC plate.

RESEARCH METHODS

Research methods was done through the collection of materials, searching for chemical specific reagent, identification of Rhodamin B in cosmetics and foods with UV Vis Spectrophotometer, identification of Rhodamin B in cosmetics and foods with Thin Layer Chromatography.

1. Raw materials and sample collection

Rhodamin B standard solution created in several concentrations. Samples of food and cosmetic that are suspected of containing Rhodamin B purchased from traditional markets in Bandung.

2. Searching for specific chemical reagent for Rhodamin B

Rhodamin B standard solution with a concentration 1000 ppm, 100 ppm, 10 ppm and 1 ppm was reacted respectively with chemical specific reagent as follows :

a. Biuret test to find out the group-CO and-NH :

Dissolve 150 mg of copper (II) sulfate (CuSO4. 5H2O) and sodium potassium tartrat (KNaC4H4O6. 4H2O) in 50 ml aquadest in 100 ml flask. Then add 30 ml 10% sodium hydroxide while shaken, beaten, add aquades further up the line markings.

b. Testing with Marquis and mandelin reagent to know a group of Xanten.

Mandelin reagent made from 1.0 grams of ammonium vanadat in 100 mL concentrated sulfuric acid to produce the color orange, Marquis reagent made from 1 part formaldehyde and 9 parts concentrated sulfuric acid. Xanten cluster reactions with the color orange (Nihilanth, 2011).

c. Xantoproteat Test to find out the clusters of benzene (aromatic ring)

sample was reacted with concentrated HNO3 to give a yellow color (Harper, et.al., 1980).

d. Testing to determine the cluster functions amines:

Diazotation reaction (for primary amine): Substance + 2 drops of 2 N HCl and NaOH, and drops of water + 0.1 g solution of beta-naftol in 2 ml of NaOH to produce sediment

orange and blood red. If the use of alpha naftol produces red purple. Safety match reaction: Substance + aqueous HCl and dipped into it matches, orange yellow colour will be produced (Praeparandi, 1979).

3. Identification Rhodamin B with UV-Vis spectrophotometer

a. **The determination of the maximum wavelength Rhodamin B**
Determination of Rhodamin B wavelength was performed by measuring the standard solution of Rhodamin B in the wavelength range 400-800 nm.

b. Method Validation of UV-Vis spectrophotometry

In this research, experimental validation is also performed to see the truth test methods used include liniearity with correlation coefficient values as parameters, test the accuracy with recovery percentage as parameters, precision test with the parameter value of coefficient of variation (CV), the determination of the detection limit (LOD) and limit of quantification (LOQ).

RESULTS

1. Raw materials and sample collection

Rhodamin B standard solution prepared in various concentrations, ie 1000 ppm, 100 ppm, 10 ppm, and 1 ppm. Samples of food and cosmetic samples examined is 7 crackers, 7 samples of shrimp paste, 7 samples of lipstick, and 9 samples of eyeshadow and rouge.

2. Rhodamine chemical specific reagent

The chemical reagent that can be used to detect rhodamine B are reagent Mandelin, Marquis, biuret, xantoproteat, and diazotation. The results of the color reaction can be seen in table 4.1.

Tabel 4.1 reaction colour of specific chemical reagent with Rhodamin B 2%

NO	Specific reagent	Reaction colour	comment
1	Mandelin	Orange	+
2	Marquis	Orange	+
3	Biuret	purple	+
4	Diazotation	Red purple	+
5	Xantoproteat	yellow	+

3. Identification Rhodamin B by UV-Vis spectrophotometer

a. Determination of the Maximum Wavelength

Rhodamin measurement standard solution with a concentration of 8 ppm B performed in the wavelength range 400-800 for the standard solution.

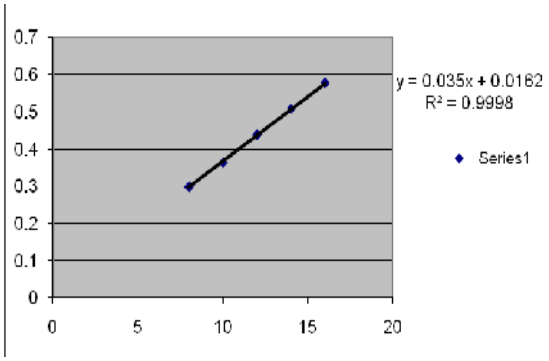
The measurement results for Rhodamin B standard solution with a concentration 8 ppm gave maximum absorption at a wavelength of 544 nm. The maximum wavelength of absorption is different from the literature obtained 543.355 nm (Aldrich, 1992). The difference of 1 nm is within the permitted tolerance in Indonesian Pharmacopoeia Fourth Edition 1995, ie less than 3 nm. This means that the wavelength of 544 nm can still be used for the analysis Rhodamin B on the sample.

b. Validation Methods

In this study also conducted validation tests to see the truth of the methods used include test linearity with correlation coefficient values as parameters, accuracy test with recovery percentage as parameter value, precision test with a parameter value of coefficient of variation (CV), the determination limit of detection (LOD) and limit of quantification (LOQ).

i.Linearitytest

Standard curve created with concentration 8 ppm, 10 ppm, 12 ppm, 14 ppm and 16 ppm. The concentration of standard solution was made in order to make Rhodamin absorption ranged from 0.2 to 0.8 because the linearity between absorbance and concentration can be achieved according to Lambert-Beer law.Liniearity test result can be seen at the Picture 4.1



Picture 1. Standard Curve of Rhodamine B

Standard curve equation obtained was Y = 0.035x + 0.0162 withcorrelation coefficient value of 0.9998 which states that there is a correlation between concentration and absorbance. The acceptance criteria are the correlation coefficient R ≥ 0, 995 (Shargel, 1985).

ii. Accuracy test

Determination of accuracy is done by making three variations ofconcentration and each concentration was made three times replication. Accucary test was done to measure the closeness of an analytical results obtained using these methods with the actual price. Accuracy of analytical methods are usually expressed as percent recovery (% recovery) of the samples whose levels are already known for sure (Susidarti etal., 2008).

Table 4.2 Accuracy Test Results

Real Conc. (ppm)	Calculated conc. (ppm)	% *recovery*	Mean
8	8.04	100,5	100,15%
	7.97	99,64	
	8,026	100,32	
12	12,023	100,19	100,097%
	12,037	100,31	
	11,97	99,79	
16	16,014	100,089	99.89%
	15,977	99,857	
	15,95	99,714	

Table 4.2 shows that the average percent recovery was 100.15%, 100.097% and 99.89%. These results indicate that the percent recovery obtained meet the test criteria for accuracy range between 98-102% (Harmita, 2004), so it can be said that the ultraviolet-visible spectrophotometric method used in this study has good accuracy to determine levels of Rhodamin B.

iii. Precision determination

Precision is a measure of the proximity of data values to one another in an analysis of measurements on the same conditions. Precision shows the distribution of each of the test results around the average value. Precision is often expressed as a percent Relative Standard Deviation (RSD) or Coefficient of Variation (CV) (Susidarti et al., 2008).

Tabel 4.3 Precision Test Results

Real Conc. (ppm)	Calculated Conc. (ppm)	Standard Deviation	Coefficient of Variation (%)
8	8.04 7.97	0.037	0.46
12	8.026 12.023 12.037	0.035	0.29
16	11.97 16.014 15.977 15.95	0.032	0.2

The result gives the value of standard deviation for 0037, 0035, 0032 and an average coefficient of variation of 0.46%, 0.29% and 0.2%. These results indicate that the coefficient of variation obtained meets the criteria of <2% (Harmita, 2004). Thus the ultraviolet visible spectrophotometric method used in research has good precision for determination of rhodamine B.

iv. Determination of detection Limit and Quantification Limit

Limits of detection (LOD) and limit of quantification (LOQ) is determined from a calibration curve linear regression equation. Limit of detection (LOD) was 0,268 ppm and limit of quantification was 0,8123 ppm.

4. Rhodamine B identification on Cosmetics and Food Samples by using TLC

TLC method was done using silica gel as a plate and ethyl acetate : methanol : (ammonium:water = 3:7) = 15:3:3 as an eluent and UV 254 nm as detector.

Table 4.4 TLC Identification of Shrimp paste

Spot	Rf	Colour in UV 366	Comment
Rhodamin B	0.8125	Light yellow	+
Shrimp Paste A	0.0375	Blue	-
Shrimp Paste B	0.8125	Light yellow	+
Shrimp Paste C	0.125	Blue	-
Shrimp Paste D	0.8125	Light yellow	+
Shrimp Paste E	0.125	Blue	-
Shrimp Paste F	0.8125	Light yellow	+
Shrimp Paste G	0.125	Blue	-
Shrimp Paste H	0.8125	Light yellow	+

Table 4.5 TLC Identification of Lipstic

No	Spot	Rf	Colour at UV 366	Comment
1	Baku rhodamin B	0.8125	Yellow	+
2	Lipstic A	0.425	Yellow	-
3	Lipstic B	0.425	Yellow	-
4	Lipstic C	0.425	Yellow	-
5	Lipstic D	0.425	Yellow	-
6	Lipstic E	-	-	-
7	Lipstic F	0.675	Yellow	-
8	Lipstic G	-	-	-
9	Lipstic H	0.675	Yellow	-

Table 4.6 TLC Identification of Crackers

No	Spot	Rf	Colour at UV 366	Comment
1	rhodamin B	5.9/8= 0.7375	Yellow	+
2	Crackers A	-	-	-
3	Crackers B	0.7375	Yellow	+
4	Crackers C	0.7375	Yellow	+
5	Crackers D	-	-	-
6	Crackers E	0.7375	Yellow	+
7	Crackers F	-	-	-
8	Crackers G	0.75	Yellow	+

Table 4.7 TLC Identification of Eye Shadow and Rouge

No	Spot	Rf	Colour at UV 366	Comment
1	rhodamin B	0.8125	Yellow	+
2	Eye Shadow and Rouge A	0.0	Purple	-
3	Eye Shadow and Rouge B	0.5375	Purple	-
4	Eye Shadow and Rouge C	0.3625	Yellow	-
5	Eye Shadow and Rouge D	0.475	purple	-
6	Eye Shadow and Rouge E	0.3625	Orange	-
7	Eye Shadow and Rouge F	0.3625	Orange	-
8	Eye Shadow and Rouge G	0.375	yellow	-

5. RhodamineB identification on Cosmetics and Food Samples by using Spectrophotometer UV Vis

The results of the identification of Rhodamin B in some samples by means of UV Vis spectrophotometer in the wavelength range 400-700 nm can be seen in table 4.8.

Tabel 4.8 Identification results of Rhodamin B in a sample by using spectrophotometer UV Vis

Sample	Max Wavelength (nm)	Absorbance	Results
Lipstick G	528	0.2246	-
Lipstick A	484	0.67245	-
Lipstick E	484	0.1089	-
Lipstick F	526	1.8151	-
Lipstick B	510	0.0584	-

Lipstick C	514	0.5175	-
Lipstick D	485	0.2439	-
Crackers B	545	0.1054	+
Crackers E	543	0.0747	+
Crackers G	546	0.0312	+
Crackers A	482	0.2021	-
Crackers C	546	0.1613	+
Crackers F	400	0.0101	-
Crackers B	400	0.0151	-
Shrimp paste H	544	0.1518	+
Shrimp paste G	439	0.1296	-
Shrimp paste F	546	0.5229	+
Shrimp paste E	400	0.3518	-
Shrimp paste C	545	1.5806	+
Shrimp paste D	546	1.5828	+
Shrimp paste A	534	0.2214	-
Shrimp paste B	468	0.0818	-
eyeshadow D	552	0.5713	-
eyeshadow E	506	0.3556	-
eyeshadow F	523	0.5018	-
eyeshadow i	511	0.2179	-
rouge B	553	1.0596	-
rouge C	553	0.6876	-
Rouge H	476	0.0678	-
rouge G	501	0.1568	-
rouge A	534	0.2376	-
rouge j	553	0.5877	-

Table 4.8 shows that there are some positive samples containing Rhodamin B because it has a maximum absorption around 544nm, ie destitute crackers C, crackers B, Crackers E, Crackers G, shrimp paste H, shrimp paste F, shrimp paste C, and shrimp paste D.

Acknowledgement

Ministry of Higher Education Indonesia through Hibah Bersaing grant 2011.

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Conversion of Sugarcane Bagasse to Bioethanol Using Different Acids Pretreatment and Commercial Yeast

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Abstract

Recently, sugarcane bagasse is drawing many interest in bio-fuel research, as it have a potential use for production of bio-ethanol as well as a way to reduce the waste in sugarcane processing. In this study, we evaluated several pretreatment of sugarcane bagasse using various organic acids such as hydrochloric acid (HCl), sulfuric acid (H_2SO_4) and acetic acid (CH_3COOH). The changes of functional group before and after pretreatment were investigated by Fourier Transform Infrared (FTIR) analysis. After acid hydrolysis, we convert the fermented the sugar with a commercial yeast, Instaferm. The result revealed that the pretreatment process using H_2SO_4 1.25% w/w was the most effective process for producing reducing sugar as it was obtained 0.027 g/l on the fermented solution.

Keywords: sugarcane bagasse, bioethanol, fermentation, Infrared analysis, reducing sugar

Introduction

Energy is the most important thing to human life in order to survive. Several sources of energy up to now have relied on petroleum and natural gas, whose existence cannot be renewed, which means one day it may be vanished. To solve this problem, various attempts to obtain alternative sources of renewable energy actively encouraged. Besides natural energy such as geothermal, solar, wind, and water energy, energy from biomass sources is one of the field in renewable energy that attracting the attention of researchers. Biomass such as corn, switch grass, wood [1,2] and soybean molasses [3] are several example of the primary commodities as raw material for bioethanol production, moreover, the utilize of this biomass for energy source will reduce the global warming effect caused by nonrenewable energy.

However, in developing countries such as South Asia countries, the use of raw materials from soybean, corn or palm oil still raises the pro and contra [4]. This is because the plants are still used as food's sources. The use of foods as energy resources may make an anxiety in food's stock safety. Sugarcane bagasse is one of the alternative materials that can be used as a source of raw material for producing bioethanol. This is because sugarcane bagasse still contain hemicellulose and cellulose that can be converted into sugar and then

fermented into alcohol. The use of sugarcane bagasse also provide other benefits, such as reducing the waste from the sugar factory. Therefore, the utilization of sugarcane bagasse, besides producing bioethanol as fuel also helping the waste management of sugar factory. Several research in utilizing of sugarcane bagasse had been done such as Pessoa et al. that studied acid hydrolysis on hemicellulose of sugarcane bagasse [5], and Dawson et al. that studied cellulosic ethanol production from sugarcane bagasse without enzymatic saccharification [6].

In the city of Yogyakarta, Indonesia, there is a sugar manufactory that produces sugarcane bagasse as its waste, so the purpose of this research is to conduct utilization of agricultural waste, i.e. sugarcane bagasse as an alternative energy source for bioethanol production. As it is known, sugarcane bagasse is one of lignocelluloses wastes containing lignin, thus lignin must be removed first in order to obtain its cellulose and hemicellulose. Removal of lignin was carried out with pretreatment method. In this study the initial pretreatment using several types of organic acids such as hydrochloric acid (HCl), sulfuric acid (H_2SO_4), and acetic acid (CH_3COOH) will be evaluated. The fermentation process is done using the commercial yeast Instaferm, that available in the market. The use of this commercial yeast is in order to provide simple technology of fermentation to the society.

Materials and Methods

Chemicals are from Sigma-Aldrich, except that mentioned. HCl, H_2SO_4, CH_3COOH, NaOH, acetone, aquadest, Instaferm yeast (from Liquid Sunshine Distillery), urea, NPK, Nelson reagent, arsenomolybdat reagent, sugarcane bagasse waste material was collected from PT. Madukismo, Yogyakarta, Indonesia. It was drying under the sun and sieved ~0.2-0.5 cm long.

Pretreatment method

10 grams of bagasse was weighed and added with 150 ml of aquadest and added with HCl 1.25% w/w. The mixture then refluxed for 4 hours at temperature of 150^oC. The mixture then filtered, the residue was washed with water until neutral while the filtrate is stored in a bottle. The same pretreatment were conducted using H_2SO_4 1.25% w/w and CH_3COOH 1.25% w/w.

Fermentation Process

The filtrate of initial treatment was neutralized with NaOH 3M, and then 100 ml of solutions (50 ml of filtrate and 50 ml of aquadest) was added with 0.65 grams of urea and 0.01 grams of NPK (solution A). Yeast solution was prepared by weighing 0.25 grams of Instaferm added with 20 mL of aquadest (solution B). Solution A and solution B were mixed and fermented for 6 days. Reducing sugar analysis was done using Nelson-Somogyi method [7,8]. Alcohol contents was roughly determined using alcoholmeter.

FTIR Analysis

FTIR spectroscopic analysis was carried out in Department of Chemistry, Gadjah Mada University. FTIR spectrum was recorded between 4000 and 400 cm^{-1} using a Shimadzu spectrometer with detector at 16 cm^{-1} resolution and 10 scan per sample

Results and Discussion

Characterization

The sugarcane bagasse before and after pretreatment were characterized using FTIR spectroscopy as shown in Figure 1 (before pretreatment), Figure 2 (after pretreatment using HCl), Figure 3 (after pretreatment using H_2SO_4), and Figure 4 (after pretreatment using CH_3COOH). The FTIR measurement was used to investigated the changes of cellulose structure during acids pretreatment. Generally, the broad absorption around 3400 to 3500 cm-^{1} was related to the stretching peak of –OH groups, and the peak at 2924 cm-^{1} was obtained from C-H stretching. Two peaks arose in 1056 and 1120 cm-^{1} were the C-O-C stretching from β-(1–4) glycosidic linkages [9]. C-H bending peaks arose around 1381-1371 cm-^{1} and 1327 cm-^{1}. The bands at 1249 cm-^{1} was the O-H bending. The FTIR spectra for sugarcane bagasse before and after pretreatment were different, this indicates that the structural of bagasse were altered. The changes were observed in the broadening of –OH stretching bands. For the raw bagasse, it has the broad peak around 3425 cm-^{1}, while after acid pretreatment, it shifted to 3433 and 3441 cm-^{1}. The peak around 2924 cm-^{1}, that came from $–CH_2$ stretching also different for bagasse before and after acid pretreatment. The enhancement of peaks around 1000-1150 cm-^{1} were related to the cellulose content that increased in the residue after acid pretreatment [9].

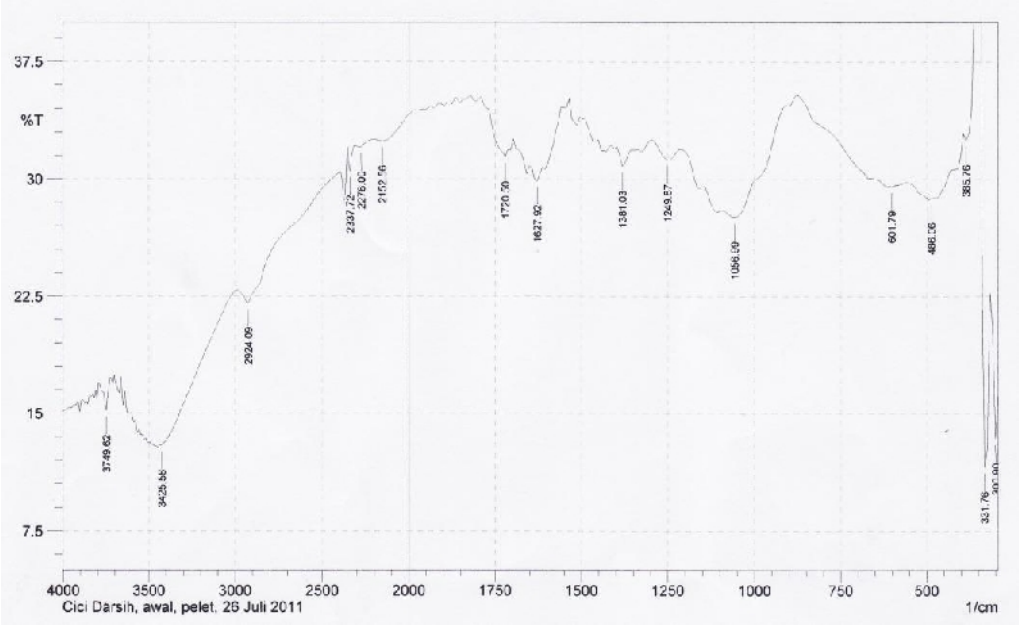

Figure 1. FTIR spectra of sugarcane bagasse before pretreatment.

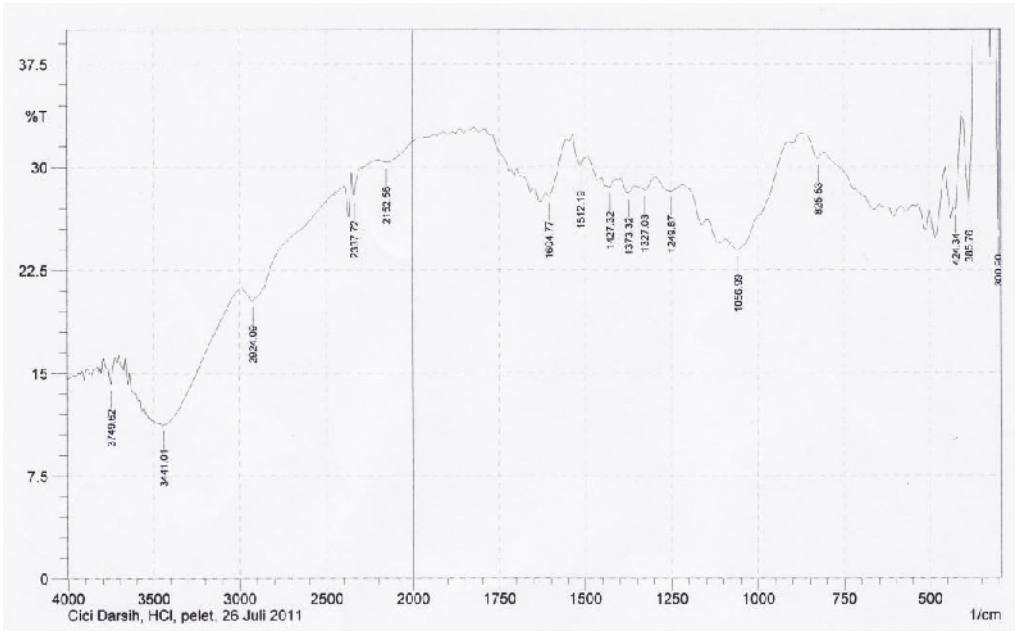

Figure 2. FTIR spectra of bagasse after pretreatment using HCl 1.25%w/w.

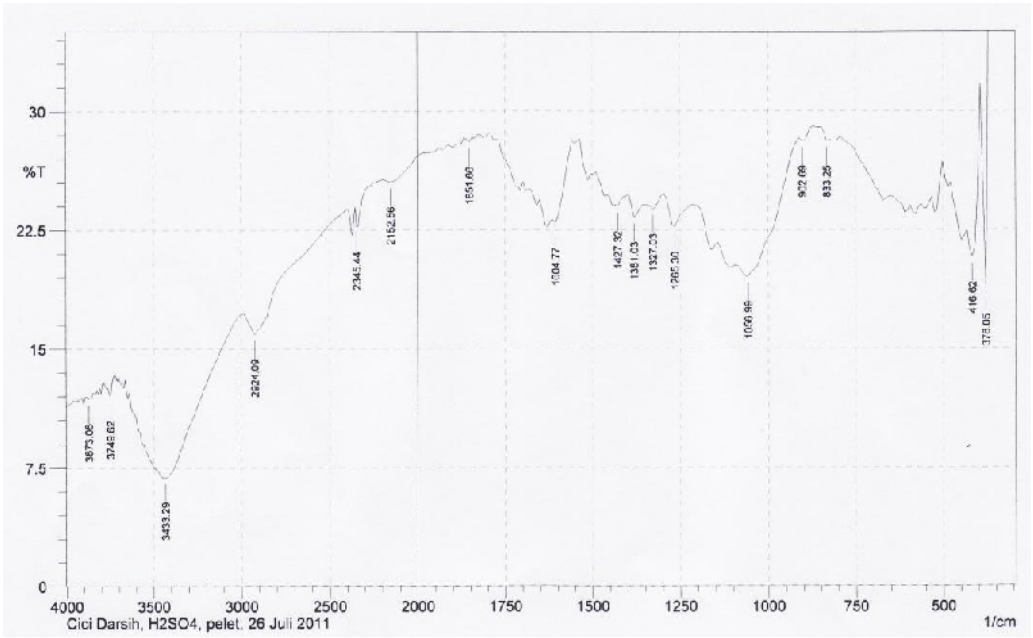

Figure 3. FTIR spectra of bagasse after pretreatment using H_2SO_4 1.25%w/w.

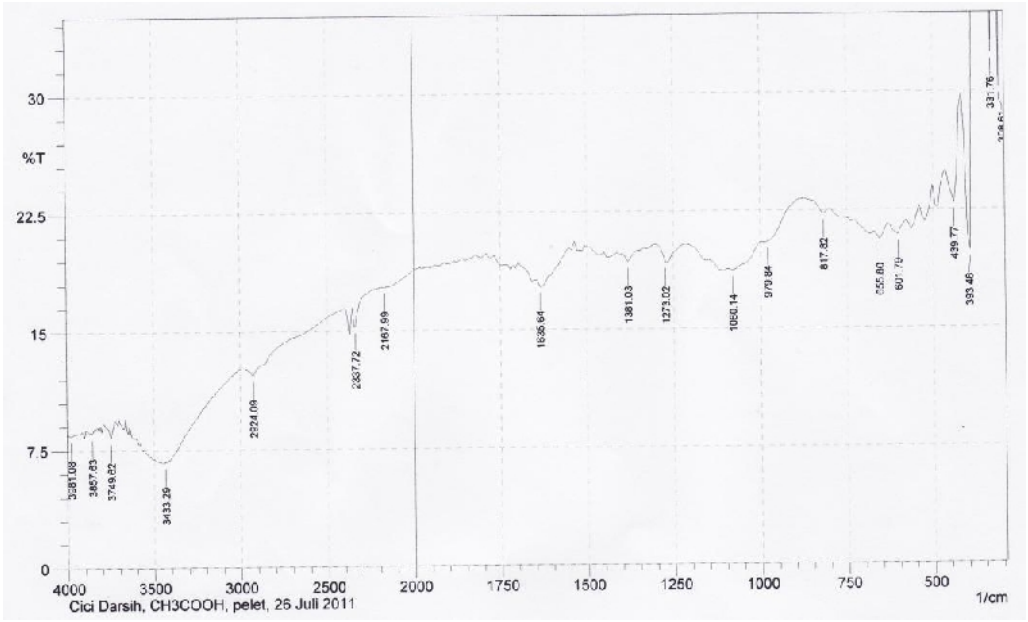

Figure 4. FTIR spectra of bagasse after pretreatment using CH_3COOH 1.25%w/w.

Reducing Sugar

The reducing sugar was analyzed using Nelson-Somogyi method. The standard curve was shown in Figure 5.

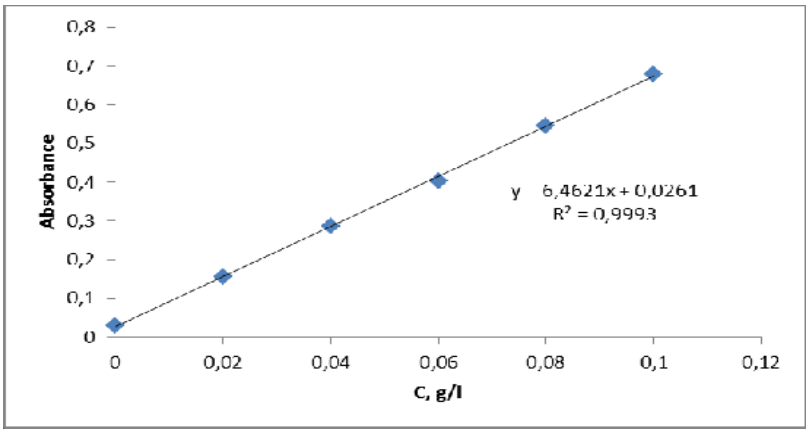

Figure 5. Standard curve for reducing sugar analysis.

The reducing sugar of the filtrate resulting from pretreatment after fermentation using Instaferm was presented in Table 1.

Table 1. Reducing sugar content on the fermented solution

Acid	C, g/l
HCl	0.019
H_2SO_4	0.027
CH_3COOH	0.020

Based on the results, there was still reducing sugar content after 6 days of fermentation process that not yet transformed into alcohol. Since we used the same amount of commercial

yeast for fermentation, it could be possibly that based from the result we can conclude that H_2SO_4 1,25% w/w produce the most reducing sugar in amount that is 0.027 g/l. Further optimization of Instaferm yeast using in fermentation process also should be considered to make all reducing sugar convert to alcohol. The roughly result from alcoholmeter measurement it revealed that all of the fermented solution contain alcohol around 1%. However, the further analysis using gas chromatography is needed to know exactly the content of alcohol.

Conclusions

Generally, the acid pretreatment for sugarcane bagasse improve the structure of bagasse as showed in the FTIR spectra. The study also revealed that H_2SO_4 1.25% w/w was the most effective acid pretreatment to produce reducing sugar, and commercial yeast Instaferm could be potentially used in fermentation process of producing alcohol.

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Analysis of Cocofoam Microstructure and Mechanical Characteristics From the Mixture of Coconut Fiber and Latex Compound

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The research on the making of cocofoam from the mixture of coconut fiber and latex compound and its characteristics has been conducted. Cocofoam is a composite product which has a high elasticity and can be used as an alternative in natural fiber foam. Cocofoam is obtained by mixing the coconut fiber and latex compound with weight ratio (b/b): 20/222 (CF10-1), 30/55 (CF10-2) and 40/115 (CF10-3). The measured cocofoam' characteristics are density, tensile strength, elongation at break, and fixed compression at 50%. The cocofoam microstructure is analyzed by using Scanning Electron Microscope (SEM).

The result shows that the weight increasement ratio of the mixture of coconut fiber and latex compound made in constant volume produce cocofoams with density as follows: CF10-1=0,7660g/cm^3, CF10-2=0,8043g/cm^3, and CF10-3=0,8146g/cm^3. The tensile strength for each cocofoam are as follows: CF10-1=0,08N/mm^2, CF10-2=0,12N/mm^2, and CF10-3=0,16N/mm^2. The elongation at break for each cocofoam are as follows: CF10-1=41,82%, CF10-2=39,63%, and CF10-3=41,90%. The values of fixed compression at 50% after 4 hour-cocofoam pressure are CF10-1=15,43%, CF10-2=13,33%, and CF10-3=3,13%. The microstructure analysis reveals that the three cocofoam surfaces, CF10-1, CF10-2, and CF10-3, have the similar characteristics that the cocofoam cavities are not formed compactly, and the latex compound which serves as adhesive is not evenly distributed on the cocofoam surfaces.

Keywords: cocofoam, tensile strength, compression, latex compound, coconut fiber, SEM.

INTRODUCTION

Coconut has been the inseparable part of Indonesian life because every part of a coconut tree has high value to improve the quality of economic, social and cultural life. The importance of coconut for the Indonesian citizens can be seen from the tremendous increase of the coconut area in Indonesia which represents around 98% from approximately 3,89 million hectares. Further, the coconut plantation involves more than 3 million farmers. The activity of coconut processing to gain some valuable end products results in the new opening of job opportunity and increases the economic value of society (Novarianto, 2007; Allorerung dan Lay, 1998). The total coconut area in Indonesia is the highest in the world which constitutes 3,8 million hectares, followed by Philippines with 3,4 million hectares and India in the third place with 1,9 million hectares (Idrous et. al., 2010).

The abundant coconut trees in Indonesia produce approximately 15,5 trillion which is equal to 3,02 million tons copra, 3,75 million tons water, 0,75 million tons charcoal, 1,8 million tons coconut husk's fiber, and 3,3 million tons coconut husk's dust (Agustian et. al., 2003). The industry of coconut processing mainly focuses on the processing of coconut meat as the main product, while the industry which mainly focuses on the processing of by-product of coconut fruit such as water, coir, and coconut shell uses traditional method and is a low-scale industry. This condition seems so ironic because of the abundance of raw material that

would be quite enough to establish a developing processing industry. The coconut husk possesses a great potential to be grown as the composite product that has high commercial value, considering that Indonesia has great amount of coconut husk (Mahmud and Ferry, 2005; Arbintarso, 2009).

India and Sri Lanka have transformed to be countries to have more sophisticated technology in the processing industry of coconut husk. Many by-products have been produced and provided the nation with the high income from products. Meanwhile, the level of utilization of coconut husk for commercial products in other countries which become big producers of coconut trees is still low. Indonesia and Philippine sells the products made from coconut husk to international market in the form of raw fiber and the main destination is China. The export value of Sri Lanka in 2009 reach USD 40,2 million coming from the selling of semi-raw material products and the selling of end product contributes national income for USD 78,7 million (Idrous et. al., 2010).

Besides vast coconut areas, Indonesia also has vast rubber plantation which constitutes 3,4 million hectares, with estimated 85% of which are small-scale plantation and other 15% are big-scale plantation. Natural rubber is one of reliable non-oil and fuel productions from the plantation sector upon which the government obtain great amount of national incomes. Nowadays, Indonesia is the second largest rubber producer after Thailand (Dirjenbun, 2007). The low consumption of natural fiber in local level is resulted from the difficulties that hamper the development of end-product made from rubber. The main difficulty in the development of rubber's end-product in local level is the absence of the efforts in increasing the value of product in most of rubber industries in Indonesia. This phenomenon emerges because of the high dependence on the recycle technology. Further, the main root of the problem also lies on the inconsistency in the quality of natural rubber, the less guaranteed-supply of natural rubber due to the more dominant role of big vendor, and the rapid flow of imported rubber product so the local product is less competitive either in domestic or international market (Arizal et. al., 2000).

The explanation above entails that one of the natural resources potential which is going to be explored by the writer is the utilization of coconut husk with liquid latex as the deposits for natural composite. The making of composite from the mixing of coconut husk with latex produces new material called Cocofoam. Cocofoam is a type of composite with high elasticity and flexibility so it can be used as the alternative product for natural fiber-foam. Cocofoam has more distinctive feature compared to synthetic foam for cocofoam looks so cool and comfortable because it is made of natural ingredient with bigger pores. Cocofoam becomes very prospective new product that has promising development in Indonesia. The fact that cocofoam is abundant, renewable, biologically degraded and eco-friendly natural resources underlines this possibility.

RESEARCH METHOD

Materials

Coconut fiber was obtained from PT Tropica Nucifera Industry Bantul Yogyakarta and liquid latex brand 'Cap Jempol' obtained from Toko Liman Malioboro Yogyakarta. Potassium oleat, Potassium Hidroxide, Zinc diethyl-dithiocarbamate (ZDEC), Zinc Mercaptobenzothiazole (ZMBT), Butylated Hydroxytoluene (BHT), and Sulfur which was obtained from PT Bratako Yogyakarta.

Equipments

Standard glass equipment, analytical scale AND GR-200 SER. 14214919 Japan, magnetic whisk, oven Memmert 854 Schwabach Tel. 09122/4031 0-24 h. 220^{o}C Western

Germany, Jeol JSM – 6360LA analytical Scanning Electron Microscope, Tokyo Testing Machine MF6 Co. LTD Tokyo-Japan, Template and One Set Compressor.

Procedures

Preparation of Coconut Fiber

The first step was to separate coconut fiber from dirt and dust in order to get clean coconut fiber. Then, this fine fiber was washed using water, and dried up under the sunlight. The fiber was then twisted before heated in the oven under the temperature of 95^{o} – 100^{o}C for approximately 6 h, then cooled off under room temperature for 2-3 days. A ball of twined fiber was reopened in order to get a spiraled shape fiber.

Preparation of Latex Compound

Latex used in this research was cleaned to cleanse rough particles such as dust and sand. All chemical substances are dissolved into liquid latex. The composition of chemical substances was 100 g of liquid latex, 2 g Potassium Oleat solution 20%, 3 g of KOH solution 10%, 2 g of ZDEC solution 50%, 2 g of ZMBT solution 50%, 4 g of ZnO solution 50%, 1 g BHT solution 50% and 3 g Sulfur solution 50%. Latex compound mixed with all solutions is stirred by using magnetic whisk for 30 minutes. Then, this ingredient is kept for 2 days before it is ready to use.

Preparation of Cocofoam

Coconut fiber was spread over wooden plate (l x w x h = 24 x 15 x 5 cm). This coconut fiber should be well-managed in the plate. The fiber surface was evenly sprayed by using latex compound with the distance between fiber surface and nozzle sprayer was approximately 20 cm. The initial spraying of latex compound was 1/3 part of overall dosage. The blend of coconut fiber and latex compound begins with oven heating in the temperature of 80-90^{o} C for 15-20 minutes. Then, cocofoam was taken out from the oven and cooled off for several minutes before having sprayed with 2/3 remaining part of compound with the distance with nozzle spray was 5 cm. Cocofoam was taken out from the plate then vulcanized for the second time in the oven, shaped it and labeled with CF10-1. The preparation of cocofoam CF10-2 has similar process with that of the cocofoam CF10-1. The assessment process of physical and mechanical natures includes its density, tensile strength, elongation at break, fixed 50% compression and the of cocofoam microstructure analysis bay using *Scanning Electron Microscope.*

RESULT AND DISCUSSION

This research involves density test of *p*olyurethane foam (PF) which serves as comparative agent to cocofoam. The graphic of density of compound latex (CL), coconut fiber (CB), and cocofoam (CF10-1, CF10-2, and CF10-3) is shown in Figure 1

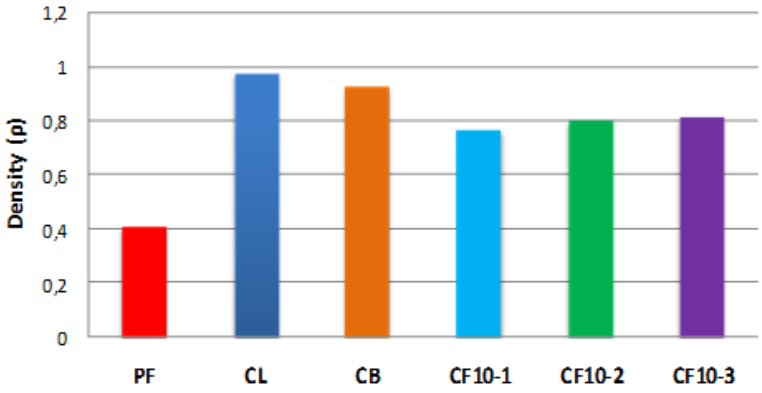

Figure 1. Density graphic : PF, CL, CB, F10-1, CF10-2 and CF10-3

The research result shows that density as follows: PF = 0,4075 g/cm^3, CL = 0,9727 g/cm^3, CB = 0, 9283 g/cm^3, CF10-1 = 0,7660 g.cm^3, CF10-2 = 0,8043 g/cm^3 and CF10-3 = 0,8146 g/cm^3. Cocofoam which have density value 0,7660-0,8146 g/cm^3 is categorized into high density cocofoam. The different value in cocofoam's density is resulted from the higher ratio between coconut husk and latex used while the volume of cocofoam remains unchanged. Therefore, it increases the density of cocofoam. In addition, the difference found in cocofoam's density probably rooted from unfairly distribution in spraying activity, thus, the amount of latex scattered upon the surface of coconut husk is not equally distributed. Latex mixed with other chemicals has higher density which eventually hampers the nozzle to freely spray the coconut husk's fiber. Another reason is the low quality of mixing making the molecules of latex is easily to agglomerate and it allows bigger possibility for the blockage in the nozzle valve. As a result, each sample receives different amount of which at once influences the density. The combination between coconut husk's fiber and latex produces hollow composite with high elasticity.

Wijaya and Tahir (2001) state that composite is a solid entity made up of the fusion between two or more distinct materials in order to produce better properties not present in any individual ent. Composite whose characteristics inclines to have more resemblance with pore polymers such as foam possesses higher density approximately 0,4 to 0,8 (Mills, 2007; Anggaravidya, 2008; Najib et. al., 2009). According to Ben and Shoji (2003), phenolic foam has density ranging from 0,40-0,42. Cocofoam is new type of composite distinguishable from its individual ents, latex and coconut husk's fiber, in its density. Cocofoam has higher density than polyurethane foam meaning that cocofoam is heavier.

The tensile strength test serves as the parameter of physical strength of composite product. The value of tensile strength test of cocofoam is presented in Figure 2. The research shows that the values of tensile strength for each cocofoam are as follows; CF10-1 = 0,08N/mm^2, CF10-2 = 0,12N/mm^2, and CF10-3 = 0,16N/mm^2. The tensile strength's value depends on the material's density.

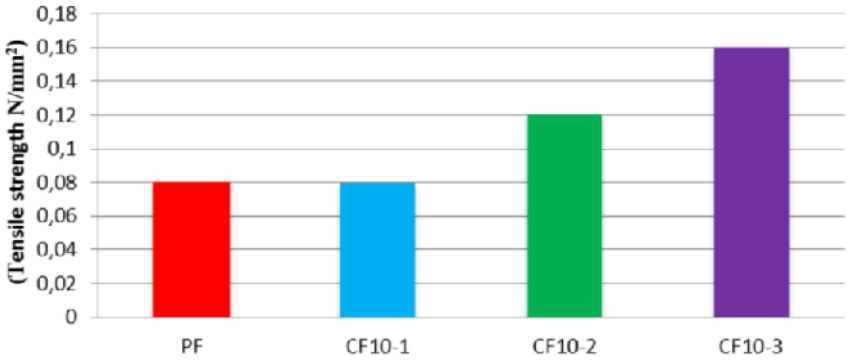

Figure 2. Tensile strength graphic : PF, CF10-1, CF10-2 and CF10-3

The higher the cocofoam's density, the higher the tensile strength's value. In the making process of cocofoam, the greater amount of coconut husk and latex and the consistent volume of cocofoam produce more solid material thus cocofoam emerging from this process will have bigger tensile strength. The elongation at break test is another parameter in testing physical strength of one composite product. The value of elongation at break test of cocofoam is presented in Figure 3. The value of elongation at break for polyurethane foam are as follows: PF = 85,99%, CF10-1 = 41,82%, CF10-2 = 39,63%, and CF10-3 = 41,90%. The ratio of the mixing between coconut husk and latex is intentionally altered while the volume of cocofoam remains unchanged. The value of elongation at break test for CF10-1, CF10-2, and CF10-3 is mostly influenced from the orientation of random wavy fibers which is unequally distributed and the dissimilarity in cocofoam cavities. The heterogeneous cocofoam cavities lead to various value of elongation at break test. The insufficient number of fiber orientation for homogeneous fiber weakens the interface bonding between fiber and latex.

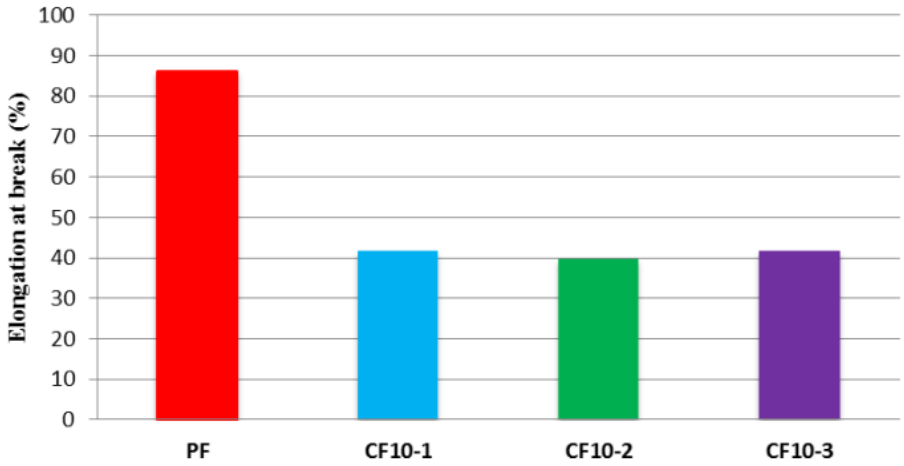

Figure 3. Elongation at break graphic : PF, CF10-1, CF10-2 and CF10-3

Further, the heterogeneity in cocofoam cavities alters the possibility of deformation, elongation at break, the breakage of interface bonding between latex surface and fiber. The value of cocofoam's elongation at break may also be influence from the distinct tensile strength that cocofoam receives during the test. The breakage, consequently, is found only in

some parts receiving more strains than other parts. Forces required to pull the cocofoam is not equal in overall surface.

One test for measuring the elasticity of composite product is so called fixed compression test. The compression test is conducted in room temperature for one to four hours. The graphic of the mean value of fixed 50% compression is presented in Figure 4. Figure 4 shows that the increasing compression value for each cocofoam is congruent with the length of compression time. The higher the weight upon, the more decreasing the strength cocofoam has.

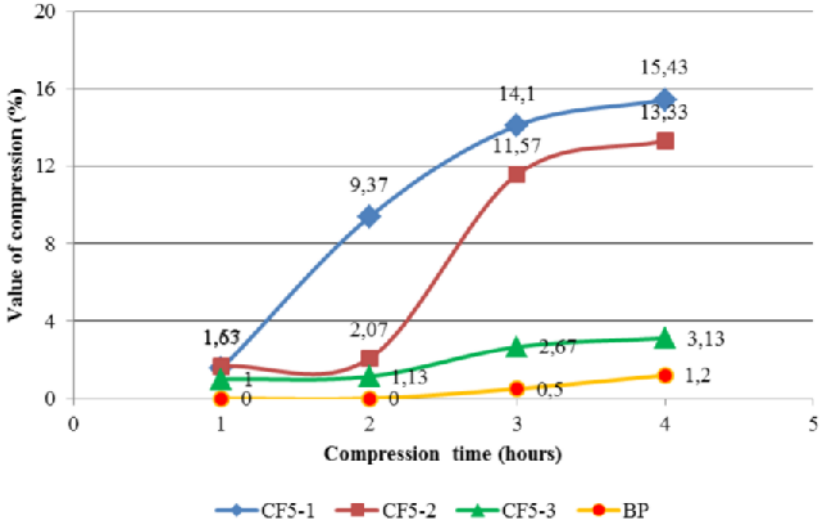

Figure 4. The mean value of fixed 50% compression graphic : PF, CF10-1, CF10-2, and CF10-3.

As a matter of fact, the similarity in the length of time during compression process results in the difference of compression value for each cocofoam. It is due to the compression and the ratio of coconut husk's weight compared to latex is getting higher while the volume of cocofoam receives no difference, as a result, the density of cocofoam is also undoubtedly increasing. Cocofoam is a type of composite which have big and heterogeneous air pores. When cocofoam undergoes compression by giving it higher pressure, the air pores becomes smaller. Compression value is defined as the percentage of cocofoam's thickness changes as a result of temporary weight pressure when a heavy substance is imposed upon cocofoam and it will retain its former state after the weight is removed from the cocofoam (Anom et. al. 2010).

Compared to compression value of polyurethane foam, cocofoam has higher value. The compression value of each cocofoam CF10-1, CF10-2, and CF10-3 after 4 hour pressure is subsequently as follows15,43%, 13,33%, and 3,13%. Meanwhile the value of polyurethane foam after 4-hour compression is reaching 1,20%. This fact reveals that polyurethane foam is stronger and has more endurance in coping with heavy substance than cocofoam. The material used in compression test is fixed or static load imposed on cocofoam for certain length of time. Fixed compression test is aimed at examining the changes in cocofoam's thickness after restraining a heavy load. Moreover, it is useful to predict the longer use of product. The higher compression value indicates that the ability of cocofoam to restraint load is getting lower. When a heavy load is imposed upon cocofoam in a longer time, the thickness of cocofoam is reduced which eventually it loses its elasticity.

The morphology of polyurethane foam, cocofoam CF10-1, CF10-2 and CF10-3 is shown in Figure 5.

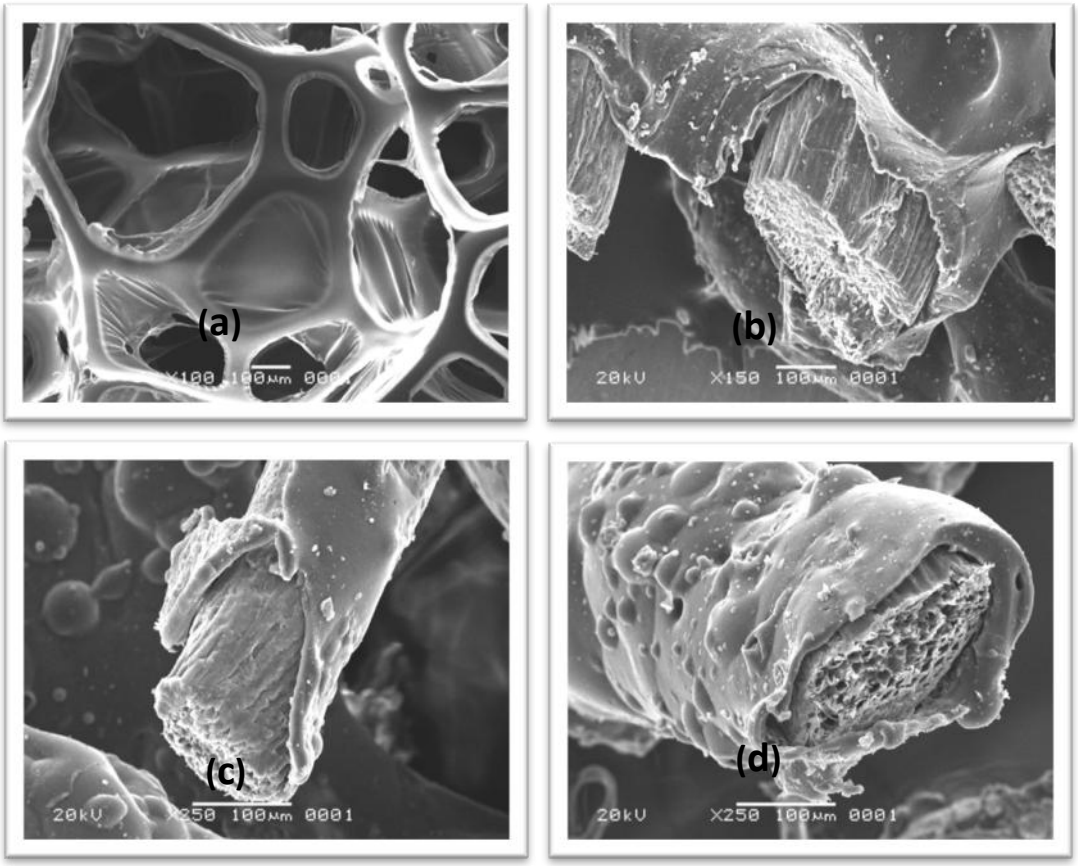

Figure 5. Cocofoam SEM Micrograph : (5a) PF, 5(b) CF10-1, (5c) CF10-2 and (5d) CF10-3.

Figure 5a exposes that the morphology of microstructure shows no deformation on the surface of polyurethane foam. The interaction of filling material and polymer matrix seems absolutely compact leading to fair distribution of air pores and it looks nearly homogeneous. The surface's morphology of cocofoam CF10-1, CF10-2 and CF10-3 shown in Figure 5b, 5c, 5d shows scattered distribution of latex upon the surface of cocofoam resulting in the difference of thickness of latex that coat and bind fibers. The distinction in density of cocofoam CF10-1, CF10-2 and CF10-3 does not give any indication of significant difference in cocofoam morphology. It means that fibers and latex bond together within cocofoam are not distributed well, therefore, the microstructure of cocofoam CF10-1, CF10-2 and CF10-3 is quite similar.

The length of fiber and the non-homogenous latex mixing give significant on the compactness level of solid material. It is possible to get the fiber-latex interaction that shows slight compactness and to reveal the agglomeration of latex or additives granule attached to the surface of cocofoam. Due to microstructure analysis, it can be concluded that those three cocofoam share similar characteristics as SEM photo has revealed.

CONCLUSION

1. The increasing weight ratio of the mixing between coconut fiber and latex in the constant volume results in cocofoam with density as follows: CF10-1 = 0,7660 g/cm^3, CF10-2 = 0,8043 g/cm^3, and CF10-3 = 0,8146 g/cm^3 . The value of polyurethane foam's density is 0, 4075 g/cm^3. The lower value of polyurethane foam shows that it is less heavier than cocofoam.

2. The value of tensile strength test for polyurethane foam is 0,08N/mm^2 with the value of elongation at break is 85,99%. Meanwhile, The highest value of tensile strength test for cocofoam found in CF10-3 with 0,16N/mm^2 with the value of elongation at break is 41,90%.
3. The value of fixed 50% compression for polyurethane foam is smaller than that of cocofoam CF10-1, CF10-2 and CF10-3. It means that polyurethane foam is stronger and has more endurance to receive heavy load than cocofoam.
4. The microstructure analysis conducted on CF10-1, CF10-2 and CF10-3 reveals similar characteristic in which the surface of cocofoam is not compact and latex serving as adhesive is not fairly distributed over the surface of cocofoam.

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Genetic Diversity on Goffin's Cockatoo Bird (*Cacatua goffini*) Inferred from Cytochrome B Gene Sequences

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ABSTRACT

Genetic variability among 18 individuals of Goffin's cockatoo bird (*Cacatua goffini)* was investigated using DNA sequences of cytochrome b gene. Two DNA fragments were amplified using two nucleotide primer pairs in the PCR. Eight hundred base pairs of cytochrome b gene were used in this analysis. The results showed that within the birds, there were 20 variable sites and 18 haplotypes (Hcg1- Hcg18). There were no identical DNA sequence and no dominant haplotype. Nucleotide (Pi) and Haplotype (Hd) diversities were 0.005437 and 1.0000, respectively.

Key words: DNA sequences, cockatoo bird, haplotype diversity, nucleotide diversity.

INTRODUCTION

The biological diversity of the planet is being rapidly depleted due to the direct and indirect consequences of human activity (habitat destruction and fragmentation, over exploitation, pollution and movement of species into new locations) (Frankham R et al., 2004). Since, the human population and human impact on natural habitat have significantly increased in the past few decades, therefore, it is well recognized that extinction now threatens a large number of species in the world (Ryder et al. 2000). Many endangered species require captive breeding to save them from extinction, as they are incapable of surviving in inhospitable natural environments due to direct or indirect human impacts in the form of habitat loss, overexploitation, pollution, or introduced predators, competitors or diseases (Millennium Ecosystem Assessment 2005; IUCN 2006).

Therefore, conservation of biodiversity becomes an urgent task for both governments and the public. With a good knowledge of the underlying processes of extinction, conservation efforts will be much more effective (May et al., 1994). In the scientific effort for conservation of biodiversity, there has often been an emphasis on the importance of genetic factors in influencing extinction (Hedrick, 1996).

Goffin's cockatoo (*Cacatua goffini*) is one of the white cockatoo species found in Indonesia and originally ditributed only at Tanimbar island so that the bird is also called as Tanimbar cockatoo. The bird has a body length of about 32 cm with white plumage colors and white a movable crest on their head (Forshaw, 1989). Currently, the bird population in the nature tend to be decreased, so it was recorded and became a priority in the World Conservation Union, IUCN 2006 and included in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Therefore, some activities were carried out both of ex-situ action in captive breedings and zoos and, as well as an in-situ action in their original habitat to support their conservation programme. One of its activities was study on molecular level to know its genetic diversity.

As the size of animal and plant populations decreases, loss of genetic diversity reduces their ability to adapt to changes in the environment, with inbreeding and reduced fitness inevitable consequences for most species. Genetic diversity is typically described using polymorphism, average heterozygosity, allelic diversity, DNA sequences variation, amino acid differences, and protein variation. Such protein variation may result in functional biochemical or morphological dissimilarities that cause differences in reproductive rate, survival or behaviour of individuals (Frankham R et al., 2002).

Genetic diversity is reflected in the differences among individuals for many characters, including eye, skin and hair colour in humans, colour and banding patterns of snail shells, flower colours in plants, and protein and DNA sequences (Frankham R et al., 2002). Genetic diversity primarily refers to variation within the genetic make up of an individual. It also is variation in genetic make up among individuals within a population, genetic variation among different populations of a species, and genetic differences among species (*Cothran E.G, http//printfu.org/ genetic-diversity).* Genetic diversity is, therefore, a key component for conservation efforts associated with population management (e.g., Andayani et al., 2001).

The use of molecular characters to investigate genetic, relationships, ecological and behavioral aspects of wildlife populations has gained immense popularity in recent years. It can significantly contribute to captive breeding and reintroduction strategies for the conservation of various endangered animals (Russello and Amato, 2007).

Mitochondrial DNA (mtDNA) is regarded as an important tool in studying evolutionary relationships among various taxa due to its conserved protein-coding regions, high variability in non-coding sequences and lack of recombination (Olivo et al., 1983; Ingman et al., 2000). Because of these genetic features, mtDNA is thought to be very useful genetic markers to study the origin, the genetic diversity and differentiation of both closely related species and individuals within species (Brown et al., 1986; Hauswirth et al., 1984; Ishida et al., 1994). mtDNA contains 13 protein-coding genes, and cytochrome b (cyt b) is one of them.

Mitochondrial cyt b is expanding very rapidly and have used as a tool in studies of molecular evolution (e.g: Kocher et al., 1989; Montgelard et al., 1997; Prusak et al., 2004), genetic diversity within and between species and genetic relationships of some bird groups (e.g. parrots: Astuti D et al., 2006; oryx : Russello and Amato, 2007).

For this work I am focusing on variation among individuals bird. This research was conducted to find out genetic diversity among individuals of Goffin's cockatoo (*C. goffini*) bird based on DNA sequences of the mitochondrial cyt b gene.

METHODS

Blood sample collection and DNA extraction.

Blood samples were used in the present study, taken from living birds in the zoos and captive breeding. Eigtheen blood (18) samples were collected from each individual cockatoo bird. Samples were taken using 1 ml of syringe and preserved in 96% ethanol, sealed with tape and stored in room temperature. Genomic DNA was extracted from blood with the QIAamp® Mini Kit (QIAGEN®), following the instructions from the manufacturer.

DNA amplification and sequencing.

Two internal parts of mitochondrial cyt b gene were amplified using two pairs of internal primers : 1) L: 5'-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3 / H: 5'-AATAGGAAGTATCATTCGGGTTTGATG-3' (Kocher et al., 1989) and 2) L: 5'-ATCCCATTCCACCATACTACTC-3' / H: 5'-ATGAAGGGATGTTCTACTGGTTG-3' (Edwards et al., 1991), at PCR condition: 1 cycle of 94 º C for 5 min, continued with 35

cyclus of (94 º C for 1 min, 52 º C for 1 min, 72 º C for 2 min) and finished by 72 º C for 10 min. PCR products were separated at 2 % of agarose by using elctrophoresis and then soaked in the ethidium bromide solution, then visualized under UV polaroid camera. Each primer pair amplified one DNA fragment, clearly. Two cyt-b fragments were around 760-bp and 370-bp.

PCR products were purified using QIAquick (Qiagen) and then used as a DNA template for DNA sequencing process. Each PCR product was then sequenced in an automatic ABI Prism 3100 DNA Sequencer. Each PCR product was sequenced from both direction (forward and reverse) to validate the results.

Cyt-b gene sequences analyses.

The aligned cyt b gene sequences were subjected to analyses of haplotype and nucleotide diversities, gene variation, and sequence divergence at the individual level. First, the individual variability within each of the mitochondrial polymorphic sites was used to define sequence divergence between all haplotypes, where haplotypic divergence was calculated as the number of mutational differences between haplotype sequences.

DNA sequences were aligned using BioEdit (Hall, 1999). Nucleotide composition and genetic distance were analyzed using MEGA 3 (Kumar et al., 2004). Haplotype and nucleotide diversity, and other genetic diversity characters were calculated with DnaSP (Rozas and Rozas, 1999) and MEGA 3 (Kumar et al., 2004). Neigbor-joining tree was performated by MEGA3 and the bootstrap values were consensus inferred from 1000 replicates (Felsenstein, 1985). Estimates of average evolutionary divergence over all sequence pairs were computed by averaging the number of base substitutions per site. *Cactua alba* was used as an outgroup species in constracting neighbor-joining tree.

RESULTS

In all sequences data of 18 birds analyzed, there were no stop codons. In the 800-bp of cyt-b contained 26.00 % of adenine, 25.30 % of tymine, 14.50 % guanine, and 34.30 % of cytocine. First and thirs codon positions were dominated by cytocine, while second codon position was dominated by tymine, and guanine was the lowest (6.61 %) at third codon position.

Genetic distances were calculated based on Kimura 2-parameter model. Pair wise distances were presented in Table 1. Overall mean genetic distance from entire 18 birds was 0.008 ± 0.001 (0.002 to 0.015).

Nucleotides variation, variable sites and type of haplotypes were presented in Tabel 2. Tweenty variable sites, covering 2.50 % of the entire length of the sequence were observed, and occurred at 3, 28, 39, 117, 145, 159, 186, 216, 246, 247, 345, 432, 459, 489, 600, 624, 636, 668, 694, 738 site positions. From these variable sites defined 18 haplotypes (Hcg1-Hcg18). There was no dominant haplotype. The values of ucleotide diversity (Pi) and haplotype diversity (Hd) estimated from mtDNA cyt-b in the 18 individuals were 0.005437 and 1.0000, respectively. Among nucleotide pair frequencies; there six transitions and only two transversions substitution were found.

Clustering was performed based on neighbor joining (NJ) analysis with Kimura 2-parameters model, and NJ tree was presented in Figure 1. According to the NJ phylogenetic tree, 18 individuals of cockatoos were divided into five clades: 1) First clade contained 4 individuals (MZB1-MZB7, MZB2-MZB14), 2) second clade contained 3 individuals (MZB8, MZB9-MZB40), 3) third clade contained 4 individuals (MZB12, MZB15, MZB6-TMR41), 4) fourth clade contained 3 individual (TMI1, MZB3, MZB11), and 5) fifth clade contained 3 individuals bird (MZB10, MZB4, MZB5).

ISSN: 2088-9771

Table 2: The numbers and the position of nucleotide variable sites in the 800-bp of mitochondrial cyt-b sequences.

		Nucleotide variable sites																				
samples code	species	3	8	39	117	145	159	186	216	246	247	345	432	459	589	600	624	636	668	694	738	Haplotype
MZB1	*Cacatua.goffini*	A	A	C	C	A	T	T	A	A	A	G	C	A	C	C	A	C	C	C	G	Hcg1
MZB2	*C. goffini*	.	.	.	T	C	.	C	.	.	.	A	.	C	.	.	.	.	.	.	.	Hcg2
MZB3	*C. goffini*	.	.	.	.	.	.	.	.	.	T	A	.	C	.	.	.	.	.	.	.	Hcg3
MZB4	*C. goffini*	.	.	.	.	.	.	.	.	.	.	A	.	C	.	.	G	.	.	.	.	Hcg4
MZB5	*C. goffini*	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	G	.	.	.	.	Hcg5
MZB6	*C. goffini*	.	.	T	.	.	.	.	.	T	.	A	T	C	.	.	.	.	T	G	.	Hcg6
MZB7	*C. goffini*	.	.	.	T	.	C	.	.	.	.	A	.	C	.	T	.	.	.	.	.	Hcg7
MZB8	*C. goffini*	G	.	.	.	.	.	.	.	.	.	A	.	C	T	.	.	.	.	.	.	Hcg8
MZB9	*C. goffini*	.	.	.	.	.	C	.	.	.	.	A	.	C	.	.	.	.	.	.	.	Hcg9
MZB10	*C. goffini*	.	.	.	.	.	.	.	.	.	.	A	.	C	.	.	.	.	.	G	.	Hcg10
MZB11	*C. goffini*	.	.	.	.	.	.	.	.	.	.	A	T	C	.	.	G	.	.	G	.	Hcg11
MZB12	*C. goffini*	.	.	.	.	.	.	.	G	.	.	A	.	C	.	T	.	.	T	.	A	Hcg12
MZB13	*C. goffini*	.	.	.	.	.	.	.	.	.	.	A	.	.	T	.	.	T	.	.	.	Hcg13
MZB14	*C. goffini*	.	.	.	T	.	C	C	.	.	.	A	.	C	.	.	.	.	.	.	.	Hcg14
MZB15	*C. goffini*	.	.	T	.	.	.	.	.	.	.	A	.	C	.	.	.	.	.	.	.	Hcg15
TMI1	*C. goffini*	.	.	.	.	.	.	.	.	.	.	A	.	C	.	.	.	T	.	.	.	Hcg16
TMR40	*C. goffini*	G	G	.	.	.	.	.	.	.	T	A	.	C	.	.	.	.	.	.	.	Hcg17
TMR41	*C. goffini*	.	.	T	.	C	.	.	.	.	.	A	.	C	.	.	.	.	.	.	.	Hcg18

ISSN: 2088-9771

Table 1: Kimura 2-parameter distances among individuals of Goffin's Cockatoo (*Cacatua goffini*) inferred from 800-bp of cyt-b gene sequences

No.	sample code	species name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	MZB1	*Cacatua goffini*																	
2	MZB2	*C. goffini*	0.01																
3	MZB3	*C. goffini*	0.007	0.008															
4	MZB4	*C. goffini*	0.01	0.011	0.008														
5	MZB5	*C. goffini*	0.006	0.009	0.006	0.007													
6	MZB6	*C. goffini*	0.012	0.013	0.01	0.013	0.011												
7	MZB7	*C. goffini*	0.006	0.009	0.008	0.011	0.009	0.013											
8	MZB8	*C. goffini*	0.009	0.01	0.004	0.01	0.008	0.012	0.01										
9	MZB9	*C. goffini*	0.008	0.009	0.006	0.007	0.007	0.011	0.007	0.008									
10	MZB10	*C. goffini*	0.007	0.008	0.004	0.006	0.003	0.008	0.008	0.007	0.006								
11	MZB11	*C. goffini*	0.011	0.01	0.007	0.01	0.01	0.01	0.012	0.011	0.01	0.007							
12	MZB12	*C. goffini*	0.007	0.008	0.004	0.008	0.006	0.008	0.006	0.007	0.006	0.004	0.009						
13	MZB13	*C. goffini*	0.011	0.015	0.011	0.008	0.008	0.015	0.015	0.011	0.012	0.011	0.013	0.011					
14	MZB14	*C. goffini*	0.009	0.006	0.007	0.01	0.008	0.012	0.006	0.007	0.006	0.007	0.011	0.007	0.013				
15	MZB15	*C. goffini*	0.006	0.007	0.003	0.007	0.004	0.007	0.006	0.004	0.003	0.008	0.003	0.01	0.006	0.006			
16	TMI1	*C. goffini*	0.008	0.007	0.006	0.009	0.007	0.009	0.008	0.007	0.006	0.01	0.006	0.012	0.008	0.008	0.002		
17	TMR40	*C. goffini*	0.008	0.007	0.006	0.009	0.007	0.009	0.006	0.004	0.006	0.01	0.006	0.012	0.008	0.008	0.004	0.007	
18	TMR41	*C. goffini*	0.009	0.012	0.004	0.01	0.01	0.01	0.007	0.01	0.009	0.011	0.009	0.01	0.011	0.011	0.008	0.01	0.008

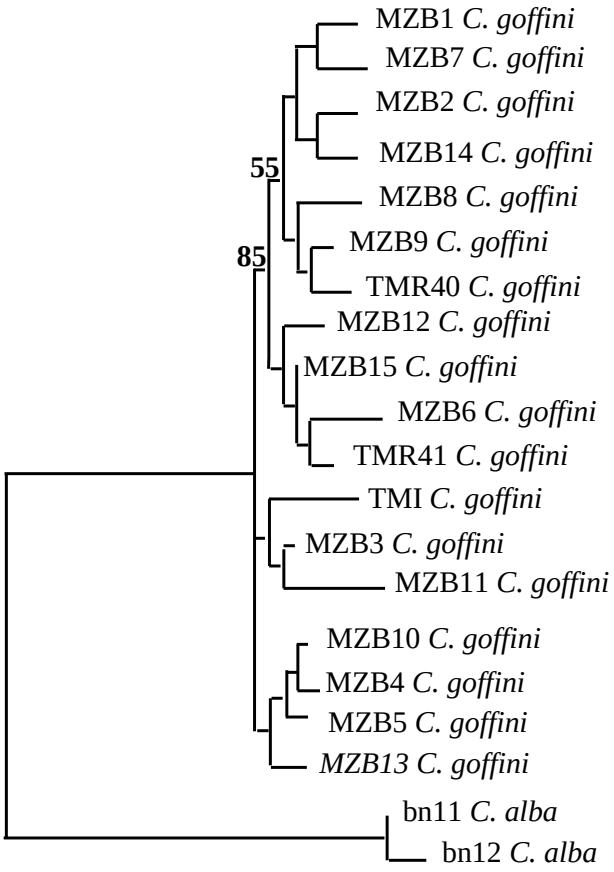

Figure 1: A neighbor-joining (NJ) tree constructed from 800-bp of cyt-b sequences based on Kimura 2-parameter model. Numbers above indicated bootstrap values > 50 %. *Cacatua alba* is outgroup species.

DISCUSSION

Pattern of base composition in the cyt-b sequences of Goffin's cockatoo (C. goffini was similar to that found in previous others avian studies (e.g: Miyaki et al., 1998; Zalzburger et al., 2002; Barhoum and Burns 2002; Perez-Eman, 2005; Astuti et al., 2006; Pan et al., 2007) and others animal taxa (e.g. Abol-Munafi et al, 2007) in that cyt b sequences is rich in cytosine and adenine. Lowest guanine compositions at third codon position occurred in DNA sequences of cytb gene of Goffin's cockatoo are commonly found in the DNA, particularly in protein-coding genes in the mitochondrial DNA such as ND2 (Zuccon, D. *et al*. 2006), cyt-b (Astuti, D. et al., 2006), and also on COI in other bird groups (Weibel and Moore 2002).

Our result showed that overall mean distance among Goffin's cockatoos was 0.08 (0.002- 0.015). In others birds species, the overall mean distance in Oryx found to be 0.140 (Khan HA et al., 2008), sequence divergence among individual within a species was 0 % (*Parus dichrous*) until 2.8 % (*Aegithalos concinnus)* of cyt b (Lijtmaer et al., 2004), and intraspecific divergence in a same population was only 1 %, and 0.25 % to 8 % sequence divergence for individuals at different population (Dai et al., 2010).

According to Liu RY et al. (2006), haplotype diversity and nucleotide diversity of mtDNA are the important indices for assessing population polymorphism and genetic differentiation. The value of haplotype diversity and nucleotide diversity of mtDNA are bigger, the population polymorphism is higher.

Our results showed that cyt b in Goffin's cockatoo exhibited a low nucleotide diversity (Pi) = 0.005. In contrast to its low nucleotide diversity, haplotype diversity was relatively high (Hd = 1.0000). Low Nucleotide diversity and high haplotype diversity were also occurred in other birds *Alectoris chukar* (Germin et al., 2007) and Taiko (Lawrence & Tailor, 2008), and other animal taxa Neotropical otter (*Lontra longicaudis*) (Trinca et al. 2007) and *Lontra felina* (Valqui et al., 2010), black muntjac (Ran J. et al., 2008). LI XL et al. (1999) revealed that the average nucleotide diversity was 0.0487%, suggesting the poor genetic diversity and lower differentiation level in Chinese goats. In small populations, the individuals are likely to be genetically, anatomically, and physiologically more homogeneous than in larger populations and less able to adapt to different environmental conditions. The wild population exhibited a low nucleotide diversity (pi = 0.00562), which suggests that the black muntjac had a small effective population size historically (Ran J. et al., 2008). While, high value of haplotype diversity (0.9320), indicating high genetic diversity (Wu and Fang, 2005). Relatively high genetic diversity in a rare species can indicate that decline in numbers is recent (Moritz 1994).

From NJ tree in the present study (Figure 1), there were some clades, but each clade did not supported by bootstrap value. In this case, bootstrap values only support relationships between one clade to another clade ; for instance first clade and second clade clustered together and supported by 55 %, and third clade with first and second clades was supported by 80 % bootstrap values. Because of blood samples in the present study were taken from cockatoo birds living in the cages; not from original habitat in the nature, so I only estimate that individual birds in the same clade might be come from the same population or location in the nature.

ACKNOWLEDGEMENTS

This work was partially supported by JICA & JSPS Core University Program (Hokkaido University-Indonesian Institute of Sciences). Thank to members of Laboratory of Genetics, Division of Zoology, R.C for Biology- Indonesian Institute for Sciences for their kind help. Thank to reviewers for helpful comments.

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GENETIC DIVERSITY OF STEVIA (*Stevia rebaudiana* (Bertoni) Bertoni) BASED ON MOLECULAR CHARACTERS

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ABSTRACT

Stevia (*Stevia rebaudiana* (Bertoni) Bertoni) produces stevioside that may be up 300 times sweeter than sugar and used as non caloric natural sweetener. Medicinal Plant and Traditional Medicine Research and Development Office has 6 accessions of stevia: Jumbo Ungu, Jumbo Putih, Super Hijau, Super Kuning, Keriting, and Mini. The aim of this study was to assessed variation of stevia based on molecular marker ISSR (*Inter Simple Sequence Repeats*). Seven ISSR primers generated 60 total DNA bands which were scored for presence (1) or absent (0). Similarity matrix was calculated using Dice coefficient. *Unweighted Pair Group Method Using Arithmetic Mean* (UPGMA) cluster analysis was performed to develop a dendrogram. This data analysis was performed by NTSYS software ver. 2.02. This research revealed that six accessions divided into two clusters with similarity coefficient 76,1%, Super Kuning and Keriting were the most closely similar (85,4%) based on molecular characters.

Key words: Stevia (*Stevia rebaudiana* (Bertoni) Bertoni), ISSR *(Inter Simple Sequence Repeats)*

INTRODUCTION

Stevia rebaudiana Bertoni is a plant originated in Paraguay and northeast Brazil (Andolfi et al., 2006). It is a small hurb perennially growing up to 65 cm tall, with sessile, oppositely arranged lanceolate to oblanceolate leaves, serrated above the middle (Sairkar *et al.*, 2009).

The leaves of the stevia shrub contain specific glycosides which produce a sweet taste but have no caloric value. Stevioside is the primary glycoside involved in this effect. Dulcoside and rebaudioside are also major glycosides contained in the herb (Elkins, 1999). The extracts of stevioside may be up to 300 times sweeter than sugar. Stevia can be used for many purposes; as medicinal plants and sweeteners (Heikal *et al*., 2008).

Medicinal Plant and Traditional Medicine Research and Development Office (B2P2TO-OT) has 6 accessions of stevia: Jumbo Ungu, Jumbo Putih, Super Hijau, Super Kuning, Keriting, and Mini based on morphological characters (Sugiarso, 2006).

Molecular markers have been shown to be useful for diversity assessment in a number of plant species. These markers, based on the polymerase chain reaction (PCR) technique, are the most commonly used for this purpose. Several molecular techniques are available for detecting genetic differences within and among cultivars (Heikal *et al*., 2008). The commonly used polymerase chain reaction (PCR)-based DNA marker systems are random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and more recently, simple sequence repeats (SSRs) or microsatellites (Reddy *et al*., 2002). The major limitations of these methods are low reproducibility of RAPD, high cost of amplified fragment length polymorphism (AFLP) and the need to know the flanking sequences to

develop species specific primers for SSR polymorphism, ISSR-PCR (Inter Simple Sequence Repeats) is a technique that overcomes most of these limitations (Jabbarzadech *et al*., 2010).

ISSR is a dominant marker like RAPD (scored using presence or absence of band at a locus) but with greater robustness in repeatability and extremely high variability. These features make ISSR better than other readily available marker systems in investigating the genetic variation among very closely related individuals and in crop cultivar classification (Dje *et al*., 2006).

The aim of this study was to assessed variation of stevia based on molecular marker ISSR (*Inter Simple Sequence Repeats*).

MATERIAL AND METHODS

Plant Material

Six accessions of Stevia in B2P2TO-OT (Jumbo Ungu, Jumbo Putih, Super Hijau, Super Kuning, Keriting, and Mini) were used in this study. Fresh and young leaf samples were collected to isolate genomic DNA

Methods

Genomic DNA Extraction

About 0.1 mg fresh leaf was grind for DNA extraction. Total DNA was extracted using a modified CTAB procedure.

ISSR Analysis

Seven ISSR primers {$(CT)_8$GC/ISSR 844B, $(CA)_6$AC/ISSR 17898A, $(GA)_6$CC/ISSR HB 10, $(CA)_6$GT/ISSR 17898B, $(GT)_6$CC/ISSR HB 11, $(CAC)_3$CC/ISSR HB 12 dan $(GTG)_3$GC/ISSR HB 15} were used in this study. Amplification was carried out in a 25 µL reaction consisting of 2 µl (20 ng) of template DNA, 12.6 µl Go Taq Green (Promega), 0,6 µl primer (100 µM), and 9.8 µl free nuclease water. DNA was amplified using C 1000 Bio-Rad Thermocycler. Amplification was performed under the following conditions: 5 min at 94°C for 1 cycle, followed by 1 min at 94°C, 45 s at annealing temperature (depend on primer used), and 2 min at 72°C for 40 cycles, and 8 min at 72°C for final extension.

PCR products were electrophoresed on 1,8% (w/v) agarose gel in 1X TBE buffer at 70 V or 90-110 min depending on the size on amplified fragment from each primer. The gel was stained in 0,01% ethidium bromide for 20 min and visualized with Gel Documentation (Bio-Rad).

Data Analysis

Each DNA fragment generated was treated as a separate character and scored as a discrete variable, using 1 to indicate presence and 0 for absence for each primer. Similarity matrix was calculated using Dice coefficient. *Unweighted Pair Group Method Using Arithmetic Mean* (UPGMA) cluster analysis was performed to develop a dendrogram. This data analysis was performed by NTSYS software ver. 2.02.

RESULTS AND DISCUSSION

Seven primers resulted in the amplification of 60 fragments, of which 36 fragments (60%) were polymorphic while 14 fragments (40%) were monomorphic, indicating the presence of a high degree of genetic variation in the studied accessions. The mean number of amplified bands per primer was eight, ranged in size 100 bp-1369 bp. From the 7 primers tested based on Heikal's *et al*. (2008) research on stevia accessions, two primers (17898B and

HB 11) were monomorphic and five others were polymorphic. The highest number of polymorphic fragments was detected by primer 17898A (90%) (Table 1).

Table 1. The amplification products by 7 ISSR primers in 6 stevia accessions

No	*Oligo Name* (Primer)	*Sequence*	Total fragments	Monomorphic fragments	Polymorphic fragments	Polymorphism (%)
1	ISSR 844B	$(CT)_8GC$	9	3	6	66,7
2	ISSR 17898A	$(CA)_6AC$	10	1	9	90
3	ISSR 17898B	$(CA)_6GT$	4	4	0	0
4	ISSR HB-10	$(GA)_6CC$	9	4	5	55,6
5	ISSR HB-11	$(GT)_6CC$	7	7	0	0
6	ISSR HB-12	$(CAC)_3CC$	7	1	6	85,7
7	ISSR HB-15	$(GTG)_3GC$	14	4	10	71,4
Total			**60**	**24**	**36**	
Average			8,6	3,4	5,2	**60**

Primers 17898B and HB 11 were not suitable to analize genetic diversity of stevia accession in B2P2TO-OT. Gonzalez *et al*. (2005) reported that the level polymorphism of ISSR is dependent on the plant species and the type of simple sequence repeat incorporated in the ISSR primer used. In most genetic diversity analyses, selection of suitable primers is a very important step for the PCR reaction. Needed properties for suitable ISSR primers are that then be able to give highly polymorphic PCR products, high reproducibility and are more informative (Denduangboripant *et al*., 2010).

ISSR Primer 844B amplified 9 fragments in a range from 253 bp to 1333 bp (Figure 1). Two specific markers were produced with size 631 bp in stevia Super Kuning and 1333 bp in stevia Keriting by using this primer.

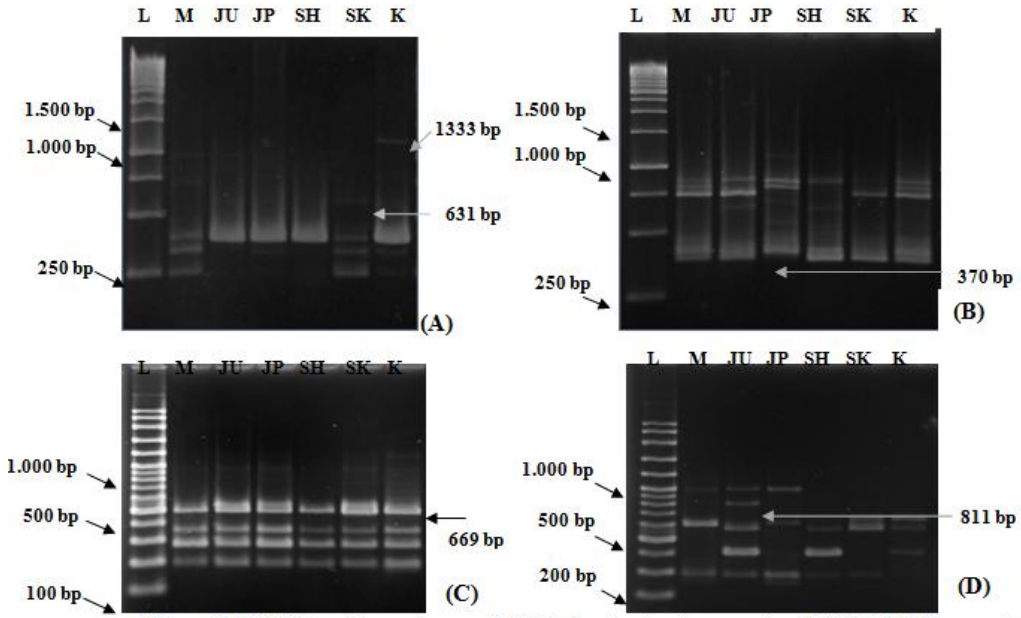

Figure 1. ISSR banding patterns of DNA for six stevia accessions B2P2TO-OT generated by ISSR primer 844B (A); 17898A (B); ISSR HB 10 (C); HB 12 (D) (L: DNA Ladder 1 kb & 100 bp; M: Mini; JU: Jumbo Ungu; JP: Jumbo Putih; SH: Super Hijau; SK: Super Kuning; K: Keriting)

ISSR primer 17898A generated 10 DNA fragments ranged in size 370 bp-1206 bp. DNA fragment 370 bp was not found in stevia Jumbo Putih, mean negative specific marker to identify this accession (Figure 2B) if amplified using primer 17898A. ISSR primer HB-10 generated 9 DNA fragments ranged in size 169 bp-1369 bp. DNA fragment 669 bp was only found in stevia Keriting, mean positive specific marker to identify this accession (Figure 2C) if amplified using primer HB-10.

The results also show that six stevia accessions had differences banding patterns and between accessions got same patterns, mean accessions share same genotype. Differences primers produces differences fragments pattern, this is because of the differences of sequences of the primers (Hasan et al., 2010)

The fragments data was also used to study the genetic diversity among accessions, a similarity matrix was generated using Dice's similarity coefficient leading to preparation of dendrogram constructed by UPGMA cluster analysis (Figure 3).

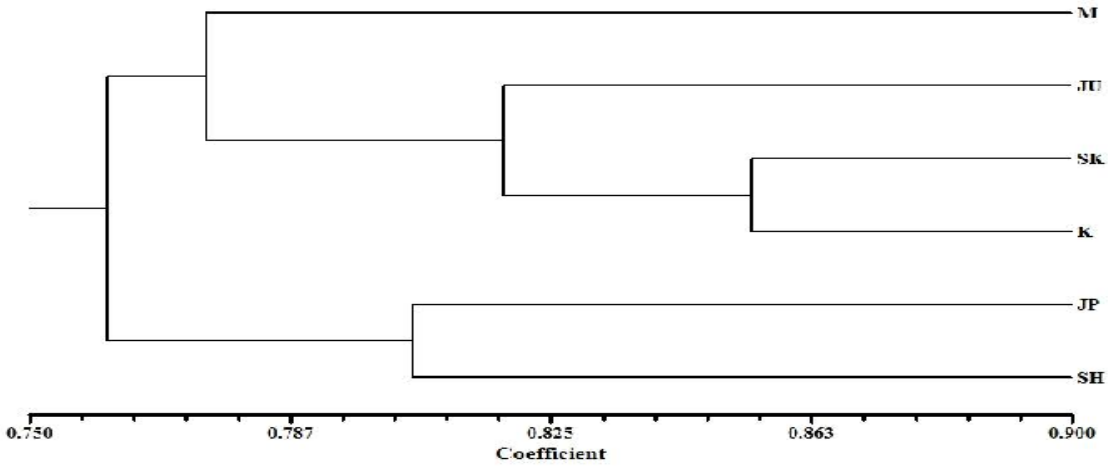

Figure 3. Dendogram of 6 stevia accessions (*Stevia rebaudiana* (Bertoni) Bertoni) in B2P2TO-OT based on ISSR analysis (M: Mini; JU: Jumbo Ungu; JP: Jumbo Putih; SH: Super Hijau; SK: Super Kuning; K: Keriting)

Based on dendogram (Figure 3), 6 stevia accessions can be separated in to two main clusters with similarity coefficient 76,1%. Cluster I consists of 2 stevia accessions (Jumbo Putih and Super Hijau), Cluster II consists of 4 stevia accessions (Jumbo Ungu, Super Kuning, Keriting and Mini). Stevia Super Kuning and Keriting were the most closely similar (85,4%) based on ISSR analysis. The similarity indices show the relationship of the individual in each sample. Higher similarity indices suggest that the individuals in the population have closer genetic relation among them, while lower similarity indices suggest that individuals in the population have farther genetics relation (Hasan *et al.*, 2010)

Plant groups for which all OTUs have similarities between 85%–100% might be recognized as part of the same species, while a 65% criterion might be used for genera. However, the ultimate interpretation of the dendrogram is dependent upon the taxonomist's knowledge of the operational taxonomic units (Chaveerach *et al.*, 2008).

In conclusion, six stevia accessions in Medicinal Plant and Traditional Medicine Research and Development Office (B2P2TO-OT) had genetic diversity based on ISSR molecular marker. This research also demonstrate that ISSR markers were potential to identify and discriminate accessions specific marker of 4 stevia accessions in B2P2TO-OT. ISSR markers were chosen because the technique is very simple, cost effective, highly

discriminative, reliable and require small quantity of sample DNA, also do not need any prior primer sequence information.

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Isolation, Fermentation and Antidiabetic Endophytic Fungi A.AP.3F of the Stem Sambiloto Plant *(Andrographys paniculata Ness)*

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Abstract

Isolation ,fermentation and test antidiabetic A.Ap.3F of endophytic fungi from plant stems Sambiloto*(Andrographys paniculata Ness*) have been done. The isolation of endophytic fungi was done directly from the stem part of Sambiloto . Identification of endophytic fungi A.Ap.3F done microscopically. Fermentation is carried out using 2 methods; static fermentation and dynamic fermentation; fermentation was conducted over 14 days by using the media *Potato Dextrose Broth* (PDB).The product of fermentation process was extracted with ethyl acetate. The antidiabetic assay was performed using α-glucosidase. The results shown that with static fermentation method produced 0.19 g (9.5%) of mass of filtrate and biomass weight 0.56 g(28%); and with dynamic fermentation produced mass of filtrate 0.68 g (34.17%) and biomass weight 0.77 g (38.50%). The filtrate of endophytic fungi showed antidiabetic activity against A.Ap.3f endophytic fungus filtrate against α-glucosidase by 81.40% and for A.Ap.3F biomass by 98.84%. Microscopic identification A.Ap.3F endophytic fungi included in the class *zygomycetes.*

Keywords: *Andrographys paniculata* Ness, fermentation, *Potato Dextrose Broth*, α-glucosidase

INTRODUCTION

Endophytes are microorganisms that include bacteria and fungi living within plant tissues without causing any immediate overt negative effects have been found in every plant species examined to date and recognized as the potential sources of novel natural products for exploitation in medicine, agriculture and industry with more bioactive natural products isolated from the microorganisms (Strobel G.A and Bryn D, 2003)

Endophytic fungi is one of potential natural resources for new antidiabetic compound sources. Endophytic microbes are bacteria including *Actinomycetes*, or fungi which spend part or whole of its lifespan inside intra- or intercellular tissue of its healthy host without giving any symptoms (Tan and Zou, 2001).

Andrographys paniculata Ness is an herbaceus plant, commonly as a King bitter, in the family Acanthaceae. It is widely cultivated in southern Asia. Mostly the leaves and roots have been traditionally used over the centuries for different medicinal purpose in Asia and Europe as a folklore remedy for a wide spectrum of ailments or as an supplement for health promotion (Kanokwan Jarukmajorn, *et al*., 2008).

This study was aim to isolate and test the antidiabetic activity of endophyt fungi from stem of sambiloto plants and to identify and production endophytic fungi A.Ap.3F with static and dynamic fermentation process.

MATERIAL AND METHOD

Research Material

Research materials are stem of Sambiloto plants, collected from Bogor areas. This research was conducted during December 2009-October 2010.

Isolation and Purification of Endophytic Microbes

Plant's stem in 2 centimeter for length and 1 centimeter for diameter were washed by water followed by sterilization using ethanol 70 % for 1 minute and ethanol 75 % for 30 seconds. Stems were cut longitudinally into 2 pieces then were placed on petri disc filled with Corn Meal Malt Agar (CMMA) mixed with 0.05 mg/ml chloramphenicol (Theantana, 2007). Others were placed on Potato Dextrose Agar (PDA) mixed with 0.05 mg/ml chloramphenicol (Croizer, 2006). Those agar were incubated at 25 ºC for 3 days. Purification were done by transferring colony to fresh PDA followed by incubation at 25 ºC for few times until we got pure colony. Pure colony are kept on slanted PDA in the refrigerator (-80 ºC) for further treatment.

In vitro Antidiabetic Activity Test of Active Compounds

In vitro antidiabetic activity test were done using α-glucosidase method (Saijyo *et al*, 2008). An amount of 1 mg α-glucosidase were dissolved into 100 ml phosphate buffer (pH 7). 1 ml of enzyme solution were then diluted into 50 ml phosphate buffer before testing. 250 µl of 20 mM-pnp-α-D-glucopyranoside, 475 µl of 100 mM phosphate buffer and 25µl of sample solution were dissolved in DMSO. After homogenization, the solution were incubated at 37 ºC for 5 minutes. 250µl of α-glucosidase enzyme solution were added and incubation were continued for 15 minutes. Reaction were stopped by adding 1 ml of 0.2 M Na_2CO_3. Quantity of p-nitrophenol released were measured by spectrophotometer at χ= 400 nm. Inhibition capability were calculated using formula {(C-S)/C}x100. S showed sample absorbance and C showed blank absorbance with quercetin solution as standard.

Fermentation of Endopytic fungi A.Ap.3F with dynamic method.

Fermentation of endophytic fungi A.Ap.3F used dynamic fermentation method is done by cutting 1 week old endophytic microbe colony in medium agar using cork borer tobe inoculum disc with diameter of 1.8 centimeter. Inoculum was inoculated into 250 mL Potato Dextrose Broth (PDB) medium in Erlenmeyer flask size 1000 ml, fermentation done by using the 2L PDB and fermented for 14 days with agitation 150 rpm at room temperature (Onifade, 2007). Fermented fungi were extracted using ethylacetate and filtrated using Whatman filter paper number 41 until we got filtrate and biomass of fungi. Filtrate collected were evaporated using evaporator and were dried using oven. Dried biomass were extracted again using ethylacetate to get the extract (Sunil, *et al*., 2009).

Fermentation of Endopytic fungi A.Ap.3F with static method.

Static fermentation method done the same as dynamic fermentation method, the difference in static fermentation done without agitation.

Identification of Endophytic Fungi A.Ap.3F

Endophytic fungi that has been isolated and identified based on the Barnett guidelines (Barnet.,1955).Identification of the endophytic fungi is done by observing several morphological characters, both macroscopically and microscopically.

RESULTS

Isolation of Endophytic Fungi

Isolation and purification of endophytic fungi from Sambiloto (*Andrographis paniculata* Ness), gave results 9 fungi isolates. Complete results of isolation can be seen on Table 1.

Screening of Endophytic Fungi Isolates Using α-Glucosidase Test

The results of the screening were summarized in Table 2.

Identification of Endophytic Fungi A.Ap.3F

The macroscopic characterization of endofit A.Ap.3F obtained identification results as follows;

1. Forward side :
    - The color of the colony: - old hyphae: brown and young hyphae: white.
    - Textures : smooth
    - Dametre : 5,5 Cm
    - (+) Zoning
    - (-)Radial lines
    - (+)Concentric lines

    Shown in Figure.1A.
2. Back side :
    - The color of the colony: - old hyphae: brown and young hyphae: white.
    - Textures : smooth
    - Diameter : 5,5 Cm
    - (+) Zoning
    - (-)Radial lines
    - (+)Concentric lines

    Shown in Figure.1B.

The microscopic characterization of endofit A.Ap.3F obtained identification results as follows;

- hyphae: hyaline without septa
- (+)relations crab
- (+)Spore
- (+)Stem spores

Microscopic identification A.Ap.3F endophytic fungi included in the class *zygomycetes.*
The results of the microscopic identification were shown in Figure 2.

Production of Endophytic Fungi A.Ap.3F large scale using static and dynamic fermentation methods.

The production of endophytic fungi A.Ap.3F using dynamic and static fermentation method carried out in 2L Potato Dextrose Broth (PDB) solution and incubated for 14 days, shown in Table.3

DISCUSSION

α-glucosidase is an enzyme in intestine which can hydrolyze carbohydrate into simple sugar (glucose). Compound which can inhibit this enzyme activity will be very potential to be

an antidiabetic drug since it can decrease blood sugar level by slowing the absorption of carbohydrate *post-prandial* (Suarsana *et al.*, 2008).

Screening was done to get isolates which can produce a chemical compound with inhibitory activity to α-glucosidase enzyme. In this screening we were having test to all ethylacetate extracts of endophytic fungi (both filtrate and biomass extracts). As control we used quercetin, a well known flavonoid compound which have inhibitory activity to α-glucosidase enzyme. All samples and standard solution were measured at concentration of 50 ppm (Suarsana, *et al.,* 2008). In this test, we compared absorbancy of standard with samples. DMSO solution was used as blank solution while quercetin as standard. Absorbancy value of DMSO solution 0.1183 and absorbancy value of quercetin solution 0.1007. So inhibition percentage standard 14.88%.

Inhibition values against α-glucosidase enzyme of biomass extract from endophytic fungi A.Ap.3F ranged between 30.94-98.84% and inhibition values of filtrate extract ranged from 20.29-87.49%. Inhibition values of biomass extract greater than inhibition values of filtrate extract because the endophitic fungi A.Ap.3F produced more secondary metabolites in the intra cellular then extra cellular.

The results shown that with static fermentation method produced 0.19 g (9.5%) of mass of filtrate and biomass weight 0.56 g(28%); and with dynamic fermentation produced mass of filtrate 0.68 g (34.17%) and biomass weight 0.77 g (38.50%).

Production of endophytic fungi A.Ap3F using dynamic fermentation method greater than using static fermentation method. This is due to the agitation makes all nutrients in the medium ingredients evenly so that maximal metabolic formation of secondary metabolites.

CONCLUSION

Isolation and purification of endophytic fungi of Sambiloto plant obtain 9 fungi isolates. The results shown that with static fermentation method produced 0.19 g (9.5%) of mass of filtrate and biomass weight 0.56 g(28%); and with dynamic fermentation produced mass of filtrate 0.68 g (34.17%) and biomass weight 0.77 g (38.50%). The filtrate of endophytic fungi showed antidiabetic activity against A.Ap.3f endophytic fungus filtrate against α-glucosidase by 81.40% and for A.Ap.3F biomass by 98.84%. Microscopic identification A.Ap.3F endophytic fungi included in the class *zygomycetes.*

ACKNOWLEDGEMENT

The writer would like to thanks Research Center for Biotechnology Indonesian Institute of Sciences (LIPI) for their help in this research.

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Appendix

Table 1. Endophytic Fungi Isolated from Sambiloto Plants (*Andrographis paniculata* Ness)

No	Plant	Fungi Isolate	Ethyl acetate extract weight	
			Filtrate (g)	Biomass (g)
1	Sambiloto (*Andrographis paniculata* Ness)	A.Ap.1F	0,1300	0,0711
2	Sambiloto (*Andrographis paniculata* Ness)	A.Ap.2F	0,0675	0,0027
3	Sambiloto (*Andrographis paniculata* Ness)	A.Ap.3F	0,0919	0,0605
4	Sambiloto (*Andrographis paniculata* Ness)	A.Ap.4F	0,0750	0,0598
5	Sambiloto (*Andrographis paniculata* Ness)	B.Ap.1F	0,0649	0,0557
6	Sambiloto (*Andrographis paniculata* Ness)	B.Ap.2F	0,0683	0,1104
7	Sambiloto (*Andrographis paniculata* Ness)	B.Ap.3F	0,1022	0,0602
8	Sambiloto (*Andrographis paniculata* Ness)	B.Ap.4F	0,0905	0,0681
9	Sambiloto (*Andrographis paniculata* Ness)	B.Ap.5F	0,0967	0,0634

(Edward J.Dompeipen, *et al*, 2011)

Notation "A" is refers to endophytic fungi isolates planted on CMM (Corn Meal Medium), Notation "B" is refers to endophytic fungi isolates planted on PDA (Potato Dextrose Agar),Ap are code name for plant's botanical name.

Table 2. The results of the screening of endophytic fungi isolates using α-glucosidase test

No	**Plant**	**Fumgi Isolates**	**Absorbans**		**α-Glucosidase (100%)**	
			Filtrate	**Biomass**	**Filtrate**	**Biomass**
1	Sambiloto (*Andrographis paniculata* Ness)	**A.Ap.1F**	**0,0250**	**0,0225**	**78,87**	**80,98**
2	Sambiloto (*Andrographis paniculata* Ness)	**A.Ap.2F**	**0,0934**	**0,0817**	**20,29**	**30,94**
3	Sambiloto (*Andrographis paniculata* Ness)	**A.Ap.3F**	**0,0220**	**0,0018**	**81,40**	**98,84**
4	Sambiloto (*Andrographis paniculata* Ness)	**A.Ap.4F**	**0,0220**	**0,0037**	**81,40**	**96,87**
5	Sambiloto (*Andrographis paniculata* Ness)	**B.Ap.1F**	**0,0148**	**0,0018**	**87,49**	**98,48**
6	Sambiloto (*Andrographis paniculata* Ness)	**B.Ap.2F**	**0,0301**	**0,0092**	**74,56**	**92,22**
7	Sambiloto (*Andrographis paniculata* Ness)	**B.Ap.3F**	**0,0767**	**0,0206**	**35,16**	**82,59**
8	Sambiloto (*Andrographis paniculata* Ness)	**B.Ap.4F**	**0,0817**	**0,0111**	**30,94**	**90,62**
9	Sambiloto (*Andrographis paniculata* Ness)	**B.Ap.5F**	**0,0301**	**0,0206**	**74,56**	**82,59**

(Edward J.Dompeipen, *et al*, 2011)

Absorbancy of DMSO solution 0,118, absorbancy of quercetin solution 0,1007 :

$$\text{Inhibition percentage of sample (\%)} = \frac{DMSO - Sample}{DMSO} \times 100\ (\%)$$

Table.3. Production of endophytic fungi A.Ap.3F using dynamic and static fermentation method.

No	Ethylacetate Extract	Fermentation Method	
		Dinamic	Static
1	Filtrate	0,68 g (34,17%)	0,19 g (9,50%)
2	Biomass	0,77 g (38,50%)	0,56 g (28,0%)

1A 1B

Figure 1A and 1B .Forward side (1A)and back side (1B) of Endophytic Fungi A.Ap.3F

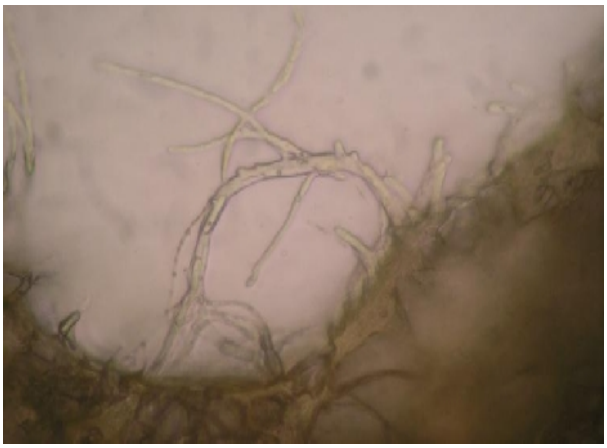

Figure 2. Microscopic picture of endophytic fungi A.Ap.3F

Identification of Genetic Markers Associated with Twinning Birth Trait in Cattle

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ABSTRACT

This research was aimed to identify the possibility of FH and Ongole descendant (PO) cows bearing the genetic markers associated with twinning birth trait. The research used 41 individual DNA samples collected from fresh blood that consisted of 31 FH and 10 PO and applied four genetic markers of AGLA254, BM8230, ETH-10 and IGF-1. Those DNA samples were amplified by polymerase chain reaction (PCR) in 30 cycles with annealing temperature at 58.9^{o}C for all primers. PCR products were visualized on 2% agarose gel. Therefore, it was obtained 164 fragments of those 41 DNA samples with applied 4 genetic markers. Results showed that all applied genetic markers emerged on the right sizes of 158-214bp (AGLA254); 212-224bp (ETH10) and 225-231bp (IGF-1); though BM8230 (103-106bp) in FH cows showed a bit blur band compared to other three genetic markers. This study concludes that all DNA samples bear those 4 genetic markers. It indicates that the used genetic markers are less specific for twinning birth trait and it seems that winning traits are influenced by multi genes.

Keywords: Genetic marker, twinning trait, cattle

INTRODUCTION

Up to 2009 Indonesia still imported 22.8% livestock, 14.4% frozen meat and 24.2% milk product of total import values derived from animal products (Livestock statistics, 2010). This reality of livestock and its products condition challenged researchers to create urgent research to solve our nation problem and to improve our local domestic animal production in order to fulfill the national needs in animal protein sector. Entering global market, Indonesia has to compete in providing foodstuff source especially from livestock sector (dairy and beef cattle) quickly both in term of quantity and quality. It is intended to release from dependency of imported values and to be a livestock producer in our own home country. Concerning to such crucial needs, Indonesia should improve local livestock production to accelerate in animal population with approach of sustainability strategy in order to achieve food safety national program from livestock sector in 2014.

To achieve national needs of animal production, it can be achieved through conventional and advanced technology. In the case of improving animal population, it can be accelerated through twinning production in livestock producers. Even though, selecting an increase rate of twinning has been debated by pessimistic and optimistic group. Based on success of selection for multiple births in sheep, it has initiated to be applied in cattle. It has been known that cattle are a type of uniparous animal where female animal only delivers a single birth in each calving. Twinning birth is naturally very rare accidence. It was reported that it is no more than 1% twinning birth in beef cattle (Komisarek and Dorynek, 2002). In dairy herd, the average of twinning rates is higher than in beef cattle (4-5%). Parity of twinning birth is influenced by age and calving of female where heifer is about 1% and

increased into 10% in older cows. Echternkamp (1992) stated that uterus capacity can be exploited to bear 3 fetuses in each uterus and even more. Twinning rate increases in the age period of 10-year cows (Berry *et al*., 1994; Cady and Van Vleck, 1978; Kinsel *et al*., 1998; Nielen *et al*, 1989; Ryan and Boland, 1991). In four-season country, twinning rate is influenced by the season and twinning birth tends to happen in spring associated with availability of feed (Cady and Van Vleck, 1978; Karlsen *et al*, 2000) or in fall (Gregory *et al*, 1990). The effect of season is naturally affected by temperature changes, the length of daylight and the availability of feed source when conception is termed (Komisarek and Dorynek, 2002).

Twinning birth trait can be sorted through genetic material. Genetic differences which distinguish one animal to another are encoded by an animal genetic material, termed as Deoxyribonucleic Acid (DNA). This DNA lies in a pair of chromosomes while gene encodes specific trait or character is a specific segment of each chromosome. Breakthrough in advanced technology especially in marker technology, quantitative traits associated with twinning birth can be sorted by applying of genetic markers. This genetic marker can inform the content of animal's genes. Marker-assisted selection (MAS) offers the advantage of reducing the number of years in selection. There are some genetic markers associated with twinning rate and higher ovulation rate. Those genetic markers lie on DNA genome and flank the desired genes. Therefore, when genetic markers associated with twining birth are obtained then can be used as genetic marker to select the cows with desired gene of twinning birth trait.

Selection of twinning trait was conducted both in sires and cows (Kappes *et al*, 2000). Twinning trait is indicated as complex traits. Linkage mapping of genes influencing complex traits is complicated in fact that genotype cannot be predicted from certainty of phenotype. It is due to other genes and environment effects influenced the phenotypes. Quantitative Trait Loci (QTL) for twinning trait is predicted at bovine chromosome 5 (Lien *et al*, 2000). Fine mapping of QTL combined with information of linkage analysis and Linkage Disequilibrium (LD) to calculate unknown gene background and cattle pedigree was conducted by Meuwissen *et al*. (2002). Their report showed that possibility of putative QTL for twinning trait is very small and lies throughout bovine chromosome 5 with region of <1cM in the middle of bovine chromosome 5. Detection of QTL associated with twinning trait was also investigated in daughters of Israeli FH cattle and reported that the QTL locates at bovine chromosome 1, 6, 7, 8, 14, 15 and 23 (Willer *et al*, 2008). QTL of twinning rate was also conducted in North American Holstein families (Cobanoglu *et al*, 2008).

The occurrence of twinning birth is also related with IGF1 analysis for candidate genes associated with twinning rate. Echternkamp *et al*. (1990) approved that twinning birth in cattle is associated with the increase of IGF-1 concentration both in serum and follicular fluid. This finding indicates that IGF-1 plays important role in folliculogenesis regulation and might be associated with multiple ovulations in cattle. Based on DNA analysis it was reported that twinning production in Chinese FH cattle chances up to 10% associating with a higher ovulation rate and locates at bovine chromosome 5, 7 and 19 (Chen *et al*., 2006). Kappes *et al*. (2000) and Allan *et al*. (2009) suspected the existence of QTL at BTA5 for a higher ovulation rate.

This research was therefore designed to identify genetic markers associated with twinning trait in cattle. This result would be useful for efficiency of breeding program since this finding in genetic markers could be as a clue in cow selection for twinning trait.

MATERIALS AND METHODS

DNA Collection. Blood of Fries Holstein (FH) and Ongole descendant (PO) cows were collected through tail base of cows into 10ml vacutainer tubes containing 15% ETDA.

Individual DNA was collected from white blood cells according a modified high salt method of Montgomery & Sise (1990). The DNA was colleted in TE Buffer with pH 8 and stored at -20^{o}C.

Polymerase Chain Reaction (PCR). A commercial PCR kit of DreamTaq^{Tm} Green PCR Master mix (Fermentas) was used for amplification of individual DNA samples. Composition of the PCR reaction was 50ng/µl DNA template, 20pmol for each forward and reverse primer of AGLA254, BM8230, ETH10 and IGF-1. PCR program was set up for 35 cycles with initial denaturation at 94^{o}C for 3 min, then followed a series of denaturation, annealing and elongation at 94^{o}C for 1 min, 50-60^{o}C for 1 min and 72^{o}C for 1 min, respectively, ended with 72^{o}C for 7 min and stand by at 4^{o}C. Each primer has specific annealing temperature therefore annealing temperature was within 50 to 60^{o}C. PCR optimization was conducted in advance for each primer to obtain annealing temperature for PCR work

Visualization of PCR Products. Visualization was conducted by electrophoresis on 2% agarose gel in 1xTBE buffer and run by 100V electric current for 60 min then stained in 1µg/ml Ethidium Bromida solution for 15 min. The stained gel was then documented by gel doc system with underneath ultraviolet exposure. The emerged bands were analyzed for their sizes based on the primer used.

RESULTS

Annealing Temperature. Four genetic markers of AGLA254, BM8230, ETH10 and IGF-1 used for twinning identification were listed and presented with base sequences (Table 1). In prior to PCR works, all primers were optimized at three different annealing temperatures (57.6, 58.9, 59.7^{o}C) using one DNA sample. The result of PCR optimization using 4 primers was visualized on 2% agarose gel (Figure 1). The result showed that the average of optimal temperature was obtained at 58.9^{o}C. Therefore following PCR was set up at 58.9^{o}C for annealing temperature in all applied genetic markers.

Genetic Markers Association with Twinning Birth Trait. Amount of 41 DNA samples (31 FH DNA; 10 PO DNA) were successfully amplified using four genetic markers (AGLA254, BM8230, ETH10 and IGF-1) associated with twinning birth trait. All fragments based on those genetic markers were presented in the right sizes (Figure 2 and Figure 3). The shown fragments (Figure 2 and Figure 3) were representative of those four genetic markers applied on 41 DNA samples, for instance BM8230 in PO cows (Figure 2a: samples no. 1 to no. 10) with fragment size of 103-106bp; AGLA254 in FH cows (Figure 2b: samples no. 11 to no. 21) with fragment size of 158-214bp with exception of sample no. 19, 20 and 21 did not show their fragments. In Figure 3 presented fragments using ETH10 in FH cows (Figure 3a: sample no. 1 to 10; 212-224 bp) and using IGF-1 in FH cows (Figure 3b: sample no. 11 to 21; 225-231bp). Four DNA samples of well no. 1, 2, 4 and 8 using ETH10 were not amplified (Figure 3a).

DISCUSSION

Annealing Temperature. A series of PCR program consisted of denaturation, annealing and elongation steps for DNA amplification. Establishment of annealing temperature in prior to PCR running is therefore to be important since primer would anneal at such temperature and proceed to elongation step of PCR work. In this study, PCR optimization was conducted at 3 different temperatures of 57.6, 58.9 and 59.7^{o}C and applied 4 primers of AGLA254, BM8230, ETH10 and IGF-1 with one DNA template. The result showed that all primers averagely emerged in right sizes though primers of BM8230 and AGLA254 emerged a bit blur band at all tried temperatures (Figure 1). Further PCR work however, we decided to implement annealing temperature of 58.9^{o}C for all four primers

associated with twinning birth trait. This applied annealing temperature met to the previous PCR work of Lien *et al*. (2000). However, annealing temperature for BM8230 and AGLA254 needs to be more optimized and might use a better commercial PCR kit since both primers showed blur bands. As stated by Ihara *et al* (2004), annealing temperature of BM8230 is 60^{o}C while for AGLA 254 is 56^{o}C.

Identification of Genetic Markers. Based on the result of DNA amplification using all 4 primers associated with twinning birth trait showed that all 164 fragments (41 DNA x 4 primers) emerged at the right sizes. Even though, there was a small number of lack bands in each primer used (Figure 3 a). Less DNA concentration was indicated the reason of none band for each primer. There are some factors influencing PCR work, one of them is DNA template concentration and purity (Altshuler, 2006). In this study, we presumed that DNA template was not enough in quantity (less than 50ng/µl) and purity for some DNA samples of both in FH and PO.

Visualization of PCR products derived from all primers was presented in Figures 2 (BM2830 in PO DNA; AGLA254 in FH DNA) and in Figure 3 (ETH10 and IGF1 both in FH DNA). Based on these results, it seems that twinning birth trait was influenced by many genes and not by a single gene. This finding supported a report of Lien *et al*. (2000) that twining birth trait is influenced by multi genes. In addition, quantitative trait loci for twinning rates located at bovine chromosome 5. Fine mapping of QTL using linkage analysis and Linkage Disequilibrium (LD) was conducted to calculate unknown gene background and pedigree (Meuwissen *et al*., 2002). Their findings indicated that there was a very small putative QTL for twinning rate and located in the middle of chromosome 5 at a region of <1cM. Chen *et al*. (2006) reported that twinning birth trait is associated with ovulation rates.

CONCLUSION

Based on the marker identification, it can be concluded that all 41 DNA samples bore all four genetic markers of AGLA254, BM8230, ETH-10 and IGF-1 with right sizes. However the BM8230 marker showed a bit blur of bands. This research needs further study to confirm more specific marker for identification of twinning birth in cattle or use of DNA sequencing technology.

ACKNOWLEDGEMENT

This research was possible conducted by RISTEK incentive research fund 2010 for capability improvement of researchers and engineers. Authors thank to M. Ridwan S.Far. and Handrie SE. for their assistant in blood collection.

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Appendix

Table 1. Sequences of genetic markers associated with twining birth trait in cattle*

No	Marker	Primer 5′ → 3′	Number of bases
1	AGLA254	F : GCTGCTTGGCACAGGCAAA	19
		R : GGATTAATTTCTGGACTCTG	20
2	IGF-1	F : GCTTGGATGGACCATGTTG	19
		R : CACTTGAGGGGCAAATGATT	20
3	ETH10	F : GTTCAGGACTGGCCCTGCTAACA	23
		R : CCTCCAGCCCACTTTCTCTTCTC	23
4	BM8230	F : GTAATTCTGGACACGACACACA	22
		R : TTAAGGATATGCAGAGGGTGTT	22

* Lien *et al*. (2000)

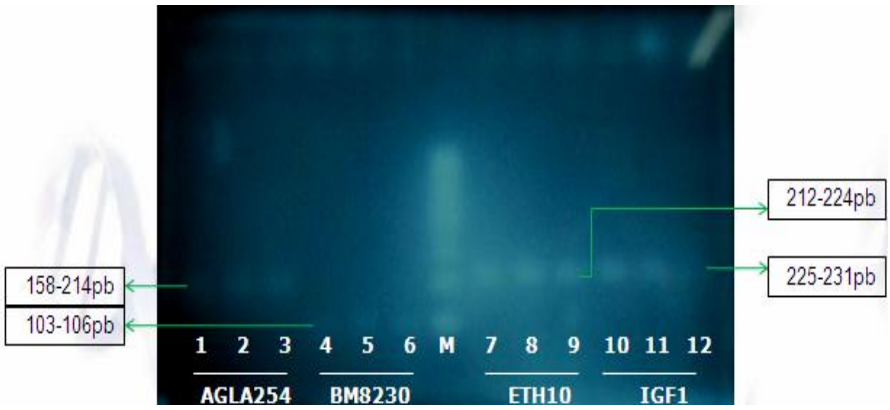

Figure1. PCR optimization at 57.6, 58.9 and 59.7^{o}C annealing temperatures in order on four primers of AGLA254 (1-3), BM8230 (4-6), ETH10 (7-9), IGF1 (10-12), M= DNA Ladder 100bp)

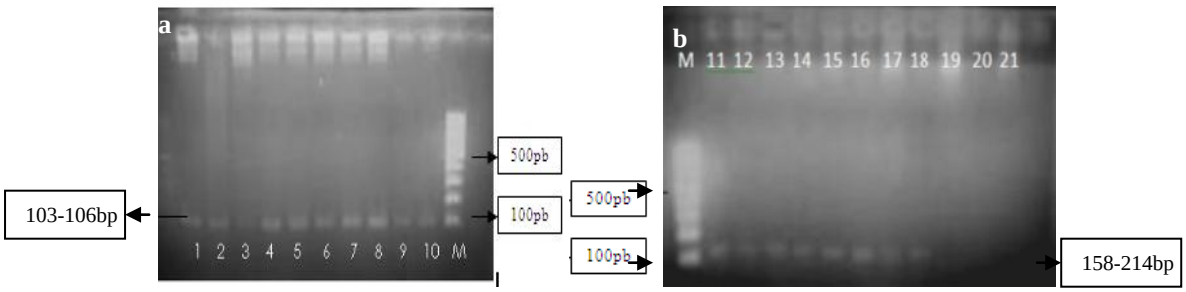

Figure 2. PCR products: (a) Ongole decendant/ PO cows using BM8230 (b) FH cows using AGLA254 (M= DNA ladder 100bp; 1-10 = PO cows; 11-21= FH cows)

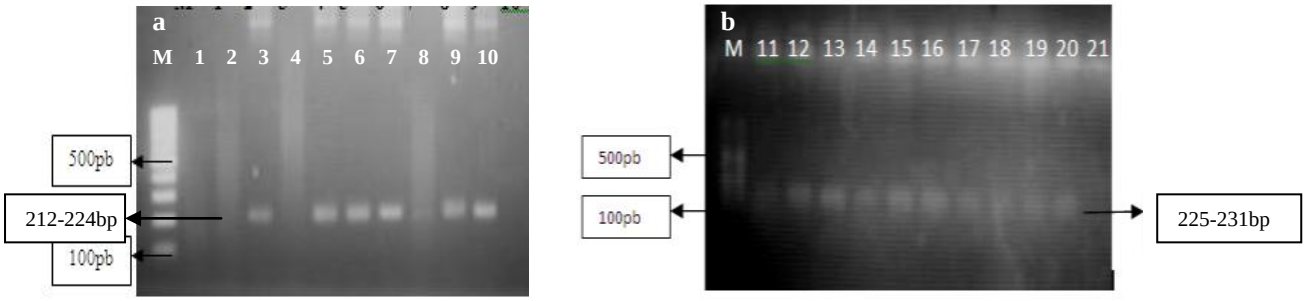

Figure 3. PCR products: (a) FH cows using ETH10; (b) FH cows using IGF-1 (M=DNA ladder 100bp; 1-10= FH cows; 11-21= FH cows)

Effect of Arbuscular Mycorrhizal Fungi and *Trichoderma* Inoculation on Growth of Oil Palm Seedling Inoculated with *Ganoderma* sp.

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Abstract

A research has been carried out to study the interactions between *Ganoderma* sp. and arbuscular mycorrhizal fungi (AMF) individually or in combination with *Trichoderma* on oil palm seedlings. In addition, the role of AMF and *Trichoderma* as a preventive or curative agents against *Ganoderma* was also observed. The time interval to see the role of AMF and *Trichoderma* as curative agents in controling *Ganoderma* is two weeks. The experiment was conducted in the greenhouse to test 12 treatments i.e.1) Control, 2) AMF, 3) *Trichoderma* DT 38, 4) AMF + *Trichoderma* DT 38, 5) *Ganoderma*, 6) AMF + *Ganoderma*, 7) AMF .... + *Ganoderma*; 8) *Trichoderma* DT 38, 9) *Trichoderma* DT 38 + *Ganoderma*, 10) AMF + *Trichoderma* DT 38 + *Ganoderma*, 11) AMF + *Trichoderma* DT 38 ....+ *Ganoderma*, and 12) *Ganoderma* .....+ AMF + *Trichoderma* DT 38. Each treatment was repeated 3 times. Oil palm seedling was first germinated on sterilized sand for 3 months. AMF used were 100 spores of *Acaulospora tuberculata* while *Trichoderma* DT 38 used was as nuch as 10 g which they are both collections of IBRIEC. Inoculation of *Ganoderma* sp. decrease the growth of seedlings significantly higher compared to controls. Mycorrhizal inoculation treatment either individually or in combination with *Trichoderma* increase significantly the number of leaves of plants inoculated with *Ganoderma* sp. .Mycorrhizae enhance the growth of the seedlings inoculated with *Ganoderma* sp. as seen from the number of leaves, fresh and dry weight of seedling. *Trichoderma* increase the growth of seedlings inoculated with *Ganoderma* sp. when they are previously colonized the plants. Interactions between AMF and *Trichoderma* improve the growth of palm seedling inoculated with *Ganoderma* sp.

Key words: oil-palm seedlings, *Ganoderma* sp, mycorrhiza, *Trichoderma*

INTRODUCTION

At this time one of the technical problems encountered by oil palm cultivation is the incidence of stem rot disease (Susanto et al., 2006). On some plantations in Indonesia, the disease causes the death up to 80% or more of the population resulting in a decrease of palm oil palm oil production sharply (Susanto et al., 2003). Several attempts have been made to control the attack *Ganoderma* sp .. Nevertheless some techniques which do not show real results. *Ganoderma* sp. live in the rhizosfer of plants. Manipulating of rhizosfer is expected to improve the community of microbial in the rhizosfer so the plants are able to control the attack *Ganoderma* sp. independently. Some microbes are known to control the *Ganoderma* sp. is a *Trichoderma* sp. and arbuscular mycorrhizal fungi (AMF). Both have different mechanisms in interacting with the plants and *Ganoderma* sp. *Trichoderma* sp. is non-symbiotic fungi while AMF are symbiotic with plants.

Some of the role of AMF symbiosis with palm is known to increase plant growth, plant nutrient uptake, and roots plant growth. Several studies have also demonstrated the role of mycorrhiza as biocontrol of soil-borne pathogens. Mycorrhizal infection occurs in the cortex and in the life cycle of plants formed the external hyphae growing outside plant roots.

The same habitat with the root pathogen causing a fairly intensive interaction between AMF and soil borne pathogens that infect plant roots. In addition, previous research also shown that *Trichoderma* sp. as a highly potential fungal antagonists as biocontrol agents several soil borne pathogens. However, the influence of *Trichoderma* sp. and AMF and its effect on soil borne pathogen *Ganoderma* sp. on palm oil has not been widely studied.

MATERIALS AND METHODS

The experiments were conducted in a greenhouse using polybags 60 x 50 cm in sized that filled with Ciomas sterilized soil. Germinated of the oil palm seed was obtained from the Indonesian Oil Palm Research Institute (IOPRI) in Medan and planted in a plastic ware containing sterilized sand for 3 months. Selected oil palm seedling are used as planting material. Preparation of microbial culture is done by previously purificate of *Trichoderma* DT 38, and *Ganoderma* sp isolates. Furthermore, pure cultures propagated for inoculum. *Ganoderma* sp. inoculated by placing inoculum (*Ganoderma* sp. colonized rubber rod, aged 2 months) on a palm trunk which then tied. Mycorrhizal inoculation (100 spores) is done in the root zone of oil palm as well as inoculation of *Trichoderma* DT 38 (10 g). Effect of mycorrhizal or *Trichoderma* DT38 as curative agents for *Ganoderma* performed with an interval periode of two weeks. Fertilization is done according to dose of recommendation, whereas in the mycorrhizal treatment performed only 25% the dose of recommendation and other treatments similar to the control of fertilization is done 100%.

Twelve treatments were tested in this research i.e. 1) Control, 2) AMF 3) *Trichoderma*, 4) *Trichoderma* + AMF, 5) *Ganoderma* sp., 6) AMF + *Ganoderma* sp., 7) AMF .... + *Ganoderma* sp., 8) *Trichoderma*, 9) *Trichoderma* + *Ganoderma* sp., 10) AMF + *Ganoderma* + *Trichoderma* sp., 11) AMF + *Trichoderma* sp. ….+ *Ganoderma* sp., and 12) *Ganoderma* sp .... + *Trichoderma* + AMF. Each treatment was repeated 3 times. Observations made after 10-month-old seedlings of high growth, number of leaves, and seed biomass.

RESULTS AND DISCUSSION

The observations are presented in Table 1. Control plants produced the highest plant height. In the also table shown that mycorrhizal inoculation, *Trichoderma*, and both of them with 25% fertilization resulted lower growth compared with controls. Though this difference was not significantly different. These results indicate that plants

Table. 1. The effect of treatment on growth of oil palm seedling

Treatment	Seedling heingt (cm)	Leave number
K (1)	200,33 b	15 ab
M (2)	191,17 ab	14,67 ab
T (3)	176,17 ab	15,67 ab
MT (4)	174,5 ab	14,67 ab
G (5)	172 ab	14 a
MG (6)	156,33 ab	16,67 b
M...+G (7)	147,87 a	16,67 b
TG (8)	157,83 ab	16 ab
T…+G (9)	175,33 ab	15,67 ab
MTG (10)	171,83 ab	15,67 ab
MT…+G (11)	157,17 ab	16,33 b
G…+MT (12)	169 ab	16,33 b

inoculated with AMF alone, *Trichoderma* alone both of them produce the similar height with the control (100% fertilization), although the treatment has been reduced by 75% fertilization.

The next treatment is to see the effect of inoculation *Ganoderma* sp. on the growth of oil palm. Inoculation *Ganoderma* sp. causes a decrease in both plant height and number of leaves compared to controls however are not significantly different as well when compared with AMF inoculation, *Trichoderma* alone or both of them together. Treatment to see the effect of AMF, *Trichoderma*, or AMF inoculated seedling with *Trichoderma* inoculated against *Ganoderma* sp. shown in subsequent treatment. Mycorrhizal inoculation treatments and *Ganoderma* together generating plant height was not significantly different compared with the inoculation of *Ganoderma* sp.. These results indicate that AMF inoculation can not fix the high growth of plants inoculated *Ganoderma* sp. . In contrast to plant height, the variable number of leaves treated either with mycorrhizal inoculation with *Ganoderma* sp. or mycorrhizal first before *Ganoderma* sp. yield leaves number significantly different compared with the number of leaves of plants inoculated with *Ganoderma* sp. . These results indicate that AMF inoculation improve growth in the number of leaves of plants inoculated *Ganoderma* sp .. The same is also found in mycorrhizal inoculation treatments before inoculation *Ganoderma* + *Trichoderma* sp. or vice versa *Ganoderma* sp. prior to AMF inoculation and *Trichoderma*. These results indicate that the influence of mycorrhizal and *Trichoderma* increase growth in the number of leaves of plants inoculated *Ganoderma* sp. . In the variables plant height, *Trichoderma* and AMF inoculation treatments did not affect plant growth inoculated with *Ganoderma* sp. .

Inoculation *Ganoderma* sp. first, followed by inoculation with AMF and *Trichoderma* showed that the number of leaves more than seedlings inoculated *Ganoderma* sp. . These results indicate that the possibility of mycorrhizae enhance the leaves number. *Ganoderma* sp + *Trichoderma* inoculation. showed that compared with controls produces a high number of leaves and lower plants. However, when compared with the inoculation treatment of *Ganoderma* sp., *Ganoderma* sp + *Trichoderma* inoculation produce a higher number of leaves, although inoculation of *Trichoderma* can not increase the height of plants infected by *Ganoderma* sp. The seedling inoculated with *Trichoderma* that were inoculated only on *Ganoderma* give high yield crops that are not significantly different compared with the inoculation treatment of *Ganoderma* sp. . The same is also found in the number of leaves. These results indicate that *Trichoderma* can not improve the growth of plants inoculated *Ganoderma* sp. .

Figures 1 and 2 show that AMF inoculation, *Trichoderma*, and both of them fertilized with 25% dose yield the fresh and dry weight of leaf, stem, shoot, and the root is lower compared with controls. Despite this difference was not significant. These results indicate that the AMF inoculated seedling, *Trichoderma* alone , or *Trichoderma* sp + AMF with 25% dose of fertilizer produces the same growth with controls (100% fertilization).

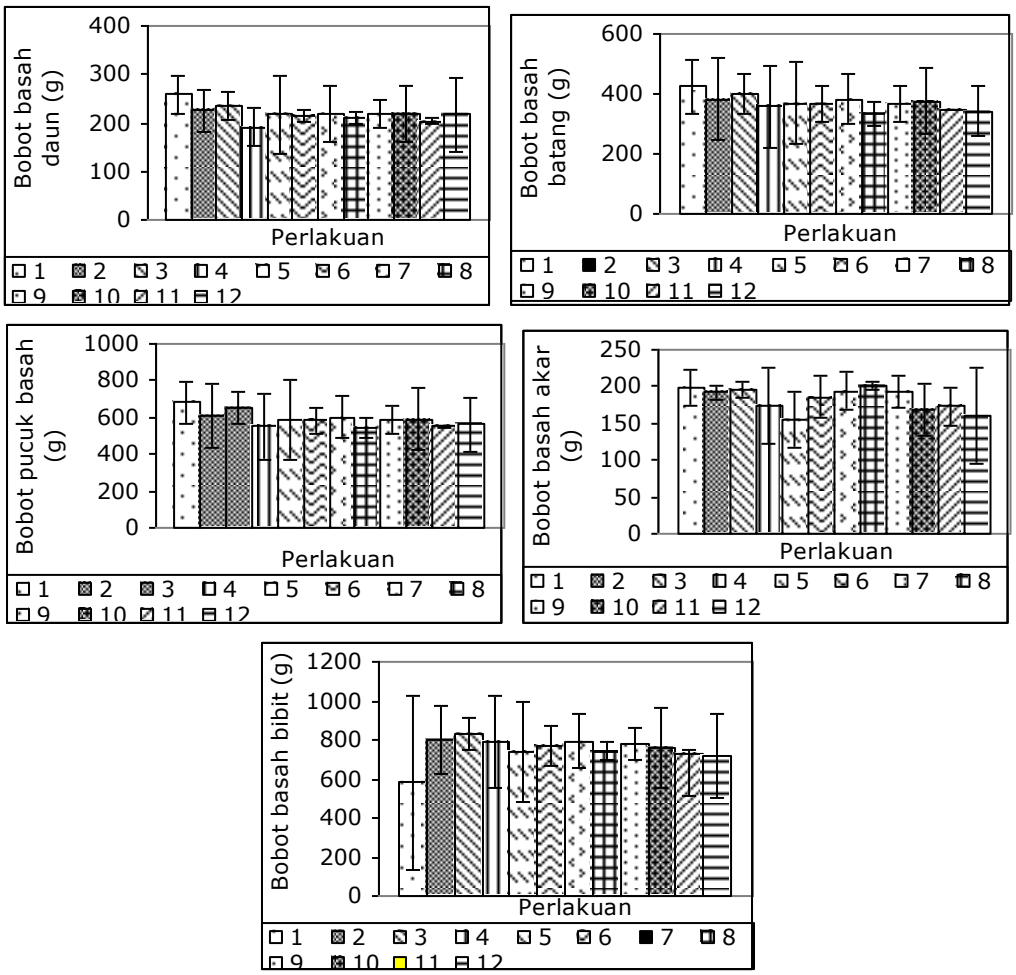

Figure 1. Seedling fresh weight of oil palm in the treatment tested

Oil palm seedling inoculated with *Ganoderma* sp. resulted fresh and dry weight of palm lower compared to those inoculated with AMF, *Trichoderma*, or both of them. These result showed that *Ganoderma* sp. inhibited oil palm seedling growth. Inoculation of AMF previously before *Ganoderma* sp. or both microbe with the *Ganoderma* sp. produces fresh and dry weight of seedlings higher than those of *Ganoderma* sp. inoculated seedlings These results indicate that mycorrhizal inoculation improve the growth of seedlings inoculated with *Ganoderma* sp.

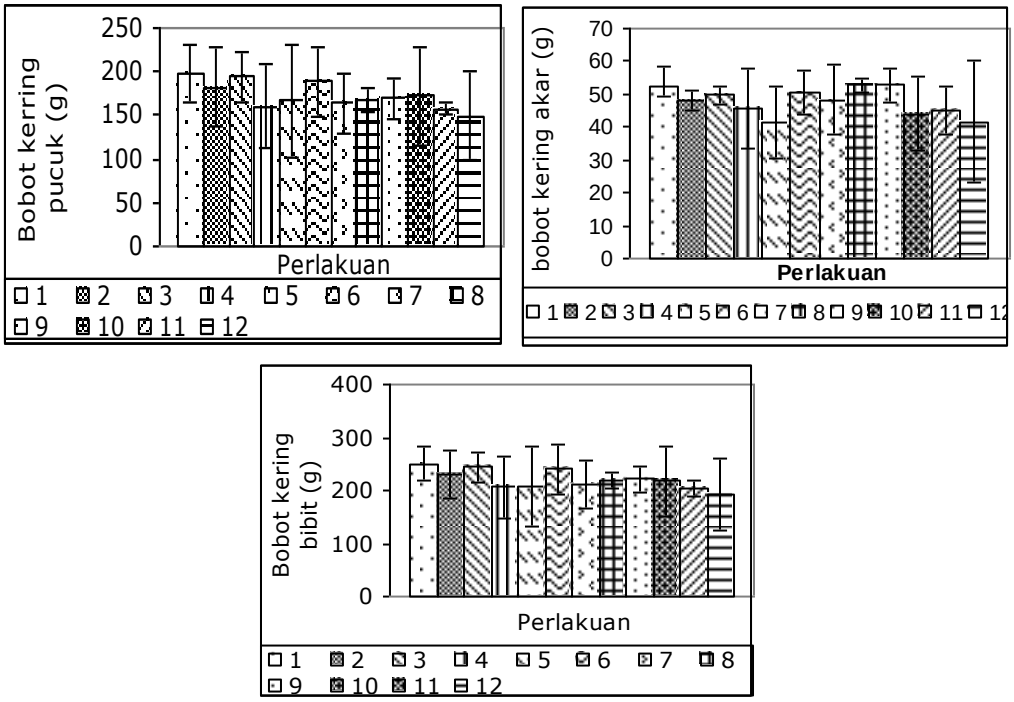

Figure 2. Dry weight of oil palm seedling in each treatment tested

Inoculation of *Trichoderma* previously produce higher fresh and dry weight of seedlings inoculated with *Ganoderma* sp. compared with the *Ganoderma* sp. These results indicate that *Trichoderma* improve oil palm inoculated with *Ganoderma* preventively. In combination treatment between AMF and *Trichoderma* are given together with the *Ganoderma* sp. able to increase plant growth of palm inoculated with *Ganoderma* sp. These results suggest that interactions between AMF with *Trichoderma* are synergies in particular to inhibit the activity of *Ganoderma* sp in oil palm seedling.

CONCLUSION

Mycorrhizae enhance the growth of the *Ganoderma* sp. inoculated seedlings of oil palm seen from the variable number of leaves, fresh and dry weight of seedling. *Trichoderma* increase the growth of seedlings inoculated with *Ganoderma* sp. when they are first to colonize plants compared with the *Ganoderma* sp. . Interactions between mycorrhizal with *Trichoderma* can improve plant growth inoculated *Ganoderma* sp.

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Phenotypic and Genotypic Identification of Lactic Acid Bacteria Isolated From an Indonesian Traditional Fermented Fish "Bakasang"

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Abstract

Bakasang is traditional fermented fish products prepared by the people in Manado, North Sulawesi Indonesia. Microbiological analysis bakasang showed the population dominated by lactic acid bacteria (LAB) ranging up to 10^8 cfu g−1. The phenotypic characterization of predominant LAB isolated from the fermented fish products was based on general morphology, physiological tests and biochemical test. The genotypic characterization of LAB was based on 16S rRNA gene sequencing. Predominant functional LAB strains associated with the fermented fish products were identified as *Pediococcus acidilactici*, *Leuconostoc mesenteroides* and *Lactobacillus paracasei.*

Key word : Bakasang, Fermented Fish ,Lactic Acid Bacteria, Phenotypic, 16S rRNA Gene Sequencing.

INTRODUCTION

Lactic acid bacteria (LAB) commonly utilized in the production and preservation of various fermented foods for example *bakasang*, such an Indonesian traditional fermented fish sauce. *Bakasang* is fermented fish products traditionally made from the guts of big fish (*Katsuwonus pelamis* L.), small fish and fish eggs which is the typical food of North Sulawesi (Manado). *Bakasang* are generally high in protein and amino acid compounds (2) besides its potential source of wide variety of LAB species because low-pH product with an added carbohydrate source, will support the predominance of LAB (2). Some of the genera that play an important role in fermentation foods commonly are *Lactobacillus, Pediococcus, Enterococcus, Leuconostoc*, and *Weissella* (2,5). Isolation and characterization of LAB from *bakasang* is scarce still.

Different phenotypic methods are used to identify LAB important for fermentation technology. However, these methods are not sufficient to characterize sub-species and strains in a genus. Thus, new methods have been developed depending on genotypical features and used effectively for the definition of the bacteria (1,4). The methods used for the current study of LAB such as 16S rRNA sequencing, a method that is universally suitable for the LAB with a high resolving power both on the species and intraspecies level. The aim of the present work was to identify the predominant lactic acid bacteria present in the traditional fermented fish product (bakasang) on the basis of phenotypic characteristics and genotypic characteristics including 16S rRNA gene sequencing.

MATERIALS AND METHODS

Isolation of Lactic Acid Bacteria

Guts of Big fish and egg fish were collected from market in Manado city, these samples were transported to the laboratory using cool box (4°C). They were cut into small pieces and mashed. Salt was added and rise was also added and mix thoroughly. The

mixture was packed into bottles, corked and then incubated at 37ºC for 7 days. LAB were isolated from sampel bakasang. 10 g samples were taken aseptically and homogenized in 90 ml of NaCl solution. Serial dilutions up to 10^{-8} were prepared and appropriate dilutions were plated onto de Man Rogosa and Sharpe Agar supplemented with $CaCO_3$ 1%, Na Azida and Syclo-hexamide. All plates were incubated at 37°C for 48 hours. Only lactic acid producing bacterial colonies were selected. This can be observed from clear zones around the colonies which indicated the dissolving of CaCO3 by an acid. Colonies with different morphology were counted, picked up and purified by restreaking on the same medium.

Cell morphology, Gram staining and catalase test, motility, non-spore forming were performed as a preliminary screening for lactic acid bacteria. The selected lactic acid bacteria were maintained as stock cultures at -80 °C in 10% skim milk and 20% glycerol (2,5)

Phenotypical characterization of isolates LAB

LAB strains used as reference strains for the characterization and identification of *bakasang* LAB isolates include *Pediococcus acidilactis* FNCC 0110, *Pediococcus pentosaceus* FNCC 0019, *Pediococcus halophillus* FNCC 0032, *Lactobacillus fermentum* FNCC 0104, *Lactobacillus brevis* FNCC 0021, *Lactobacillus plantarum* FNCC 0026 and *Leuconostoc mesenteroides* FNCC 0023 before being tested, the isolates were subcultured in MRS broth at 37°C. The following phenotypical tests were conducted: gram-reaction;gas from glucose;catalase reaction;endosphore forming;motility-test;the effects of temperature (10; 45; 50°C);different starting pH (3.5; 7.5; 9.6);different concentration of NaCl (6.5; 18%) and finally morphological character including cell form and cell arrangement.

Fermentation type of isolates: cultures were grown in 5 ml of MRS broth for 2 days. The amount of lactic acid accumulation in these cultures were calculate from titration value of 0,1 N NaOH solution.

Genotypical Characterization of isolates LAB

Genomic DNA Extraction

Extraction of genomic DNA were done as described by (8) with minor modifications to the method versatile quick prep for gram positive bacteria. Bacteria were cultivated on MRS broth. After 2-3 days of cultivation at 30 °C, 1.5 mL of biomass was collected by centrifugation (5 min, 13.000 rpm). The pellet was resuspended in 400 µL SET buffer (75mM NaCl, 25mM EDTA, 20 mM Tris, pH 7,5), 50 µL Lysozyme (10 mg/mL), 20µL Proteinase K (15mg/mL) were added and incubated at 37ºC for 1 h. 50µl SDS 10% was added and incubated at 65ºC for 1 h. 400µL cooled Chloroform was added and centrifuged (13.000 rpm 10 min). The aqueous layer was re-extracted with isopropanol (1:1v/v) and then incubated at -20ºC overnight. DNA was centrifugated and washed with cooled ethanol 70% and then supernatant was removed. The purify of DNA solution was resuspended with TE buffer and checked spectrophotometrically at λ_{260} and λ_{280} nm.

Amplification and Sequencing 16S rDNA

The 16S rRNA genes were amplified from purified DNA of the strain using a commercial kit (Mega Mix Blue ® and universal primers 27f(5'-AGAGTTTAGTCCTGGCTCAG-3') and 1492r(5'-GGTTACCTTGTTACGACTT-3') for 16S rDNA (15). The condition of amplified gene fragment : pre-denaturation of the target DNA at 96ºC for 4 min followed by 30 cycles at 94ºC for 1 min, primer annealing at 51,5ºC for 1min and 30 s and primer extension at 68ºC for 8 min. PCR was completed with 10 min elongation at 68 °C followed by cooling to 4 °C. PCR product was visualised by

electrophoresis on a 2 % (w/v) agarose gels, stained with ethidium bromide in the presence of a 1 kb ladder The parameters for the electrophoresis were 90 V for 30 min.

Phylogenetic analysis

For phylogenetic analysis, sequences strain BksC3, BksC19, BksC24 , BksC25 and strain BksC27 were aligned by using CLUSTAL X software (14). The phylogenetic tree of the 16S rDNA sequences was constructed by the neighbor-joining algoritm (10). The root position on the unrooted tree was estimated by using *Bacillus subtilis* DSM 10 as the outgroup strain.

RESULT AND DISCUSSION

A total number of 45 isolates of LAB in which production clear zone around theirs colonies were obtained from *bakasang*. The clear zone appearance is due to the dissolution of $CaCO_3$ on MRS medium by acid agent (7). Among the 45 isolates were rearrange and confirmed as LAB in amount of 27 isolates. All these isolates were gram positive, rods or cocci, appeared singly, in pair, chain, tetrad. Cell were non motile and non sporing, they gave negative reaction for catalase. These strains were then classified into genus level using profile matching method. Based on the profile matching method (Table 1.) showed that 27 isolates separated into three groups. Group I consisted of 5 isolates, 2 among them were rod-shape heterofermentative and others were represented as rod-shape homofermentative. These groups were putatively identified as genus *Lactobacillus*. Sixteen isolates were include in group II. They were represented as cocci (tetrad) homofermentative which were identified as genus *Pediococcus*. Finally, group III comprised of six cocci-heterofermentative LAB were identified as genus *Leuconostoc*. It was concluded that lactic acid bacteria isolated from *bakasang* are dominated by *Pediococcus*, *Leuconostoc* and *Lactobacillus*.

Table 1. Identification of lactic acid bacteria isolates into genera level by profile matching method.

	Group					
Characteristics	I	II	III	*Lactobacillus*	*Pediococcus*	*Leuconostoc*
Number of isolates	5	16	6			
Gram stain	+	+	+	+	+	+
Shape	Rods	Cocci	Cocci	Rods	Cocci	Cocci
Cell arrangement	Single/pair/ chain	Tetrad	Pair/chain	Single/pair/ chain	Tetrad	Pair/chain
Production gas from glucose	+/-	-	+/-	+/-	-	+/-
Catalase	-	-	-	-	-	-
Spore formation	-	-	-	-	-	-
Motility	-	-	-	-	-	-
Fermentation type	Hetero/homo	Homo	Hetero	Hetero/homo	Homo	Hetero
Growth at 10°C	+	+/-	+/-	+/-	+/-	+/-
Growth at 45°C	+	+	+	+/-	+/-	+/-
Growth at 50°C	+	+	-	+/-	+/-	-
Growth at pH 3.5	+	+/-	-	+/-	+/-	-
Growth at pH 7.5	+	+	+	+	+	+
Growth at pH 9.6	-	-	-	-	-	-
Growth at NaCl 6.5%	+	+	+	+/-	+/-	+/-
Growth at NaCl 18%	-	-	-	-	-	-

In an effort to identify bakasang LAB isolates at the species level, molecular phylogeny analysis was conducted and phylogenetic trees were constructed based on the 16S rDNA sequences from evolutionary distances by the neighbor-joining method. Based on

phylogenetic analysis, strains BksC3, BksC19 and BksC24, respectively, representative of II, were placed in the cluster making up the genus *Pediococcus* (Fig. 1). They formed a cluster together with *P.acidilactici,* supported with a bootstrap value of 93%. The group II strain BksC25 was placed in the lactobacilli cluster on the phylogenetic tree, with *Lactobacillus paracasei* subsp *paracasei* and *Lactobacillus casei* being the most closely related species in 100% of bootstrap analyses (Fig. 1). The representative strains of groups III BksC27 was clearly identified as *Leuconostoc mesenteroides* strains by forming a very welldefined cluster (100% bootstrap) with this species (Fig. 1). Nevertheless, as reported by (9,11), the 16S rDNA sequence analysis method, while very good at identifying the organisms by genus and species.

Phylogenetic trees based on the 16S rDNA sequence displayed high consistency regarding the relationships between the organisms included. All the nodes leading to BksC strain clusters are supported by high bootstrap values and should be considered significant. Also, bakasang strains were designated to the correct species with close homology.

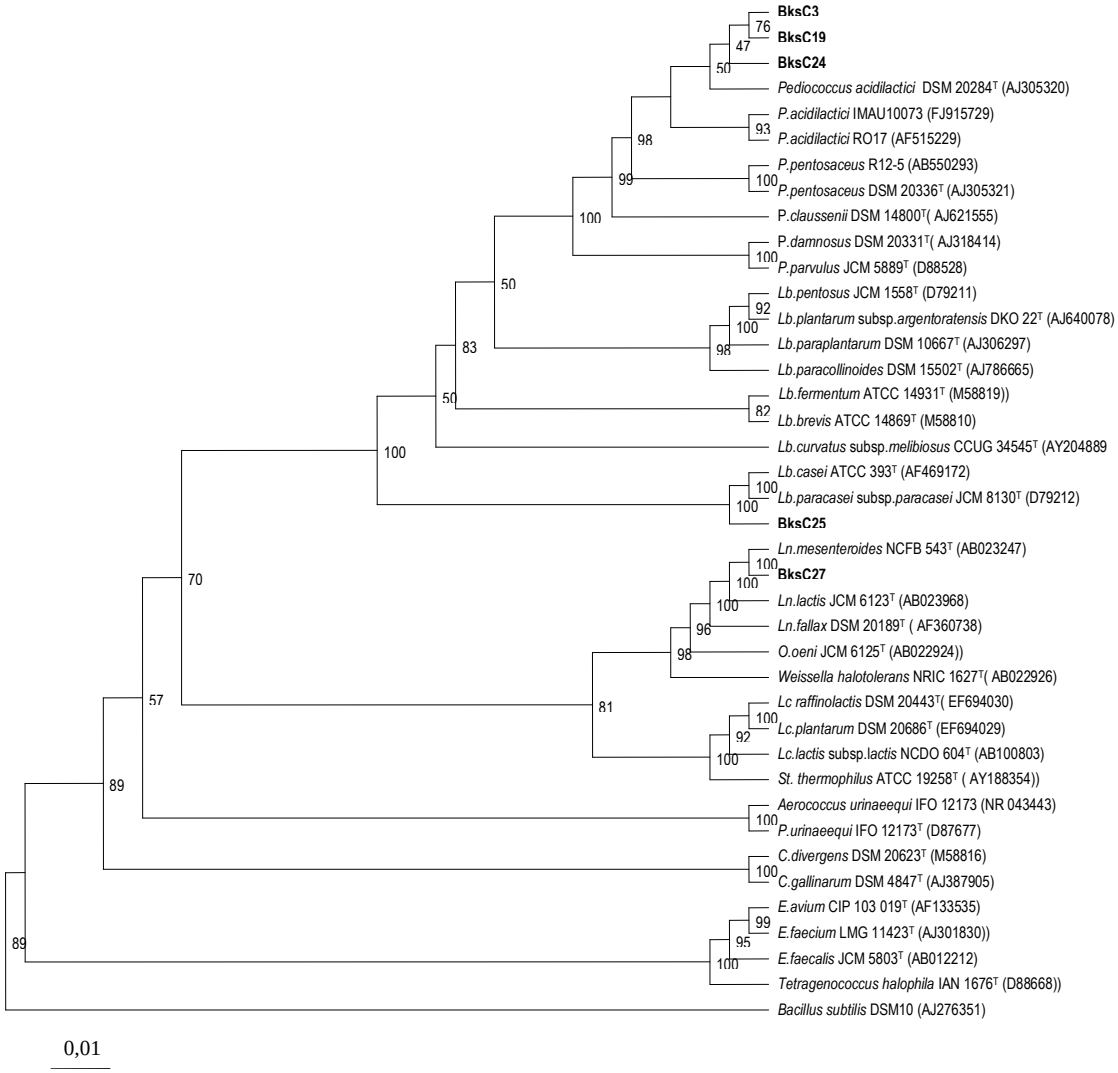

Figure 1. Phylogenetic tree showing the relationship of Bakasang isolates BksC3, BksC19, BksC24, BksC25, and BksC27 as inferred by the neighbor-joining method of complete 16S rDNA sequences. Bootstrap values for a total of 100 replicates are shown at the nodes of the tree. References of the type strains used for comparison are given, as well as the accession numbers for all 16S rDNA

Following biochemical and phylogenetic analyses, bakasang isolates fell within well-recognized groups of LAB and, for the majority of them, were clearly related to particular species. Most of the LAB characterized belonged to the genera *Lactobacillus, Pediococcus,* and *Leuconostoc.*

LAB species identified in bakasang are common inhabitants of a variety of fermented fish (fish sauce). This is in agreement with the results of other authors (5,6,12,13), which showed that the natural fermentation processes in fermented fish are dominated by species of *Lactobacillus, Pediococcus,* and *Leuconostoc*. In this research, the LAB isolated from bakasang were characterized with phenotypic and genotypic methods. We found out that *Pediococcus acidilactici* is the dominant flora. This related with raw material (gut of fish) used (1) and time of fermentation. The other strains isolated from bakasang sample consist of *Leuconostoc mesenteroides* and *Lactobacillus peracasei subsp paracasei.*

Since the isolated LAB from fermented fish (bakasang) samples were characterized with phenotypic and genotypic methods in this study, it can finally be informed that the phenotypic methods should be supported with the genotypic methods.

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Antibacterial Activity Assay of Mahkota Dewa (*Phaleria macrocarpa*) Fruit Against Pathogenic Bacteria

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ABSTRACT

Mahkota dewa (*Phaleria macrocarpa*) is an indigenous Indonesian medical plant which is known has many chemical compound that can be used as medicine for many disease such as diabetes mellitus, uric acid, acnes, exceem, insect bite, and hypertension. Mahkota dewa fruit contain of flavonoid, saponin, alkaloid, tannin, atsiri oil and lignin that believed can be used for antibacterial agents. This objective of this research was to know antibacterial activity from mahkota dewa fruits against pathogenic bacteria. The antimicrobial activity was determined by measuring the inhibition zone with well diffusion assay method using pathogenic bacteria i.e. *Salmonella thypii* O.901, *Staphylococcus aureus,* ATCC 25923 and *Escherichia coli* ATCC 25922. The result showed that juice of ripe mahkota dewa fruit has no antibacterial activity and the raw one has antibacterial activity against only *E. coli* ATCC 25922 with 2 mm diameter inhibition zone.
Keywords : mahkota dewa (*Phaleria macrocarpa*), antibacterial agent, pathogenic bacteria

Introductions

Pathogenic bacteria causing deseases that generally can be poisoned by antibiotic. Several of them that often causing infection in Indonesia are *Salmonella thypii*, *Escherichia coli* and *Staphylococcus aureus* (Supardi &Sukamto, 1999; Dzulkarnain *cit* Ajizah, 2004). *E. coli* and *S. aureus* are often causing diarrhea and enterocolitis whereas *S. thypii* can causing thypus fever. Three of them was recorded have multiresistance to many antibiotic such as chloramphenicol, amphixilin, tertaxiclin and cothrimoxazol in some area in the world (Hadinegoro, 2008; Wasitaningrum, 2007). Therefore, exploration of an antibacterial agent source from nature material being very important. Mahkota dewa (*Phaleria macrocarpa*) is an indigenous Indonesian medical plant which is known has many chemical compound that can be used as medicine for many disease such as diabetes mellitus, uric acid, acnes, exceem, insect bite, and hypertension. Mahkota dewa fruit contain of flavonoid, saponin, alkaloid, tannin, atsiri oil and lignin (Soeksmanto *et al*, 2007; Rini, 2009) that believed can be used for antibacterial agents. So that, The purpose of this research was to know antibacterial activity from mahkota dewa fruits against pathogenic bacteria.

Material and Method

Mahkota dewa (*Phaleria macrocarpa*) fruit was collected from Beringharjo traditional market in Yogyakarta, Indonesia. The ripe of mahkota dewa fruit and the raw one were washed under running tap water then blended and filtrated. The liquid was collected in the sterile bottle. Antibacterial activity was determined by measuring inhibition zone with well diffusion assay method using Mueller Hinton Agar. About 20 µl of liquid was dropped into the well within medium countaining pathogenic bacteria i.e *Salmonella thypii* O.901, *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922, then incubated at 37ºC for 24-48 h and the inhibiton zone was measured.

Result and Discussion

Bacteria has natural ability to being resist with drugs such as antibiotic although its not an direct interaction. That is their natural mecanisms to stay alive. The fruit of mahkota dewa contains saponin, alkaliod, flavonoid, tannin and atsiri oli compound that have antibacterial activtity. Flavonoid is the biggest fenol compound that can be found in the nature (Achmad, 1986) while alkaloid and atsiri oil can disturb peptidoglycan component formation. So, the cell will destruck sistemically. In this research, the liquid of raw mahkota dewa fruit and the ripe one being tested to *Salmonella thypii* O.901, *Staphylococcus aureus* ATCC 25923 and *E. coli* ATCC 25922. As the result, only the raw mahkota dewa fruit has antibacterial activity againts *E. Coli* ATCC 25922 with 2 mm diameter inhibition zone. In contrast the ripe one has no antibacterial acivity because its maybe containing more glucose. Glucose is one of the carbone source used by bacteria to growth. In addition, antibacterial compound concentration within the liquid of sample is very low. Indeed, the ability of antibacterial agent for inhibiting pathogenic bacteria is according to those compound concentration (Ajizah, 2004).

Table 1. inhibition zone of antibacterial activity assay of mahkota dewa (*P. macrocarpa*) fruit againts pathogenic bacteria

No	Pathogenic bacteria	Sample	
		Raw liquid	Ripe liquid
1	*S. thypii*	-	0 mm
2	*S. aureus*	0 mm	0 mm
3	*E. coli*	2 mm	0 mm

Acknowledgement

We thank for Dra. Darwani, M. Sc for providing strain *Salmonella thypii* O.901

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Development of a Web-based Software for Microbial Culture Collection Data Management

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Abstract

Indonesia is known for its biodiversity, including the microbial biodiversity. Many Centers or Institutes in Indonesia develop a centralised microbial culture collection in order to manage the collection better. However, many of the Centers manage their collection data paper-based, thus increasing the risk of data mismatch or loss. Therefore we are developing a web-based software for culture collection data management. The software is using a centralised database, thus preventing the risk of data mismatch and loss. The software is also built on a server-client architecture over a network, so it can be acessed on the network. The software is now under evaluation on Center of Biotehnology site's and can be accessed online through http://kultur.biotek.bppt.go.id.

Introduction

Due to its tropical location, Indonesia hosts a wide range of biodiversity. This huge number of diversity is a lucrative potency to be explored. In particular of microorganism diversity, many Centers and Institutes in Indonesia host a microbial collection center, providing an easy access of exploration of microorganism potency.

As the data grows, an effective and efficient method of managing the data is needed. Paper-based method, which record the data on a book or paper, is not an option if the data grows into thousands of records. The risk of data mismatch, duplicated, or loss are possible.

Therefore, we develop a software in order to avoid the risk. We chose a web-based and server-client platform due to their characteristic, ie availability and accessibility over a network, OS independent, and centralised data. The software is built using PHP, a web scripting language, and MySQL, a database application. Both are open source application.

International centers, such as DSMZ [1] and ATCC [2], has provided a web-based interface for clients so that they can explore their collections interactively. The interface is basically supported by the database backend of collection, which they can manage the collection data. The software that we develop hopefully will provide the information to the clients by web interface as well.

Purpose

The research aim is to develop a web-based software for management of data of microbial culture collection.

Methods

In designing the application, we separate the logic and content. The aim of the separation is for preventing the complexity of software design. Logic will basically deliver and display the information so it can be easily read by human.

The content or the data is kept on the database. One of the most important factor in designing the database is data normalisation [3]. Data normalisation, in summary, will prevent the ineffective database design.

Result

a. Software Design

The software is built on modules in order to ease further development in the future. However some of the modules are built into the software, such as authentication module and collection data module.

The software is designed that it can be accessed over the network, thus it is necessary that it has a security built into the software. This is mainly for preventing unauthorized access to the data itself, allowing only granted access has the capability of manipulating the data. Furthermore, the security provides additional advantage, it can distinguish between public access and authorize (or administrator) access. We can then separate information and privilege that can be delivered to public or admin pages. Only admin that can view all information and has unrestricted privilege in manipulating the record. Public interface is designed to delivered general information on record (Fig 1), whereas admin interface is designed to have the capability in manipulating the data (Fig 2).

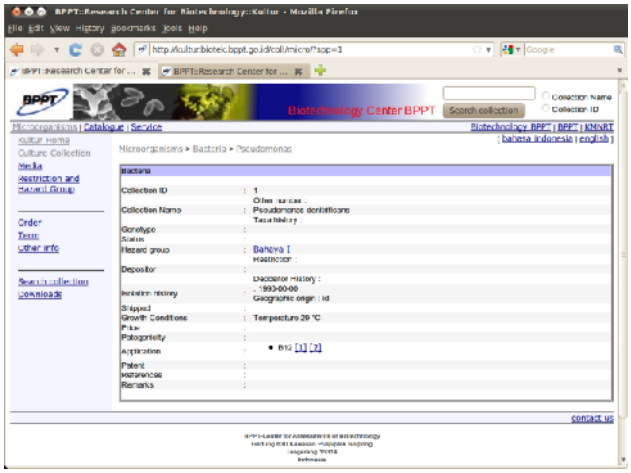

Figure 1. Public access page

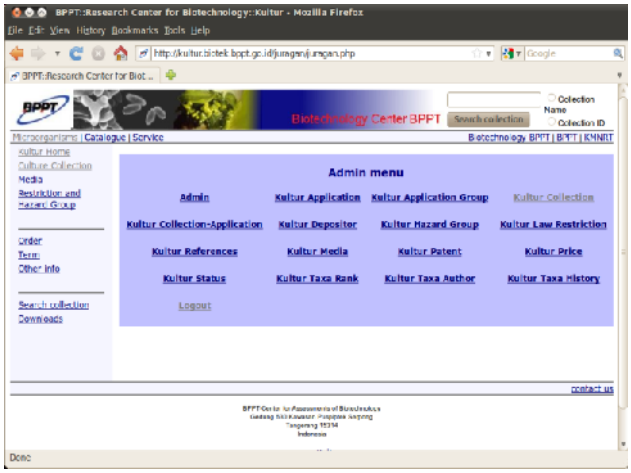

Figure 2. Administrator access page

b. Database Design

All information regarding to the collection record is kept in database. We divided the database into separate tables and each table hold a group of similar information. This is done in order to kept the data normalised, thus preventing unnecessary duplication of information. The main record are kept in main table, and any other information regarding the record are kept in separate table as illustrated in figure 3.

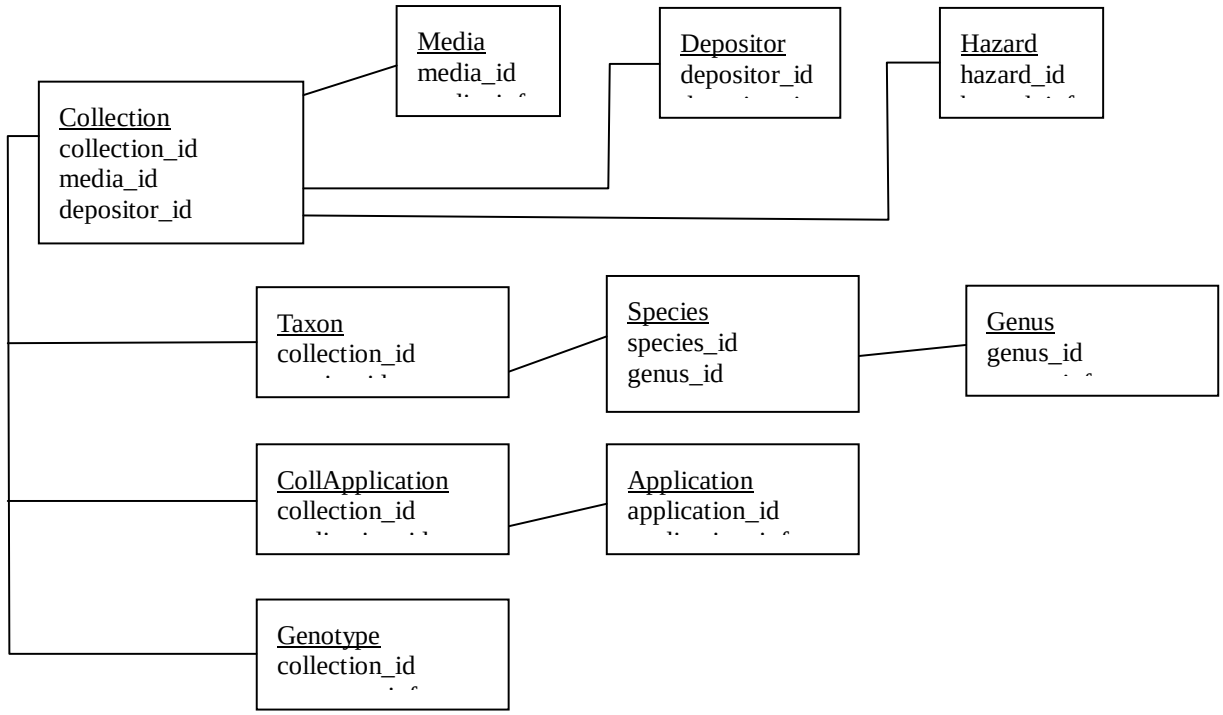

Figure 3. A simplified diagram of the table relation. Boxes are table. Names within boxes are table names (underlined) and column name.

Conclusion

The software is now being evaluated for possible bug, however public can acces the dummy records for evaluation purposes. It can be acessed on http://kultur.biotek.bppt.go.id.

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The Characteristic of Polyvinyl Alcohol-Carbon from Coconut Shell Carbon

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Abstract : The characteristic of polyvinyl alcohol-carbon from carbon as pyrolysis results of coconut shell has been studied. The process of sampling begins by calcinated coconut shell charcoal at the temperature of 873 and 1023 K flow Nitrogen. Then mix it with polyvinyl alcohol (PVA) composition includes 2.5 to 7.5 wt.% by using solvent method. Binder system is mixed with carbon powder, forming a paste with the characteristic of physical properties in different systems. The next steps molding by using a cylindrical mold with a diameter of ~ 15 mm. Compaction is performed with one direction force by the means of with the thrust. In this stage, the process produces a sample of pellets (green compact). After samples are obtained, it is dried at room temperature for one day before performing the drying process in oven for 4 hours at 383 K. The measured characteristic consisted of X-ray diffarction (XRD) and infrared spectrum (FTIR). It is suggested that polyvinyl alcohol (PVA)-coconut shell carbon can be used as a candidate of biomaterial carbon.

Keywords : *coconut shell carbon, polyvinyl alcohol (PVA), XRD, FTIR.*

1. INTRODUCTION

The advanced carbon materials such as synthetic carbon composites obtained from carbon fibers struggle to be accepted in wider practical use. Of well known carbon materials, the role of composite carbon cannot be overestimated either in modern industrial practice or in everyday life. It happens due to the unique physical and chemical characteristics of composite carbon which attract researchers to conduct certain research which explores those characteristics [1,2].

Carbon contains allotrope which different types of chemical bonding structure, including graphite, diamond, black carbon, fullerene, carbon nano tubes (CNT). Graphite is a type of carbon material obtained from carbon atoms with sp^2 orbital. One of these atoms form a new bond with 3 carbon atoms [3]. The microscopic material consists of graphite flat sheets from carbon atoms which are bonded, as the graphen. The characteristics of the bond structure and dynamic interactions between the graphene layers produce strong electrical conductivity properties [4] which functions as a lubricant.

Carbon used in the industry is an example of carbon that can be heated at temperatures up to 1000 - 1300^{o}C, while graphite is a type of carbon which can be heated at temperatures higher than 2500^{o}C. Heating at higher temperature of 3000^{o}C will produce carbon that has an irregular structure and it evolves more ordered graphite structure by removing the impurities of volatile material [5,6].

Composite carbon can be obtained by carbonizing coconut shell, and polyvinyl alcohol (PVA) in a thermal decomposition process [7,8]. The main composition of coconut shell consists of cellulose, lignite, and hemicelluloses with a formula C, O, H, and N. These organic materials contain functional groups such as hydroxyl (R-OH), alkanes (R-(CH_2) nR'), carboxyl (R-COOH), carbonyl (R-CO-R'), ester (R-CO-OR'), linear and cyclic clusters ether (ROR') with a variation of carbon [9]. In this process, the cellular anatomic features of

coconut shell and polyvinyl alcohol are retained in the new carbon material [2]. The structural changes during carbonization and the physical properties of the resulting chars have been investigated in several studies.

Polyvinyl alcohol (PVA) as an inert and organic adhesive is used to obtain high density products. PVA was chosen due to its homogeneity in functional groups, linearity and solubility in water [10]. With increasingly high-temperature treatment of adhesive polyvinyl alcohol (PVA), it further affects the growth of grain to be larger in size.

To obtain carbon from coconut shell charcoal, tar impurities and volatile material are removed. The removal of impurities is conducted by high temperature heating up to 600 - 1000^{o}C. This heating is also aimed to eliminate the compounds carried by the drainage of gas. During the heating process, all non-carbon materials must be removed to form a pure carbon as well as to arrange the structure [11, 12].

Heating process of coconut shell will produce a gradual change. In the first stage of the carbonization, the carbon samples become coke. The second stage is the stage of graphitization in which it changes coke into graphite carbon whose irregular structures tend to grow over the graphite whose structure is irregular [14, 15]. The nature of carbon is depends on the source of carbon [16], in addition to the method and conditions of synthesis [17]. The nature of carbon materials gives impact on their various use, primarily as carbon electrodes and carbon material structure.

The recent study which particularly discusses on these materials, coconut shell and polyvinyl alcohol (PVA) are used to produce carbon composite materials (carbon bio-composite).

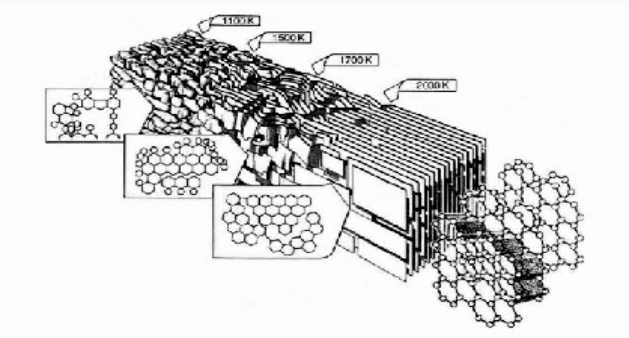

Figure 1. The diagram comprehensively models the structure changes which occur during the heat treatment of graphitizable carbon.

2. EXPERIMENTAL SECTION

Materials

This study used charcoals as the raw material produced by PT Tropica Nucifera Industry Bantul Yogyakarta, Indonesia. This charcoal is the source of carbon and polyvinyl alcohol. Further, it uses Merck stimulant and argon gas used as inert atmosphere to get air free.

Instrumentation

Calcination uses a tube-Thermolyne Furnaces (Sybron) Type 21100 with a maximum temperature 1200^{o}C. Calcinated charcoal as raw material was obtained through 100 mesh grinding and sifting. For carbon materials, it was used Tarno compacted Grocki 312 model a maximum of 20 tons. Sintering was carried out by using a Carbolite furnace-Edwards Pirani 501 A6D 1600^{o}C maximum temperature with argon gas as the atmosphere, To study the spectrum of carbon materials, it was used Fourier Transform Infra Red (FTIR) Shimadzu

model IR-Prestige 21 and X-ray Diffraction (XRD) analysis using Goniometer model difractometer with Cu K_α (α = 1.54056 Å) radiation.

Procedure

Preparation and Charcoal Calcination

Carbon powder 100 mesh sieves was calcined in an inert state through two stages: calcination at temperatures of 873 K for 3 h with the existence of nitrogen gas then, it is followed by the purification charcoal powder which was extracted for 24 hours with 1 M hydrochloric acid at room temperature [14]. The sample was washed with distilled water until it reached constant pH and was dried in an oven at temperatures at 383 K overnight [13]. Re-calcination was carried out for 3 h at temperature of 823 K with nitrogen.

The process of carbonization.

The carbon powder was calcined with 2.5 to 7.5 wt% polyvinyl alcohol then suspended in distilled water at 353 K and stirred for 60 min. Binder system was mixed with charcoal powder, forming a paste with the density in different systems. The next steps molding by using a cylindrical mold with a diameter of ~ 15 mm. Compaction was performed with one direction force by the means of Tarno Grocki with the thrust of 5 tons. In this stage, the process produced a sample of pellets (green compact). The samples were then dried at room temperature for one day before performing the drying process in oven for 4 h at 383 K. The samples were then fed into a carbollite furnace for sintering at the temperature of 1273 K. The rate of temperature was 10 K/min and reaction time was 3 h. Then, these samples were cooled in the furnace at a rate of 8 K/min, with a flow of argon gas. This process produced dense coconut shell coke or carbon-carbon composite materials [11, 15, 18].

Synthesis of carbon structures

The coke sample was heated for the 2nd time by sintering at 1723 K with a flow rate of 10 K/min, holding time of 3 h, in the furnace Carbollite, with the furnace cooling rate of 8 K/min, under argon gas flow [18].

3. RESULT AND DISCUSSION

FTIR Analysis

FTIR spectra pattern of synthesis of coke products by solvent method with PVA stimulant concentration 2.5, 5 and 7.5% of sintering (1723 K temperature conditions, reaction time 2 h, argon atmosphere, annealing) are shown in Fig. 2, and the wave number absorption bands as shown in Table 1. The spectrum shows a broad band near 3425 cm^{-1} which indicates the presence of hydroxyl groups on the carbon surface. The stretching was attributed to the absorbed water on the surface of coke and carbon black. The stretching frequencies of the aromatic C=C and aromatic C-H groups give rise to peaks at 2924 and 2862 respectively, which are originally existed as the support for the coke, carbon black and graphite. The peak at near 1700 cm^{-1} ($\upsilon_{C=O}$) along with another peak at 1458 cm^{-1} (υ_{C-O}) indicate the presence of carboxylic acid groups. The peak at 1581 cm^{-1} is assigned to a conjugated hydrogen bonded carboxyl groups, assigned by several author on coke and carbon black. The peak at 2931 cm^{-1} is due to C-H stretching of CH_2 groups. The bands near 1600 cm^{-1} indicates the fingerprint region of C=O, C-O and C-H groups that exist as functional groups of coke and carbon black. The band near 840 cm^{-1} is due to out-of-plane bending of C-H group in benzene derivatives [19-22].

Table 1. FTIR absorption band of material carbon synthesized products with PVA by sintering at 1723 K in water solvent.

Wave number (cm^{-1})			Attributes of
PVA 2,5 %	PVA 5%	PVA 7,5%	
871,82	840,96	856,39	Coke
	1072,42	1064,71	
2931,8	2924,09	2931,8	
1126,43	1072,42	1064,71	Carbon black
	1627,92		
	2862,36	2862,36	
3425,58	3425,58	3425,58	
2931,8	2924,09	2931,8	Graphite

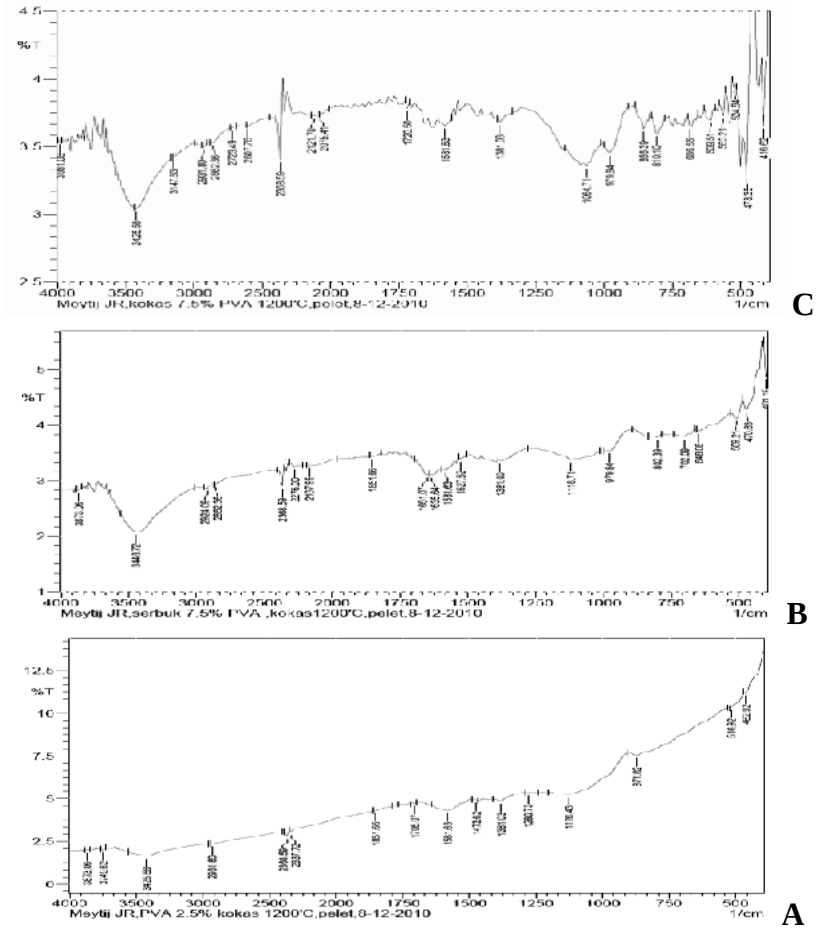

Figure 3. FTIR spectra pattern of carbon and PVA, solvent method, sintering 1723 K, argon ; 2.5 % PVA (A), 5% PVA (B), and 7.5 % PVA (C)

XRD analysis

Figure 3 showed a typical X-ray diffraction (XRD) pattern of testing charcoal calcined carbon and coke carbon material that sintered at temperatures 1273 K. XRD diffractograms of charcoal calcined carbon and coke carbon material gave the value of d(Å) as a graphite semi-crystalline structure. Yin *et al.* (2009) reported that the decrease of the interlayer spacing (d) and the increases of the crystalline diameter and average stacking

height of the aromatic carbon sheets (L_c) with increasing the temperature suggested the development of stacking structure, increased the size of crystallite as well as removal of defects and increased order in carbon materials structure. The increase of L_c with increasing temperature resulted from crystallite growth in-plane and coalescence of crystallites [23]. In this process, the structure of amorphous carbon structure was changed into semi-crystalline structure with a better degree of order, namely turbostatic structure [24]. There was the change of internal structure by setting the position of equilibrium carbon atoms.

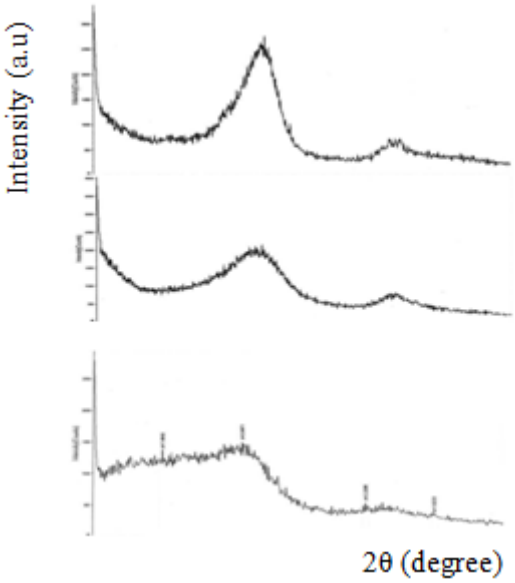

Figure 3. XRD pattern of : (a) coconut shell charcoal, (b) carbon calcined at 1023 K (N_2), and (c) carbon sintered at 1273 K (Ar).

4. CONCLUSION

Carbon composite can be prepared from carbonized coconut shell and polyvinyl alcohol (PVA). It is suggested that polyvinyl alcohol (PVA)-coconut shell carbon can be used as a candidate of biomaterial carbon.

ACKNOWLEDGEMENT

The author would like to thank the Department of Chemistry, Faculty of Mathematics and Natural Sciences; and Department of Mechanical and Industrial Engineering, Faculty of Engineering, Gadjah Mada University, Yogyakarta, Indonesia; for the provision of laboratory facilities.

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Flow cytometric analysis of MCF-7 cell line in its treatment with leaves extract of *Eugenia uniflora* L

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Abstract

The use of natural product in treatment of cancer has attracted more attention since the existing treatments have not yet given any satisfactory result. Dewandaru (*Eugenia uniflora.*L), one of promising medicinal plants in Indonesia, was reported to have suppression activity on DNA polymerase of EBV (Eipstein-Bar Virus). This study aimed to reveal the cytotoxicity of *E. uniflora* L leaves extract and examine more deeply whether or not this activity will trigger the apoptosis process of human breast cancer MCF-7.

The extraction process was conducted using petroleum ether, dichloromethane and methanol. Each extract was subjected for MTT assay for cytotoxicity analysis. The prospective compounds were then separated using vacuum column chromatography and preparative thin layer chromatography. MTT method was used to performed cytotoxicity test of each separated fraction on MCF-7 cells. The selected compound which show the most potential activity was analyze to its IC_{50} value. MCF-7 cells which treated with this toxic compound was analyze for its cell cycle using flow cytometry assay and propidium iodide staining.

The results showed that the IC_{50} of the toxic compound tested on MCF-7 cells were 10 µg/mL. Flow cytometry analysis showed that this compound has capability in inducing apoptosis. Cell cycle arrest was observed in MCF-7 cells in which cell accumulation occurred in G1 phase.

Key words: *Eugenia uniflora* L, MCF-7, flow cytometry

INTRODUCTION

Cancer is an abnormal growth of cells caused by multiple changes in gene expression leading to disregulate balance of cell proliferation and cell death and ultimately evolving into a population of cells that can invade tissues and metastasize to distant sites, causing significant morbidity and, if untreated, death of the host (Ruddon, 2007). Cancer replaced heart disease as the leading cause of death among men and women aged younger than 85 years in 1999 in the United States. The 3 most commonly diagnosed types of cancer among women in 2011 are breast, lung and bronchus, and colorectal, accounting for about 53% of estimated cancer cases in women. Breast cancer alone is expected to account for 30% (230,480) of all new cancer cases among women (Siegel, 2011)

The ultimate goal of any cancer drug discovery process is discovering and developing effective and non-toxic therapies (Collota, 2008). In this case,cancer treatment using natural products has attracted more attention as the existing treatments did not provide satisfactory results. One of the potential plants is Dewandaru (*Eugenia uniflora* L), also known as the Surinam Cherry

E. uniflora leaves extracts have been showed pronounced anti-inflammatory action (Schapoval, *et al*., 1994), considerable contractile activity, with a resulting effect on intestinal transit (Gbolade, *et al*., 1996), endothelium-dependent vasorelaxant effects (Wazlawik *et al*., 1997) and hypotensive effects (Consolini, *et al.*, 1999; Consolini & Sarubbio, 2002), and inhibit the increase of plasma glucose and triglyceride levels (Arai *et al*., 1999). Some compounds present in *E. uniflora* leaves extracts have also been shown to inhibit the Epstein– Barr virus, known to be closely associated with nasopharyngeal carcinoma (Lee, *et al*., 2000), and have antimicrobial (Adebajo, *et al.*, 1989; Holetz *et al*., 2002) and antifungal activity (Lima, *et al*., 1993; Souza *et al*., 2002). However, research on its anticancer activity has not been reported. Therefore, the objective of this research is to study the potential application of *E. uniflora* as anticancer agent, isolate the toxic compound and study its impact on cytotoxicity and apoptosis on MCF-7 breast cancer cells.

MATERIALS AND METHODS

Plant and extracts

Fresh leaves of *E. uniflora* were collected from Tawangmangu, Indonesia and was identified at Medicinal Plant and Traditional Medicine Research and Development Office, Tawangmangu. Their powder was macerated three times using petroleum ether for 24 hours. After filtration the resulting extracts were combined and evaporated to dryness. The residue from petroleum ether was macerated three times using dichloromethane for 24 hours. After filtration the resulting extracts were combined and evaporated to dryness. The residue from dichloromethane was macerated using methanol for 24 hours. Supernatant was evaporated to dryness. Each extracts was tested for cytotoxicity test on MCF-7 cell line.

Fractionation

The toxic extract was partitioned using petroleum ether, petroleum ether/chloroform {(9 :1, 8 : 2, 7 : 3, 6 : 4, 5 : 5, 4 : 6, 3 : 7, 2 : 8, 1 : 9) v/v}, chloroform, chloroform/methanol {(9 :1, 8 : 2, 7 : 3, 6 : 4, 5 : 5, 4 : 6, 3 : 7, 2 : 8, 1 : 9) v/v}, and methanol. The chemical composition of each fraction was monitored on thin layer chromatography. The sub fractions which show similar spots on TLC analysis, were then combined. Each fraction was subjected for cytotoxicity test on MCF-7 cell line.

The toxic fraction were partitioned with petroleum ether, petroleum ether/chloroform {(9 :1, 8 : 2, 7 : 3, 6 : 4, 5 : 5, 4 : 6, 3 : 7, 2 : 8, 1 : 9) v/v}, chloroform, and methanol. The fractions that showed similarity on TLC were combined. Each fraction was subjected for its cytotoxicity on MCF-7 cell line.

Cytotoxicity assay using MTT method

Cell Culture

Human breast cancer cell lines MCF-7 were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco) in an incubator with humidified air with 5% CO_2 at 37˚C.

Cell viability assay

The viability of the cells was assessed by MTT (3, 4, 5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) (Sigma) assay which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. Cells were plated onto 96-well plates ($2x10^3$ cells/well) (Iwaki). After 24 h incubation, cells were treated with each extract/fraction with various concentrations for 48 h. Then, MTT solution was added in each well and cells were incubated for 4 h at 37˚C and then incubated with 100 µl of soluble

solution at 37˚C overnight. The quantity of formazan product was measured by using a spectrophotometric microtiter plate reader (Bio-Rad) at 595 nm wavelength.

Flow cytometry analysis

Apoptosis detection and analysis of cell cycle distribution were performed by flow cytometry. Briefly, cells were incubated for 24 h in a medium without FBS to synchronize the cell cycle. Cells were then treated for 24 and 48 h in the medium containing 10% FBS with isolated compounds solution . Cells were harvested by trypsinization, washed twice with PBS, incubated with 0.125% Triton X-100, and stained with propidium iodide (PI) in PBS containing 0.2 mg/mL RNase A. Stained cells were analyzed using a FACS calibur. For each sample, cells were counted until the count reached 5 x 10^5cells. The percentages of cells in the subG1, G1, S, and G2/M phases were determined using the CELLQUEST software.

RESULTS AND DISCUSSION

Extraction and Cytotoxicity Assay

Maceration of powdered leaf of Dewandaru was conducted using petroleum ether, dichloromethane and methanol. The four extracts was then tested on T47D breast cancer cells using concentration series as follows: 500, 250, 125, 62,5 dan 31,25 µg/mL. Each treatment was observed after 48 hours incubation. Figure 1 showed a curved of cell viability versus various concentration extract which is used on the treatment. The results indicate that dichloromethane extracts has the smallest IC_{50} value of 115 µg/ml, whereas the IC_{50} for petroleum ether extract and methanol extract was 160 µg /ml and 150 µg/mL respectively.

There were no significant differences among IC_{50} values of petroleum ether extract, dichloromethane extract and methanol extract. This is due to distribution of toxic compounds in each extract. Extraction process by maceration technique enables the toxic compounds can't extracted perfectly. Dichloromethane extract was then chosen for fractionation because of its smallest IC_{50} value.

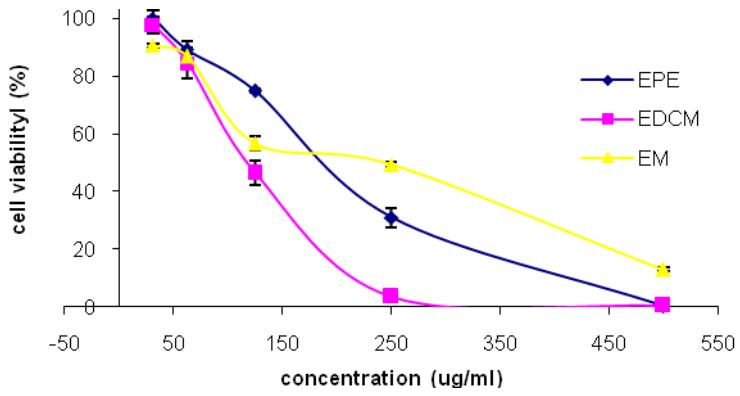

Figure 1. MCF-7 cell viability caused by treatment of petroleum ether extract (EPE), dichloromethane extract (EDCM) and methanol extract (EM) leaves of E. uniflora with MTT method

The portion of dichloromethane extract that showed the strongest cytotoxicity activity was partitioned by vacuum liquid chromatography (VLC). The fraction that showed similarity on TLC were combined to give four fractions. Each fractions were tested for cytotoxicity test. Two fraction had cytotoxicity activity (fraction II and III) and the other fraction not toxic.

The most toxic fraction (fraction II) were further were partitioned by VLC using gradient elution. The chemical composition of each fraction was monitored on thin layer chromatography. The fractions that showed similarity on TLC were combined and evaporated given two fraction. Cytotoxicity activity of F2V2 fraction against MCF-7 cells stronger than F2V1 fraction.

F2V2 fraction were further separated by preparatif thin layer chromatography. The TLC spot were scraped into two portion, upper and lower part and diluted in mixture chloroform/methanol 4:1. After filtration each fraction were evaporated and tested for cytotoxic activity. It was found that the lower part of PTLC had cytotoxic activity. The toxic compounds were purified by preparatif TLC given two portion upper and lower part. After 48 treatment of each portion, it was found that the upper part of preparative TLC had cytotoxic activity. Toxic compounds activity of *E. uniflora* against MCF-7 cells showed a linear correlation between the concentration of the test with cell viability as shown in Figure 2.

Cytotoxic activity of toxic compounds E uniflora against MCF-7 cells showed a linear correlation between the concentration of the test with cell viability as shown in Figure 2.

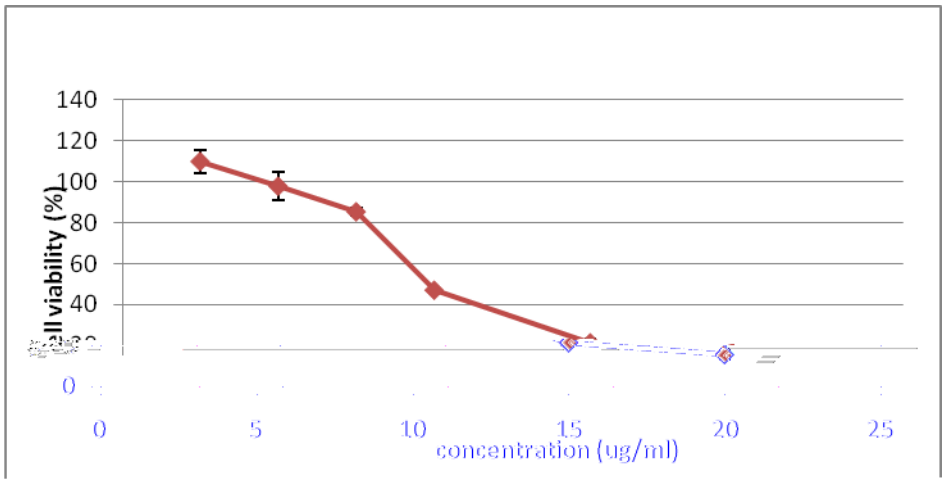

Figure 2. Treatment effects of toxic compounds extracted from *E. uniflora* against MCF-7 cell lines. The cell viability was determined by MTT method.

The calculation showed that IC_{50} of toxic compounds extracted from *E. uniflora* tested on MCF-7 cells is 10 µg/mL. There were morphological changes in MCF-7 cells due to the treatment of doxorubicin and toxic compounds extracted from *E. uniflora*. The cells look Tues looked round, flat and floats (Figure 3). Sel tampak berbentuk bulat, pipih dan mengapung (gambar 3).

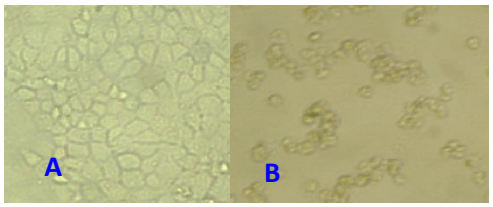

Figure 3. MCF-7 cell morphology (A) without treatment (B) treatment with toxic compounds isolated from *E.uniflora* 15 µg / mL

Flow cytometry analysis

We analyzed cell cycle distributions using flow cytometry to investigate the effects of toxic compounds extracted from *Eugenia uniflora* L. The distribution of MCF-7 cells in each phase after treatment can be observed in tables and figures below.

Table. 1. The distribution of MCF-7 cells in each phase of cell cycle after treatment using extract of *E. uniflora*. The cell cycle distribution was observed using flow cytometry method

Treatment	Concentration (μg/ml)	Incubation (hour)	Persentase jumlah sel (%)				
			Sub G1	G1	S	G2/M	Polyploidi
Control		*48*	*1.18*	*45,96*	*23.41*	*26,91*	*3,18*
Toxic compound	*8*	*24*	*0,98*	*76,05*	*12,08*	*8,33*	*2,90*
	8	*48*	*16,25*	*59,40*	*8,06*	*15,04*	*1,58*
	6,5	*24*	*1,00*	*53,63*	*21,86*	*21,42*	*2,60*
	6,5	*48*	*10,16*	*69,47*	*10,95*	*14,82*	*2,62*

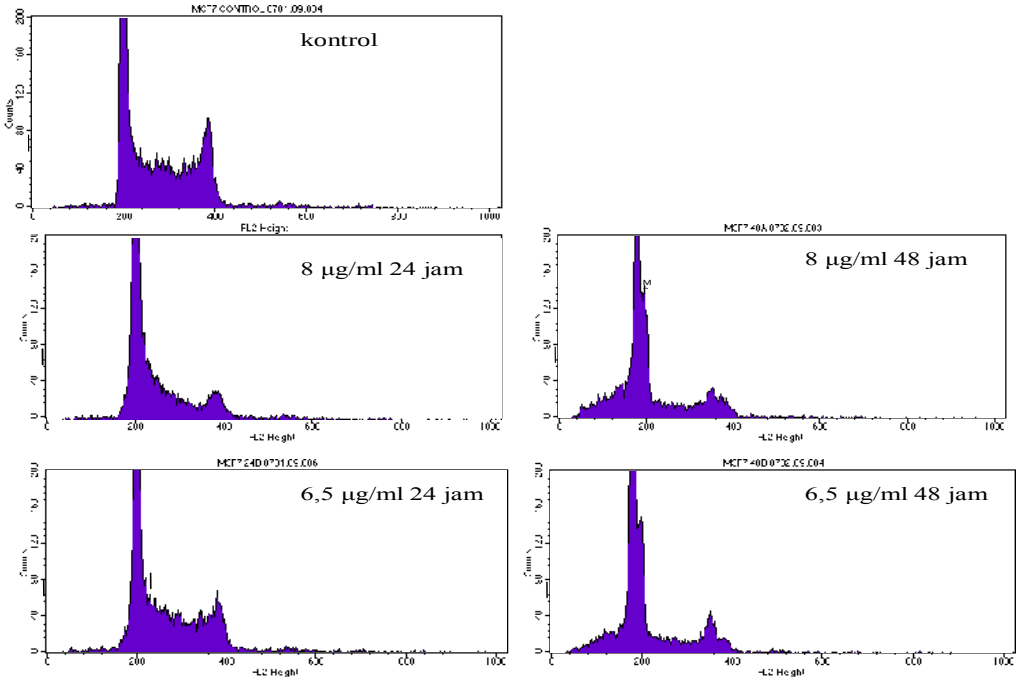

Figure 4. Flow cytometry of MCF-7 cell cancer. Cell were treated with 6.5 and 8 μg/mL of toxic compounds extracted from *E. uniflora*. Incubation process was conducted for 24 and 48 hours.

MCF-7 cell lines were treated with concentrations of toxic compounds of 8 μg/mL and 6.5 μg/mL. Incubation process was conducted for 24 and 48 hours. Both concentration were shown to induce the apoptosis process after 48-hour incubation. It was indicated by the increase of the percentage of cells in sub G1 phase. Inhibition of cell cycle which occurs in

G1 phase can reduce the percentage of cell population in S phase. As a result, the cells that enter the phase of G2 / M also reduced. According to the data above, it can be inferred that toxic compounds from *E. uniflora* arrested cell cycle at G1 phase and lead to apoptosis.

In cell cycle analysis using flow cytometry, it can be known that toxic compounds of *E. uniflora* lead to apoptosis. To support these data, the cell was also observed for apoptosis using double staining method. Here we used fluorescent compounds that can bind to DNA / RNA, ethidium bromide-acridine orange (AO-EB), which was subjected against MCF-7 cells. Apoptotic morphology of MCF-7 cells on double staining test results can be examined in Figure 5.

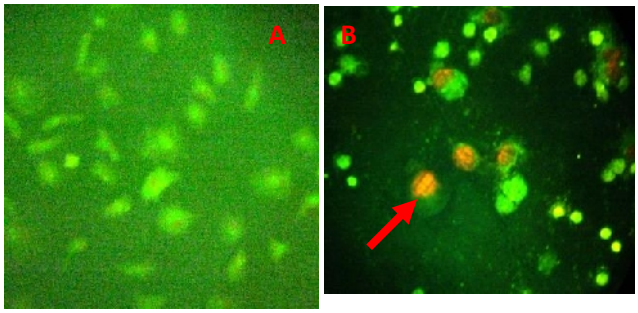

Figure 5. Apoptosis Induction of toxic compounds in the MCF-7 cells with double staining method (ethidium bromide-acridine orange). (A) MCF-7 cells without treatment, (B) MCF-7 cells in treatment with toxic compounds of 9 ug / mL.

The results showed that all apoptosis in control cells exhibit a green fluorescent. It means that there was no cell death. Treatment of MCF-7 using toxic compounds causing some cells be fluorescent which show the loss of cell membrane permeability and indicate the occurrence of apoptosis. Further observations indicate the presence of an enlarged cell nuclei size and the occurrence of multi nucleus. Some cells begin to divide the cell nucleus and form the apoptotic bodies. On one hand, acridine orange can penetrate the membrane of normal cells, binds to the DNA / RNA and cause a bright green fluorescence. On the other hand, ethidium bromide is more easily to penetrate into the decreased permeability of cell membranes and cause orange fluorescence. This method showed the presence of live and dead cells, and cells which undergo apoptosis. One of the characteristics of apoptotic cells is the fragmentation of the cell nucleus that followed by the fragmentation of cells into apoptotic bodies.

CONCLUSIONS

The IC_{50} values of toxic compounds from *E. uniflora* L tested on MCF-7 cells were 10 µg/ml, Flow cytometry analysis showed that the toxic compounds are capable of inducing apoptosis. Cell cycle arrest was observed in MCF-7 cells in which cell accumulation occurred in G1 phase.

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Petroleum Ether, Ethyl Acetate and Methanol Extracts of *Pseudocalymma alliceum* (Lam.) Sandwith Leaves and Their Antiviral Activities Against Newcastle Disease Virus

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Introduction

Newcastle Disease (ND) is a systematical exhalation disease having the character of acute, easy to catching once and very complex, causing by Avian paramyxovirus. During 2006, there were ND cases on more than 10 countries (Anonim, 2006). Conventional vaccine which is available, not yet able to overcome problematic around ND cases. The existence of ND mutation strain as found in New South Wales, Australia, generates separate problem beside the happening of resistance of virus to the common therapy (Derek, 2000).

Pseudocalymma alliaceum (Lam.) Sandwith is a tropical plant with evergreen leaves, well grown in the wet and dry forests and this species was known as garlic vine (Lhomann, 2009). The smell of the crushed leaves is nearly same as garlic (*Allium sativum*) (Taylor, 2006). This plant demonstrated to have many biological activities such as anticancer (Denn, 2008), anticholestherol (Srinivasan and Srinivasan, 1995), antifungal (Bhupendra *et al.*, 1999), antioxidant (Desmachelier *et al*., 1997) and antiviral against papaya mosaic virus (Khurana, 1970). The objectives of this research were to obtain the the petroleum ether, ethyl acetate and methanol extracts from *Pseudocalymma alliaceum* (Lam.) Sandwith leaves and to evaluate their antiviral activities against Newcastle Disease (ND) virus using embryonated chicken egg as culture medium.

Methodology

The ground dried leaves were gradually macerated with petroleum ether, ethyl acetate and methanol. Evaporation of the solvents gave petroleum ether extract (4 g), ethyl acetate extract (33.5 g) and methanol extract (42 g). The extracts were assayed for their antiviral activities against Newcastle Disease (ND) virus. The steps of antiviral assay were :

a. Preparation of the embryonated chicken eggs
   The embryonated chicken eggs were be candled to perceive the condition of the embryo inside. Only chicken eggs with alive embryo could be used in this research. Then the area of the air cavity (head of embryo) of the egg is signed to be used as virus and extract inoculation places.
b. Preparation of sample (the extracts)
   The petroleum ether, ethyl acetate and methanol extracts with concentration of 10 and 100 µg/ml were added vicillin and streptomycin antibiotic (1:1) then they were incubated on 37°C for 2 hours.
c. Preparation of the ND virus
   The virus was obtained from Medivac ND La Sota dry fost vaccine. Vaccine was dissolved in aquadest and added vicilln and streptomycin antibiotic (1:1) then be incubated on 37°C for 2 hours.
d. Inoculation of the virus and the extract into the embryonated chicken eggs
   As much as 0.1 ml of each extract was injected into the embryonated chicken eggs using injection spuit and be followed by inoculation of 0.1 ml ND virus. The eggs were then incubated on 37°C for 2 days. The embryonated chicken eggs were placed in refrigerator

at temperature 4°C to kill the embryo. The air cavity area of egg were then opened, alantoic fluid was then taken using injection spuit and would be used for hemaglutination test which conducted on microplate 96 wells to get the virus titer.

Result and Discussion

The virus titer was determined based on the maximum dilution causing hemaglutination. From the virus titer data obtained, the percentage of ND viral growth inhibition could be counted. If the erythrocite agregat was formed (hemaglutination reaction did not happen) at the coloumn number 1 (the first dilution), it could be said that the virus titer was 2^0 means alantoic fluid did not contain virus. If erythrocite agregat was formed at 2^{nd} coloumn (2 time dilution), the virus titer was 2^1 and so on.

Table 1. The virus titer of *Pseudocalymma alliaceum (Lam.) Sandwith* leaves extracts against *Newcastle Disease Virus*

Replication	The virus titer of sample groups (HA unit)						
	A	B	C	D	E	F	G
1	2^7	2^1	2^1	2^2	2^4	2^7	2^7
2	2^7	2^1	2^2	2^3	-	2^7	2^7
3	2^7	2^2	2^2	2^3	2^6	2^7	2^7

Note:
Group A = negative control (DMSO 0,25 %)
Group B = PE extract with concentration 100 μg/ml
Group C = PE extract with concentration 10 μg/ml
Group D = ethyl acetate extract with concentration 100 μg/ml
Group E = ethyl acetate extract with concentration 10 μg/ml
Group F = methanol extract with concentration 100 μg/ml
Group G = methanol extract with concentration 10 μg/ml

From table above, it could be known that the virus titer of negative control group without extract adding had the most value. Groups B to E were sample groups which having antiviral activity showed by the decreasing of virus titer in line with the increasing of extract concentration except on methanol extract which had the same virus titer for both 10 and 100 μg/ml concentration.

Table 2. The percentage of antiviral growth inhibition of *Pseudocalymma alliaceum (Lam.) Sandwith* leaves extract against ND virus

Sample groups	Percentage of inhibition (%)			Mean (%)
	1	2	3	
B	85,71	85,71	71,43	80,95
C	85,71	71,43	71,43	76,19
D	71,43	57,14	57,14	61,90
E	42,86	-	14,29	28,57
F	0	0	0	0
G	0	0	0	0

The petroleum ether of *Pseudocalymma alliaceum* (Lam.) Sandwith leaves indicated the biggest antiviral activity against NewCastle Disease virus. The percentages of ND viral growth inhibition for concentration 10 and 100 µg/ml of extracts were 76.19% and 80.95% respectively. While ethyl acetate extract showed smaller activity of antivirus growth inhibition. Methanol extract was found to be inactive. From this, there was possibility that the the chemical entity of *Pseudocalymma alliaceum* (Lam.) Sandwith leaves having antiviral activity was nonpolar compound.

Conclusion

The percentages of ND viral growth inhibition of petroleum ether and ethyl acetate extracts of *Pseudocalymma alliaceum* (Lam.) Sandwith leaves were 80.95% and 61.90%, respectively while the methanol extract was found to be inactive.

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The Test Effect of "Genjah Salak" Coconut (*Cocos nucifera* L) Water on the Heart Rate of Male Wistar Mice

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ABSTRACT

Introduction. Coconut water has been used as traditional medicine to cure various diseases, and the results of previous studies showed that coconut water can be used to replace body fluid because the sugar content found in coconut water is easily absorbed by the body. Coconut water is an excellent tonic for health. The water contained in a coconut fruit is sufficient to meet the body's daily need for vitamin C (Health Articles, 2008). Naturally, coconut water has such a perfect mineral composition and sugar that had a perfect electrolyte balance as well. It is similar to the human body fluids so that coconut water can be used as intravenous fluids. Given the composition of the minerals it contains, coconut water has a great potential to be developed as a natural isotonic beverage. (Articles of Health, 2008). One can be exposed to stress-induced hypertension due to frequent taking a part in sports, especially a dangerous sport. The stress may result from the frequence of being nervous or afraid when facing a dangerous game. Some of the sports branches that may rise such stress include rock climbing, motor racing, car racing, karate, taekwondo, and boxing. This sport can usually lead to an increase of adrenaline in a positive or negative way. Hypertension can be caused by excessive intake of sodium in extracellular fluids. To return to normal condition, patients can consume a lot of potassium which may absorb extracellular fluid and thus reducing blood pressure, and the required potassium is the highest content found in coconut water. This study used genjah salak coconut water (Cocos nucifera L) aged 5-7 months with a range of glucose levels from 0.38 to 0.61%. ***Research objectives***. This study aims to look at the effects of genjah salak coconut water (Cocos nucifera L) on the heart rate so that the results of this study is expected to know the security of consuming coconut water for people who have problems with blood pressure. Thus, the results can be developed as a potential supplement beverage with the minimal side effects. ***Methodology***. The research method used is an experimental method by observing the results of preclinical testing in male wistar rats which were divided into five groups: negative control group, positive control group, test group genjah salak coconut water, atenolol as the comparator group, and the combination of genjah salak coconut water + atenolol. The test materials were administered before induction with adrenaline and heart rate measured with a tail cuff before treatment (T0), after therapy (T1), and after induction (T2). ***Conclusion.*** Genjah salak coconut water, atenolol, and the combination of genjah salak coconut water + atenolol can inhibit an increase in heart rate caused by adrenaline administration. The measurement of heart rate after administration of adrenaline in the genjah salak coconut water, atenolol group, and the combination of genjah salak coconut water + atenolol showed that there was no significantly different increase in the heart rate compared to the frequency before and after therapy with the frequency of each group of 1.97 ± 0.53; 1.90 ± 0.57, and 1.57 ± 0.33 Hz.

Keywords: genjah salak coconut water, dangerous sports, adrenaline, hypertension, heart rate

I. INTRODUCTION

I.1. Research Background

Indonesia possesses a variety of plants and natural resources that are needed as ingredients of medicines and supplements. The medicinal plants have been used in the society for such periods of time in an effort to cure or prevent disease. The use of medicines from natural ingredients is believed to reduce the cost of treatment which is relatively expensive, one example is the Genjah Salak Coconut (*Cocos nucifera* L). This type of plant is in the category of palm species with smaller-sized fruit compared to other palm plants. Genjah salak cocnut comes from Pematang Panjang, South Kalimantan. It begins to bear fruits at the age of 24 months and to harvest at 36 months-old, and it could produce 80 – 120 fruits per tree. Coconut water has been used as a traditional medicine to cure many kinds of disease, and the previous research has shown that coconut water can be used as a replacement of body fluids. Coconut water contains simple carbohydrates which are glucose, sucrose, and fructose. It also contains some protein and mineral such as potassium, sodium, calcium, and magnesium. Moreover, vitamin C and B complex can also be found in coconut water. The glucose, electrolyte, vitamin, and protein in coconut water can be used as a substitute of body fluids, as a source of energy and to accelerate rehydration.

Hypertension is a condition where an increase of blood pressure above normal indicated by the systolic and diastolic occurs. Generally, a person is diagnosed with hypertension when the systolic/diastolyc blood pressure has gone over 140/90 mmHg. Hypertension could be specified into two categories; the primary, where the cause has not been figured out yet, and the secondary, which has certain causes like renal dysfunction, oral contraceptives, and the disruption of hormonal balance, which is a factor to adjust blood pressure. A research on hypertension-control using coconut water showed a significant decrease of the systolic/diastolic blood pressure (T Alleyne, *et al.*, 2005). Another research showed "tender" coconut water could prevent and stabilize high-blood pressure caused by fructose-rich diet that might have effects to the hypolipidemic (D Bhagya, et al., 2010). A research on mice as a biological trial in University of Kerala India showed that the mice immune system against heart disease increased after consuming coconut water. Twelve (12) out of 24 mice being tested consumed the coconut water, and as a result they were hindered from heart disease problem. The research team believes that coconut water is able to help cardiac output due to the contents of potassium, calcium, and magnesium (Indo Asian News Service, 2002). According to Oslon, et al., (1984), Karyadi and Muhilal (1988), to consume high potassium shall reduce hypertension. In physical exercise, someone could get exposed to hypertension caused by frequent stress, specialy in high risk sport. The stress is caused by frequent feeling of nervous or fearful in encountering the high risk match. Some types of sport that cause such stress are rock climbing, motor racing, car racing, taekwondo, and boxing. Hypertension can be caused by excessive intake of sodium in the extracelular fluids. A way to normalize is by enriching potassium consumption, which functionate to withdraw fluids from the extracelular so that the blood pressure is reduced. Coconut water has been proved to contain the most potassium, and this research will also prove about the effects of 5-7 months old "genjah salak" coconut water (cocos nucifera L) within a content range of glucose 0,38 – 0,61 % against the heart rate of male wistar mice.

I.2. Specification of Problems

The problems in this research are:
1. How high the heart rate frequency increase is in a group of mice fed with aquadest

2. How high the heart rate increase is in a group of mice fed with "genjah salak" coconut water.
3. How high the heart rate increase is in a group of mice fed with atenotol (comparison)
4. How high the heart rate increase is in a group of mice fed with "genjah salak" coconut water and atenotol combined.
5. How high the distinction of heart rate frequency is among the group of mice with aquadest, the group of mice with genjah salak coconut water, the group of mice with atenotol, and the group of mice with "genjah salak" coconut water – atenotol combined

1.3. Purpose of Research

The purpose of this research are:
1. To figure out the proportion of the heart rate frequency increase in the group of mice fed with aquadest
2. To figure out the proportion of the heart rate frequency increase in the group of mice fed with "genjah salak" coconut water
3. To figure out the proportion of the heart rate frequency increase in the group of mice fed with atenolol (comparison)
4. To figure out the proportion of the heart rate frequency increase in the group of mice fed with combination of "genjah salak" coconut water and atenolol.
5. To figure out the distinction of heart rate frequency among the group of mice with aquadest, the group of mice with "genjah salak" coconut water, the group of mice with atenolol, and the group of mice with "genjah salak" coconut water and atenolol combined.

II. Research Methodology

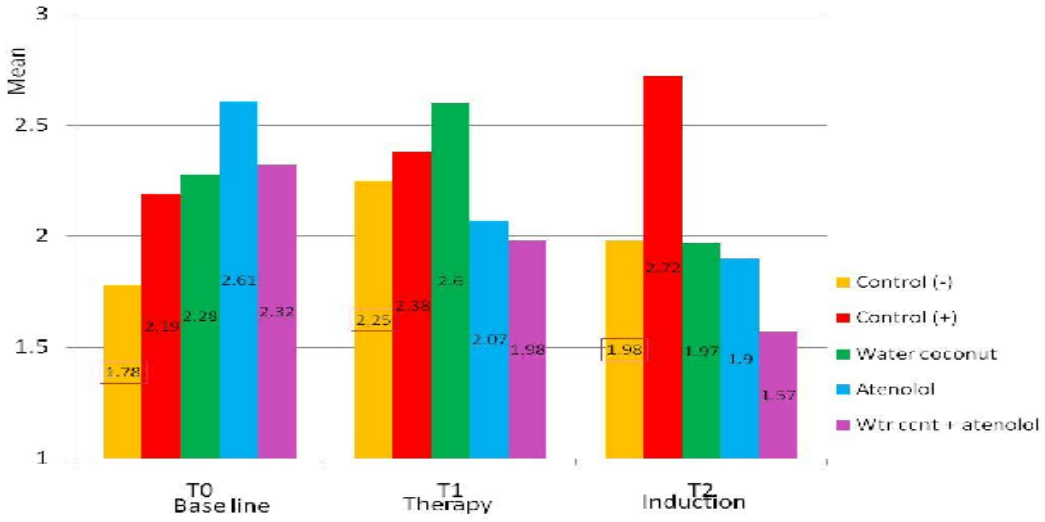
The research methodology applied is an experiment methode by observing the result of preclinical trial to the male wistar mice that is divided into five groups, namely, the negative control group, the positive control (aquadest) group, the "genjah salak" coconut water trial group, the atenolol trial as a comparison group, and the "genjah salak" coconut water and atenolol combined trial group. The trial materials is administered before the induction with adrenaline and heart rate frequency measured with a Tail Cuff before the therapy (T0), after the therapy (T1), and after induction (T2).

III. Research Results

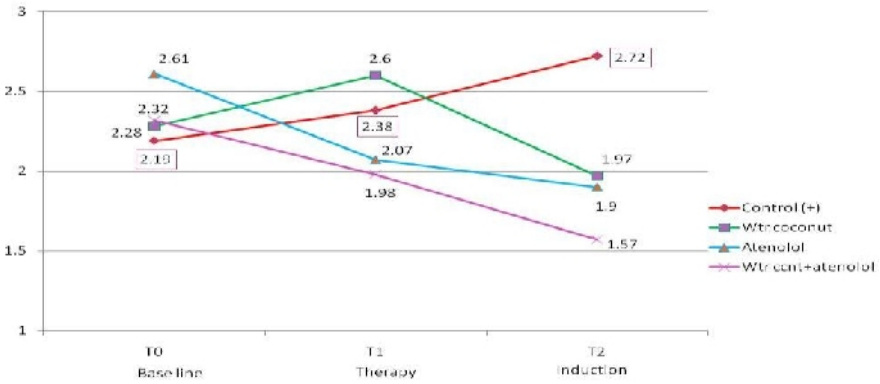
3.1. Table 1. Analysis of heart rate frequency distinction of mice after the trial material given

Group	Frequency (Hz)		
	T-0	T-1	T-2
Control negative	1.78 ± 0.23	2,25 ± 0,14	1,98 ± 0,23
Control positive	2,19 ± 0,03	2,38 ± 0,15	2,72 ± 0,25
Coconut water	2,28 ± 0,94	2,60 ± 0,32	1,97 ± 0,53*
Atenolol	2,61 ± 0,61	2,07 ± 0,68	1,90 ± 0,57*
Coconut water+Atenolol	2,32 ± 0,54	1,98 ± 0,45	1,57 ± 0,33*

3.2. Diagram 1. Heart rate frequency of mice in each group (2 minutes observation)



3.3. Diagram 2. Comparison of heart rate frequency of mice among the groups (2 minutes observation)



IV. Discussion and Conclusion

4.1. Discussion

4.1.1. A 2,72 ± 0,25 increase of mice's heart rate frequency occurs in the group of mice with aquadest given (positive control)

4.1.2. There is no distinctive heart rate frequency increase on the frequency before and after the therapy in the group of mice with "genjah salak" coconut water given within a frequency of 1,97 ± 0,53*

4.1.3. There is no distinctive heart rate frequency increase on the frequency before and after the therapy in the group of mice with atenolol given within a frequency of 1,57 ± 0,33*

4.1.4. There is no distinctive heart rate frequency increase on the frequency before and after the therapy in the group of mice with "genjah salak" coconut water and atenolol combined within a frequency of 1,57 ± 0,33*

4.1.5. There is no distinctive heart rate frequency increase on the frequency before and after the therapy amongst the group of mice with "genjah salak" coconut water, the atenolol group, and the coconut water and atenolol combined group

4.2. Conclusion

4.2.1. Genjah salak coconut water, atenolol, and the combination of coconut water and atenolol could obstruct the heart rate frequency increase that is caused by adrenaline adduction

4.2.2. A combination of coconut water and atenolol generates a better effect compared to atenolol alone, in order to obstruct the increase of heart rate frequency

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Identification on Indonesian Accessions of *Curcuma xanthorrhiza* Roxb. Using AFLP Markers

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Abstract

Study on bioactive compounds of *curcuma* and their effect on human health have been carried out in many research institutions. *Curcuma xanthorrhiza*, or Java turmeric, has been used in pharmaceutical industries in Indonesia. In spite of this commercial value, the identity of this species is commonly mistaken from other similar orange rhizomes *Curcuma.* In addition, *curcuma* has many cultivars with highly similarity to each others in term of morphology. They are hardly recognized from herbarium specimens. Correct identity of these species is crucial in pharmaceutical researches. Genetic variation of Indonesian *curcuma* could be identified using molecular biological approach based on DNA sequencing and PCR technique. AFLP marker analysis was performed by ligating DNA adapters to the ends of *Eco*RI and *Mse*I, followed by selective amplification with adapter-homologous primers. The selectively amplified samples were loaded on gels for separating DNA fragments, and then were visualized by silver staining. The polymorphisms in conjunction with DNA fingerprinting analyses will be carried out in order to recognize the elite local cultivars or ecotypes. Data were analyzed by automatic scoring using Genemapper version 3.7. The phylogenetic relationships among 13 accessions of *C. xanthorrhiza* showed that samples in our study were divided into 3 groups. The genetic diversity of all genotypes clearly showed that temu putih (*Curcuma sp.*), as control, and the accession derived from Ambon was out grouped and separated from a major group. Each primer pair resulted in various numbers of fragments from each *Curcuma* accession. A total of 2,530 fragments were detected using GeneMapper analysis. The length of fragments produced by AFLP primer combination is ranged from 50 – 500 bp. Specific fragments was identified on certain accessions which resulted from specific primers combination. The findings revealed that specific AFLP marker with 28 primer combinations could differentiate the *C. xanthorrhiza* accession from others.

Keywords: AFLP marker, *Curcuma xanthorrhiza* accession, DNA fingerprinting, genetic variation, phylogenetic tree.

Introduction

Researches on bioactive compounds of curcuma and their effect on human health have been performed intensively in Indonesia, as well as in other countries. Curcuma has many cultivars and types with highly similarity to each others in term of morphology and cytology. They are hardly recognized from herbarium specimens. In vegetative states, they are also difficult to

identify. This make curcuma is more difficult to identify and often causes misidentification of species. Many *Curcuma* species have medicinal value. In spite of this commercial value, the identity of this species is commonly mistaken from other similar orange rhizomes *Curcuma*, such as *C. soloensis, C. euchroma, C. colorata* and *C. brog.* Correct identity of these species is vital in pharmaceutical researches. One method for this purpose is accomplishing the inventory and characterization of this species phenotypic and genotypically.

Curcuma xanthorrhiza Roxb is one of the potential medicinal plant belonging to Zingiberaceae family that has not been well studied at the molecular level. *C. xanthorrhiza* is known as Java turmeric and is traditionally utilized as food essential and medicine in Southeast Asia. It has good prospect in regional and international markets. As a raw material for traditional medicine industries, curcuma is constantly used in large amount. According to Chairman of the Indonesian Herbs and Traditional Medicines Entrepreneurs Association (GP Jamu), 70% of 1,243 jamu producers are using *C. xanthorrhiza* as their main material (Charles Saerang, personal communication, 2008). This plant is broadly distributed in Asia, such as China, India, Malaysia, Sri Lanka, Burma, Thailand, Vietnam (Ardiyani and Skornickova, 2008). Furthermore in Indonesia, this species is growing widely in larger islands such as Sumatera, Jawa, Kalimantan, Sulawesi, Nusa Tenggara, Bali, and Maluku. It is very important to protect Indonesian germplasms, especially curcuma from their extinction or being claimed by other countries. *C. xanthorrhiza,* which is well-known as *temulawak,* has been extensively used in pharmaceutical industries in Indonesia.

Genetic variations contributed to phenotypic diversity in nature, and are resulted from mutation or recombination. This variation is associated to the sequences variation on DNA bases (Duran *et. al*., 2009). Genetic variation of plant species could be identified using molecular biological approach based on DNA sequencing and PCR technique (Omoto and Lurquin, 2004). Genetic variation would be recognized after genomic DNA analyzes. Whole genome sequencing is useful to observe into evolution, and the mechanisms involved, functional genomics, as well as valuable genes identification related to active compounds (Saunders *et al*., 2001). DNA markers are considered constant landmarks in the genome. Markers are identifiable DNA sequences found at specific locations of the genome and transmitted by the standard laws of inheritance from one generation to the next. Molecular markers have revealed a potential to detect genetic diversity and to assist in the management of plant genetic resources (Duran *et. al*., 2009). *Amplified fragment length polymorphism* (AFLP) is one of the DNA marker techniques, which is a reliable, highly reproducible and does not require any prior knowledge of the genome sequence itself. Using this marker, even small amounts of genomic DNA can be employed to generate polymorphisms. AFLP identifications on plants species have been studied in soybean (Lin *et al*, 1996), *Caladium* (Loh *et al*., 1999), Dioecious (Mwase, *et al.*, 2007), Zingiberaceae (Ghosh, *et al.*, 2011), as well as *Curcuma* (Islam, 2004; Keeratinijakal *et al*., 2010; Jatoi *et al*., 2010; Das *et al*., 2011).

In our study, AFLP marker was applied to recognize and elucidate the polymorphic patterns of Indonesian *C. xanthorrhiza* accessions. Therefore, the purpose of this study was to identify the variation among *C. xanthorrhiza* accessions in Indonesia. The polymorphisms in conjunction with DNA fingerprinting analyses, that is highly specific to particular accession, will be carried out in order to recognize the elite local cultivars or ecotypes.

Material and Methods

Plant Material Collection

Plant material used in this research is collected from many places in Indonesia. These accessions were Bengkulu, Lampung, Ciamis, Boyolali, Semarang, NTB, Sulawesi Utara, Buru Island, Ambon and Merauke. Some samples were taken from forest, which were marked as wild type (WT), these samples were Semarang WT, Buru Island WT, and Merauke WT. Temu putih was included into analyses as an out-group control.

Genomic DNA Isolation

Total Genomic DNA was isolated separately from young leaves of each accession samples using a modification of the cetyltrimethyle ammonium bromide (CTAB) procedure. After RNase treatment, the DNA concentration was determined both by Spectrophotometer NanoDrop (ND-1000) at wavelength of A 260/280 and by comparison with standard DNA, electrophoresed on a 0.8% agarose gel and the DNA was diluted in 1x TAE buffer to a concentration of 15 ng/µl for PCR analysis. For visualization, 0.3 µl Sybr safe was added into the agarose gel.

PCR amplification and purification

Polymerase Chain Reaction (PCR) amplification was employed using primers, which produced double-stranded DNA of approximately 800 bp length. PCR amplification was performed in a thermal cycler (Takara). The PCR reaction volume mixtures of total volume 25 ml in 0.2 ml PCR contained 34.5 ml sterile distilled water, 5.0 ml of 2mM dNTP mix, 5.0 ml of 10x Dynazyme™ reaction buffer, 2.5 ml of 50 mM $MgCl_2$, 1.5 µl of 10 mM of each primer, 0.25 ml of 5U/ml, and 0.5 µl genomic template DNA. The PCR cycle parameters for ITS amplification were as follows: initial denaturation for 3 min at 94^oC; denaturation of template DNA for 1 min at 94^oC; primer annealing for 1 min at 55^oC; primer extension for 1.5 min at 72^oC. After 30 cycles, a final extension step of 5 min at 72^oC was added.

AFLP Markers Analysis

AFLP analysis is performed as described by Vos *et al.* (1995). The digested fragments were ligated with *Eco*RI and *Mse*I adapters, followed by amplification with adapter-homologous primers. For pre-selective amplification, 25 µl of a mixture of template DNA, $MgCl_2$ 25 mM, dNTP 2 mM, 10x buffer dream Taq, dream Taq, primer *Eco*RI, primer *Mse*I, and ddH_2O was loaded into PCR. The template DNA was a product of digested ligation, after 10 times dilution. The PCR condition was 20 cycles of denaturation of template DNA for 30 sec at 94^oC; primer annealing for 1 min at 56^oC; and primer extension for 1 min at 72^oC.

For selective amplification mixture was template DNA (5 µl), $MgCl_2$ 25 mM (2.5 µl), dNTP 2 mM (0.5 µl), 10x buffer dream Taq (2.5 µl), dream Taq (0.175 µl), dye primer *Eco*RI-NNN (1 µl), primer *Mse*I-NNN (1 µl), and ddH_2O. The template DNA was a product of pre-selective amplification, after 10 times dilution. The PCR condition was 12 cycles of the first step for 2 min at 94^oC; the second step for 30 sec at 94^oC; the third step for 30 sec at 65^oC. At this step temperature was reduced 0.7^oC per cycle. The forth step for 2 min at 72^oC, and then the fifth step was returned to the second step above. The PCR process was continued for further 23 cycles, which are the sixth step for 30 sec at 94^oC, the seventh step for 30 sec at 56^oC, the eighth step for 2 min at 72^oC, and the ninth step was returned to the sixth step above. Followed by the tenth step for 10 min at 72^oC, and finally stop at 4^oC.

The selectively amplified samples were loaded on polyacrylamide gels for separating DNA fragments, and then were visualized by silver staining. Primer combinations were selected as describe in Table 1.

Data were analyzed by automatic scoring using GeneMapper® version 3.7 software, which formed scoring number and produce binary data of 1 and 0 for accessions analyzing (Chial, 2008). Numeric 1 and 0 refer to the peaks detection (DNA fragments) or no peak (without DNA fragments), respectively. Furthermore, the peaks in the electrophenogram can be distinguished by polymorphic peak and general peak.

Results and Discussion

We have collected some *C. xanthorrhiza* Roxb accessions used for mother plants which derived from many places in Indonesia. The plants were maintained in the screen house at Biotech Center BPPT, Serpong.

A genetic variation on *C. xanthorrhiza* Roxb was recognized after genomic DNA of 13 accessions were extracted and analyzed. AFLP marker analysis was performed by ligating DNA adapters to the ends of restriction fragments of *Eco*RI and *Mse*I, followed by amplification with adapter-homologous primers. The results showed that DNA concentration ranged from 398.6 – 2,880.6 ng/µl and the purity at wavelength of 260/280 nm ranged from 1.83 – 2.08.

Phylogenetic analysis using parsimony illustrated that samples in our study were clustered into 3 groups. Temu putih (*Curcuma sp*.), as control, and the accession from Ambon were out grouped and separated from a major groups (Figure 1). In addition, even the accession from Ciamis and Merauke were joined in a major group, however their sequences differences were distant from others. Semarang and Sulawesi Utara accessions created the first group, while Boyolali, Lampung and Merauke WT accessions were put together in the second group. Moreover, the other samples which were Bengkulu, NTB, Buru Island, Buru Island WT, and Semarang WT were clustered in the last group. Accessions were arranged in the same group if they have no or little variation in their sequences. These results suggest that migration has taken place for our *C. xanthorrhiza* in Indonesia. People from Jawa who immigrated to Sulawesi Utara might have brought *C. xanthorrhiza* with them and cultivated it in their new home. Accordingly, *C. xanthorrhiza* accessions derived from these areas, Jawa and Sulawesi Utara, were closely related.

Comparable study was performed on *Curcuma comosa* accessions collected throughout Thailand (Keeratinijakal *et al*., 2010). The phylogenetic tree derived from AFLP data showed that the *Curcuma comosa* accessions were divided into five clusters. Their results also suggested that there were other *Curcuma* species that were misused as *C. comosa*. The AFLP fingerprint data along with the morphological data offered the solution for the accurate identification of *C. comosa* from the other three related species.

Variations of DNA sequences among *C. xanthorrhiza* accessions may result in differences of DNA fragments sizes, and due to variation in enzyme restriction sites. The differences in size of DNA fragments occur as a consequence of DNA markers are distributed in the whole genome. DNA markers that contain differences in a single nucleotide are recognized by the restriction enzymes and specific primers. These variations contributed in DNA polymorphisms on polyacrylamide gels (Omoto and Lurquin, 2004).

The length of fragments produced by AFLP primer combinations were ranged from 50—500 bp. Number of alleles detected by each primer combination were also varied among accessions. The specific bands can be applied to identify the certain accessions of *C. xanthorrhiza*. DNA Fingerprinting analysis has been performed on *C. xanthorrhiza* using AFLP markers. Each primer pair gave rise to various numbers of AFLP fragments from each *C. xanthorrhiza* accession. A total of 2,530 AFLP fragments were detected in this study using GeneMapper analysis.

Specific fragments were identified on certain accessions which resulted from specific AFLP markers or primers combination, which are E-ACC+M-CAC, E-ACC+M-CAG, E-ACC+M-CAT, E-ACC+M-CTC, E-ACA+M-CTC, E-ACA+M-CTG, E-ACA+M-CTT, E-ACT+M-CAA, E-ACT+M-CAT, E-ACT+M-CTG, and E-ACT+M-CTT. Our result showed that specific AFLP marker with 28 primer combinations could differentiate the *C. xanthorrhiza* accession from others (Table 2). Each primer combination may identify 1 – 2 accessions. For example, E-ACC+M-CAC (primer combination 1, see Table 2) can be applied to identify accession Semarang, which produces polymorphic peak or band at allele 11. Another example, *C. xanthorrhiza* accession Ciamis can be distinguished from others by using E-ACA + M-CTT (primer combination 10), which produces specific polymorphic peak or band at allele 3.

Same finding was observed by Lin *et al*. (1996) that applying 60 AFLP primer pairs in their experiment. Fourteen identical soybean cultivars can clearly be differentiated. Likewise, 17 selective primer combinations differentiated the closely related *Caladium* species and genetic differences between cultivars can also be established (Loh *et al*. 1999). Moreover Ghosh *et al*. (2011) used 7 selected AFLP primer pairs to distinguish sixteen collections of *Zingiber* species.

In conclusion, there were 28 unique AFLP markers that can be applied to differentiate or identify specific *C. xanthorrhiza* accession that shows the polymorphic peaks or bands.

Acknowledgements

Financial support for this study was provided by State Ministry of Research and Technology under the scheme of Incentive Research Program 2010-2011. We thank Dr. Ir. Listyani Wijayanti (Deputy Chairman of BPPT for Agro-industrial Technology and Biotechnology) for initial financial support on our preliminary studies.

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Appendix

Table 1. *Eco*RI and *Mse*I primer pairs used in our study

***Eco*RI Primer**	***Mse*I Primer**						
	M-CAA	M-CAC	M-CAG	M-CAT	M-CTC	M-CTG	M-CTT
E-ACC	E-ACC+ M-CAA	E-ACC+ M-CAC	E-ACC+ M-CAG	E-ACC+ M-CAT	E-ACC+ M-CTC	E-ACC+ M-CTG	E-ACC+ M-CTT
E-ACA	E-ACA+ M-CAA	E-ACA+ M-CAC	E-ACA+ M-CAG	E-ACA+ M-CAT	E-ACA+ M-CTC	E-ACA+ M-CTG	E-ACA+ M-CTT
E-ACT	E-ACT+ M-CAA	E-ACT+ M-CAC	E-ACT+ M-CAG	E-ACT+ M-CAT	E-ACT+ M-CTC	E-ACT+ M-CTG	E-ACT+ M-CTT

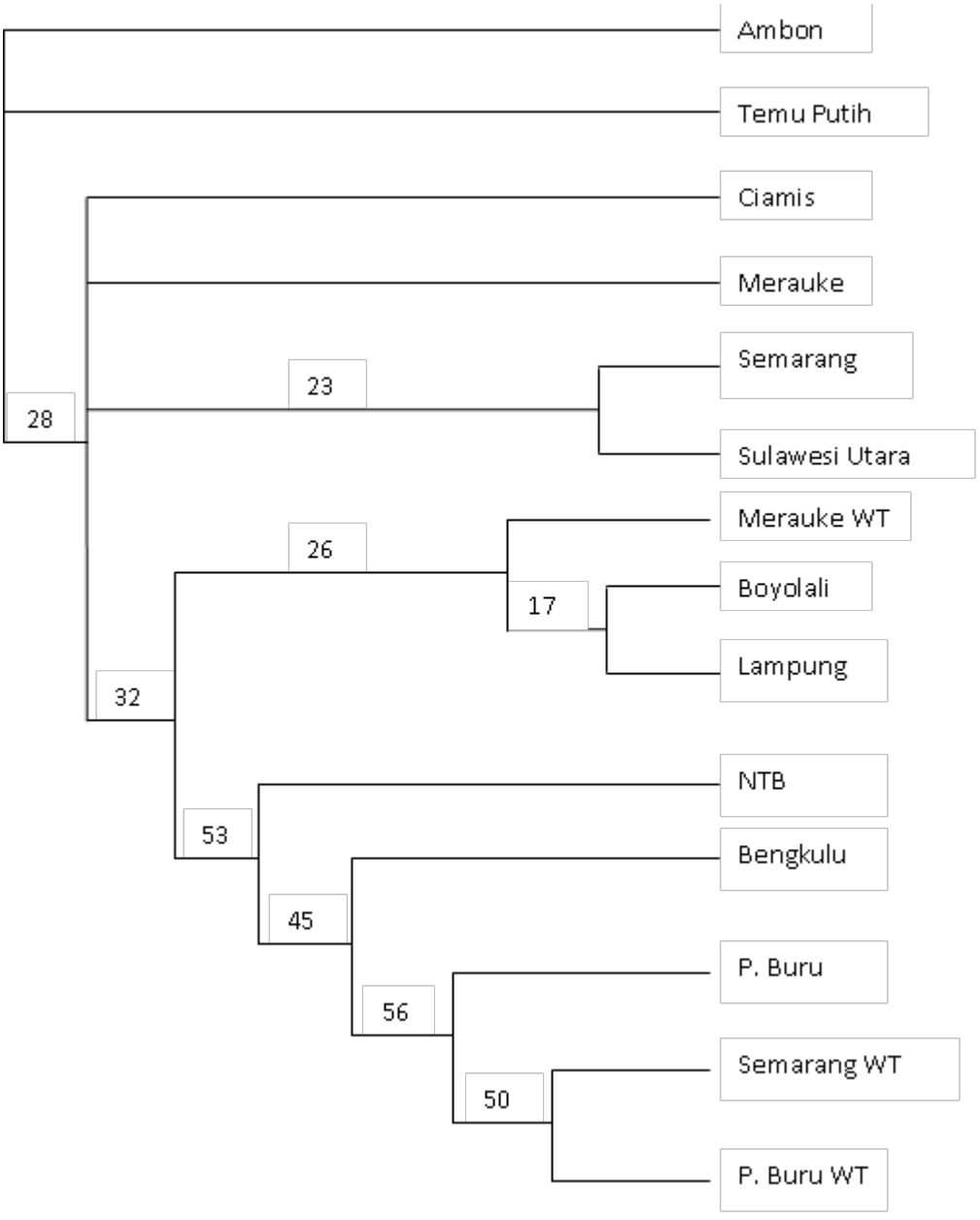

Figure 1. Phylogenetic relationships using parsimony (PAUP) on *C. xanthorrhiza* accessions

Table 2. Specific bands pattern can be applied to identify the specific accessions

Allele	Accession									
	1	2	3	4	5	6	7	8	9	10
Allele 2					2			5		
Allele 3			10							
Allele 7		4				4			2	
Allele 8	2						2			3
Allele 10	9				8					
Allele 11							1			
Allele 14									2	
Allele 16								4		
Allele 20	1	1								
Allele 24		7								
Allele 30				11						
Allele 33			3			3				
Allele 34			1				1		11	
Allele 35										3
Allele 44				4						
Allele 68						4				
Allele 132							7			
Allele 183										6

Note:

Accession

1. Bengkulu
2. Lampung
3. Ciamis
4. Buru Island
5. Merauke
6. Boyolali
7. Semarang
8. NTB
9. Ambon
10. Sulawesi Utara

Primer Combination

1.	E-ACC + M-CAC
2.	E-ACC + M-CAG
3.	E-ACC + M-CAT
4.	E-ACC + M-CTC
5.	E-ACT + M-CTG
6.	E-ACT + M-CTT
7.	E-ACT + M-CAT
8.	E-ACT + M-CAA
9.	E-ACA + M-CTG
10.	E-ACA + M-CTT
11.	E-ACA + M-CTC

Optimization Studies on Alkali Pretreatment of Biomass Waste of Oil Palm Empty Fruit Bunch Fiber for Production of Glucose

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Abstract

The use of fuel ethanol produced from biomass is promissing approach to reduce the fossil fuel and alleviate the global warming. Hence, the new technologies to produce ethanol from such as agricultural waste, are anticipated. However, there are two major technological problems for the efficient ethanol production from those materials (lignocellulosic biomass) : 1) cellulose the major component of lignocellulose is crystalline and dificult to hydrolyze; 2) pentose sugar, such as xylose and arabinose, exist in hemicellulose. To overcome those problem, the enhanced susceptibility of oil palm empty fruit bunch (EFB) fiber for hydrolysis, pretreatment should be done before hydrolysis. In this study alkali pretreatment of oil palm EFB fiber was conducted to improve hydrolysis EFB fiber for ethanol production. The oil palm EFB was crusher and chopped into small size, the alkali solution of NaOH 2 and 4 N was used to pretreated of EFB fiber. The treatment were carried in reactor at 150^0C using oil bath and residence time of 10 - 40 min. After pretreatment glucose and xylose were analysis using HPLC waters 717 plus auto sampler, however lignin content was analysis according methods of NRE. The solid treated with NaOH 4N solution show high lignin degradation and glucose availability.

Key words : *Lignocellulosic wastes, oil palm EFB, pretreatment, alkali, glucose, ethanol.*

Introduction

The use several lignocellulosic materials for ethanol fuel production has been studied exhaustively in many countries. Strong environmental legislation has been driving efforts by many enterprises, agency, university and institution to make ethanol from biomass economically viable. Production cost for ethanol from biomass has been decreasing year by year as a consequence of this massive effort. Pretreatment, enzyme recovery, and development of efficient microorganism are some promising areas of study for reducing process cost.

Biomass is basically cellulose microfibers surrounded by a mix of complex polymeric carbohydrates including xylan and glucomanan types are to some extent linked with another polymeric structure called lignin, extractives and mineral components are present in small amounts. This complex matrix makes cellulose inaccessible to hydrolytic enzymes. Many methods for making biomass more accessible to enzymes target is lignin or hemicelluloses removal (Teixeira et al,1999).

Oil palm frond constitutes the most important lignocellulosic material to be considered in Indonesia as new technology such as for production of ethanol fuel. At present most EFB is burned and because of its moisture content has a low value fuel. Ethanol production would promise in a value added product.

The present paper reported pretreatment with sodium hydroxide, the objective of this study was to develop an efficient alkaline pretreatment method for bioconversion of EFB into glucose.

Materials and Methods

Materials

Oil palm EFB used in this study was obtained from Changhae ethanol Co. LTD Korea during we trained program on Bioethanol Production Technology in May 2011, however the originaly of oil palm EFB used was obtained from global bio Diesel Sdn bhd, Sabah Malaysia. The oil palm EFB was soaked in detergent overnight to remove any residual oil, and then sun dried. The OFB then pretreated physically by chopped and hammer milled to get particle sizes of 1-3 mm, then it was stored in sealed plastic bag at room temperature until be used for chemical pretreatment.

Lab scale NaOH Pretreatment

In this study, pretreatment were carried out using a lab scale reactor. Two concentration of NaOH 2 and 4N solution were used to pretreat 5 g of ground EFB samples in oil bath at 150^0C for various lenghts duration of time 10-40 min to determine optimum prtreatment condition. After treatment and cooling, the treated EFB were washed several times with deionized water and dried at 55^0 C to fix the moisture.

Analytical methods

The soluble lignin and acid insoluble lignin content of EFB untreated as well as the solid fraction remaining after pretreatment were analysis according methods of National renewable Energy laboratory (NREL) using standard biomass analytical procedure[1)]. The carbohydrate content of untreated and pretreated EFB were determined by measuring the hemicellulose (xylose) and cellulose (glucose) derived sugars using High performance liquid chromatography (HPLC) waters 717 plus auto sampler. Before HPLC injection, all samples (derived and hydrolysate) were neutralized with calcium carbonate, centrifuge at 5000 g for 10 min and filtered through a 0.2 syring filter.

Results and Discussion

Characteristic of oil palm EFB

The chemical composition of oil palm EFB varies according to its growth location, season harvesting method, as weel as the analysis procedure. The composition of initial of EFB used before treatment is listed in Table 1.

Table 1. Major Component of oil palm EFB fiber

Component	Percentage (%)[a]
Cellulose	32.94
Hemicellulose	17.71
Acid insoluble lignin	26.44
Acid soluble lignin	3.17
Ash	1.34
Etc.	18.40

[a)] composition percentages are on a dry-weight basis

From Table 1, it can be seen that the carbohydrate fraction of EFB (cellulose and hemicellulose) were 50.61% of total biomass, and the major component were cellulose (32.94%), a polymer of glucose which is very potential as a sugar source for ethanol production. The acid insoluble lignin content of EFBs was 26.44%, comparable to the lignin

content of hardwoods (18-25%) (Syafwina et al, 2002). This high lignin content is the main reason of alkali (sodium hydroxide) pretreatment which would be applied to palm oil EFB. The main effect of alkali pretreatment on lignocellulosic biomass is delignification by breaking ester bonds cross-link lignin and xylan, thus increasing the porosity of the biomass (Silverstein et al, 2007) .

The effect of NaOH pretreatment on lignocellulosic components

An effective pretreatment is characterized by several criteria. The loss of lignin in the pretreatment is one of the most important indicators of pretreatment effectiveness because the presence of lignin impedes enzymatic hydrolysis of the carbohydrates (Mosier et al, 2005).

Alkaline pretreatment of lignocellulosic biomass is one of the most effective pretreatment methods which predominantly affect lignin content of biomass. The main effect of sodium hydroxide pretreatment on lignocellulosic biomass is delignification by breaking the ester bonds cross-linking lignin and xylan, thus increasing the porosity of biomass.

The precipitation palm oil EFB from the sodium hydroxide pretreatment was quantified for lignocellulose components. The degradation of lignin and hemicelluloses in the EFBs fiber after NaOH pretreatment are represented as the component loss of lignin and hemicelluloses, shown in Figure 1 and 3.

Lignin Component

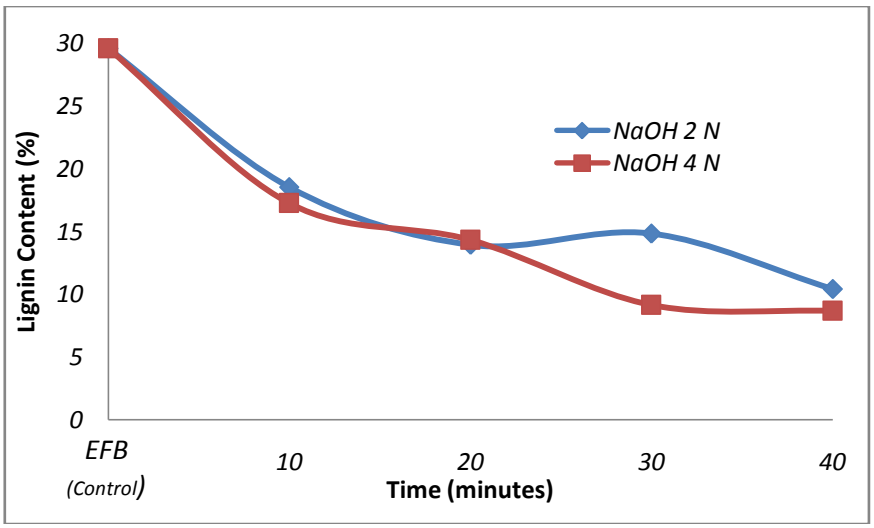

Figure 1. Loss lignin component during pretreated with NaOH 2 N and 4 N at 150 ^{o}C for 10,20,30 and 40 minutes.

Figure 1 shows the lignin component from NaOH pretreatment of palm oil EFB at 150 ^{o}C with NaOH concentrations and reaction time variables. Generally from this figure 1 occurs reduction of lignin contents as enhancing of reaction time. Pretreatment with a NaOH concentration of 4 N yielded not more significantly higher reducing lignin than 2 N. However after 20 minutes of reaction time the additional NaOH concentration can reduce effectively of lignin content. Higher NaOH concentrations means more base condition that it breaks up crosslinking among lignin in lignocellulose component. Based on this experiment, the highest reducing lignin content was obtained with 4 N NaOH for a pretreatment time of 40 minutes. These results prompted the used of higher NaOH concentrations at 150 ^{o}C.

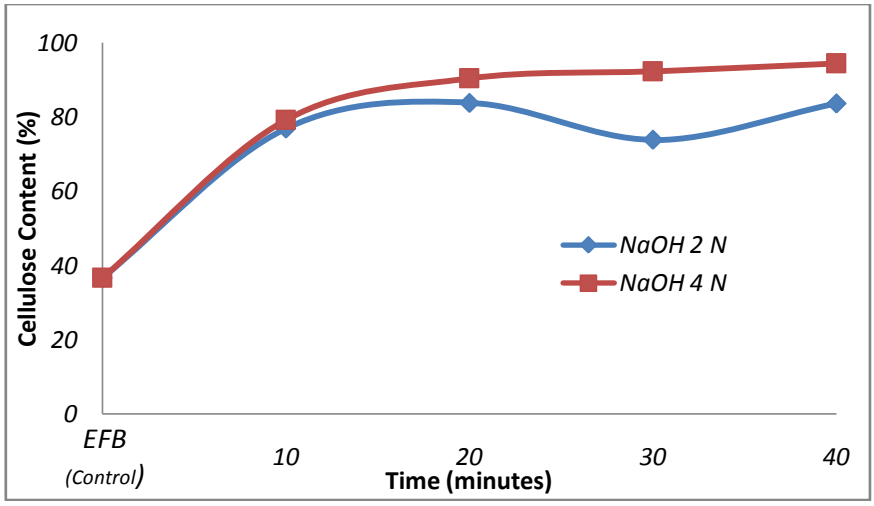

Figure 2. Cellulose component during pretreated with NaOH 2 N and 4 N at 150 ^{o}C for 10,20,30 and 40 minutes.

Figure 2 indicates that no statistical difference was observed between 2 N NaOH and 4 N NaOH for pretreatment at 20 minutes. But during reaction time of 30 and 40 minutes the NaOH concentration of 4 N yielded significantly higher of cellulose content levels than 2 N NaOH. Likely Figure 1 show that higher NaOH concentrations break up crosslinking ester bonds in lignocellulose component. Figure 2 also shows the increasing of reaction time increased cellulose content in palm oil EFB pretreated at 150 ^{o}C. Taherzadeh,M.J (2008) reported the alkali treatment appears to be the most effective method in breaking the ester bonds between lignin, hemicellulose and cellulose. The break up of lignocellulose chemical structure by alkali treatment means can reduce lignin and enhance cellulose contents in lignocellulose component.

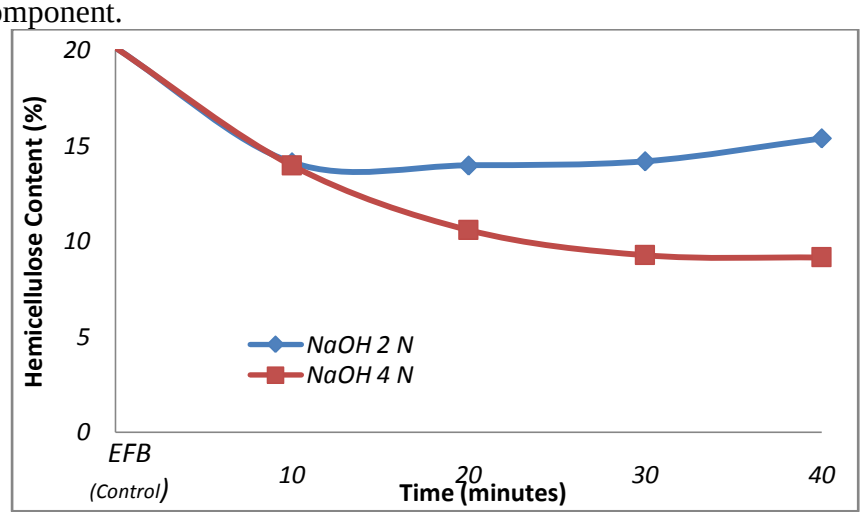

Figure 3. Loss hemicellulose component during pretreated with NaOH 2 N and 4 N at 150 ^{o}C for 10,20,30 and 40 minutes.

Hemicellulose component of palm oil EFB pretreated at 150 ^{o}C was presented in Figure 3. It can be seen that the increasing of reaction time caused decreasing of hemicellulose content. Pretreatment with NaOH concentration of 2 N and 4 N at 10 minutes have not significantly difference reducing of hemicellulose content. Extending pretreatment time beyond 10 minutes effected on hemicellulose content which 4 N NaOH concentration

was reduced up to reaction time of 40 minutes. At the same reaction time the different conditions occured on 2 N NaOH concentration that the level of hemicellulose linearly levels with additional of reaction time of 40 minutes. In this due the characteristic of hemicellulose is a semi-soluble in alkali solutions (Zheng,Yi et.al 2009). Based in this experiment prompted the used of higher NaOH concentrations at 150 ^{o}C.

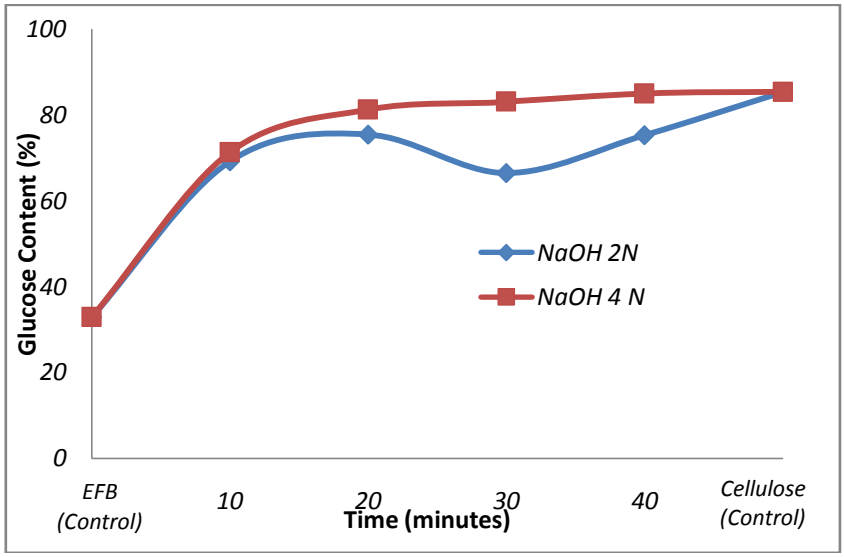

Figure 4. Glucose production for NaOH pretreatment 2 N and 4 N at 150 ^{o}C for 10,20,30 and 40 minutes

Analysis of monomeric sugars reveals that glucose production for pretreatment with 2 N NaOH were statistically similar to pretreatment with 4 N NaOH (Figure 4) for 10 minutes at 150 °C, and additional pretreatment time for 40 minutes glucose yield for pretreatment at 2 N NaOH were significantly lower than pretreatment at 4 N NaOH. Based on these results, optimal pretreatment conditions at 150 °C for glucose production are 40 minutes and 4 N NaOH. In this condition, NaOH concentration of 4 N were likely similar with raw material of pure cellulose as control for hydrolization process. The higher of NaOH concentration that means strong base , and it breaks up more chain structure of cellulose to glucose conversion.

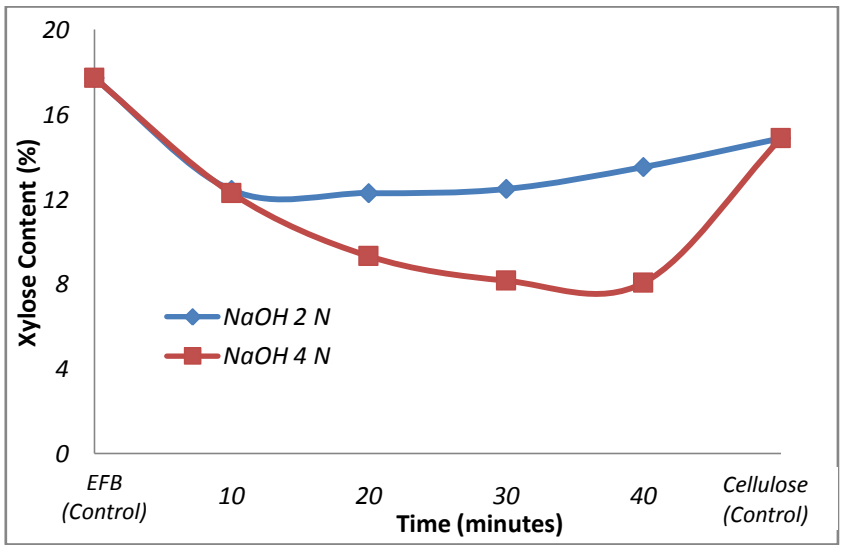

Figure 5. Xylose production for NaOH pretreatment 2 N and 4 N at 150 ^{o}C for 10,20,30 and 40 minutes

From Figure 5 shows that xylose content decreased with increasing reaction time at 150 ^{o}C which for 10 minutes there are no significantly difference between NaOH concentration of 4 N and 2 N. Extending pretreatment time were indicated NaOH concentration of 4 N more higher level decreased than NaOH concentration of 2 N. Because of this condition similar with hemicellulose production for pretratment. Based on these experiments, optimal pretreatment conditions at 150 °C for xylose production are 40 minutes and 4 N NaOH. Therefore, optimization of pretreatment conditions was conducted based on either glucose and xylose production or total reducing sugars production.

Conclusion

The efficiency of alkaline (sodium hydroxide) pretreatment of palm oil of EFB for bioethanol production was evaluated in this study. Based on the results to date, NaOH concentration of 4 N is more efficient than NaOH concentration of 2 N at 150 °C for pretreatment process. The optimal NaOH pretreatment conditions at 150 °C for glucose and xylose production are 40 minutes with NaOH 4 N.

Acknowledgement

We gratefully recognize the National Project of Indonesian Institute of Sciences (LIPI) of fiscal year 2011 for funding this research.

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ISSN: 2088-9771

Screening of Pediocin Gene Encoding Bacteriocin Isolated from Indigenous Indonesian Traditional Fermented Food

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Antimicrobial peptides called bacteriocins from lactic acid bacteria (LAB) are the interest of food industry as potential and economical natural food preservatives against spoilage and pathogenic bacteria. Bacteriocins LAB have gained recognition as safe and non-toxic natural food preservatives, additives to animal feed, and potential alternatives to conventional antibiotics. Among bacteriocins produced by LAB, instead of nisin, pediocins have gained consideration attention because of their remarkable heat stability, activity over a wide pH range, broader antimicrobial spectrum, and higher specifity and effectiveness in very low concentration. Pediocins are generally produced by pediococci strains such as *Pediococcus acidilactili* and *P. pentosaceus*. Previous studies on the antibacterial screening of LAB indigenous Indonesia from traditional fermented food carried in our laboratory revealed bacteriocin-producing LAB isolated from dadih (buffalo fermented milk), bekasam (fermented meat), and cacao fermentation have shown inhibition ability to the growth of pathogenic bacteria, mainly *Listeria monocytogenes*. An isolate producing the most efficient bacteriocin has been identified as *P. acidilactili* and *P. pentosaceus* based on 16SrRNA sequence analysis. Moreover, preliminary screening of *ped* gene encoding mature pediocin PA-1 showed PCR product of 700 bp. Since, the development of heterologous expression systems for bacteriocin production may offer several advantages over native systems such as achieving increased production levels

ISSN: 2088-9771

Isolation and cloning*Stearoyl-ACP Desaturase* (SAD) form Mesokarpoil palm (*Elaeisguineensis* Jacq.) var.Tenera

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ABSTRACT

Stearoyl-acyl carrier protein (ACP) desaturase (SAD) is a key enzyme of fatty acids synthetic metabolism in higher plants. SAD catalyzes desaturation of *stearoyl-ACP*, which introduces a double bond into the fatty acid chain to form *oleoyl-ACP* (unsaturated fatty acid). Therefore, SAD plays a key role in determiningthe ratio of saturated to unsaturated fattyacids in plants. An important objective of the oil palmto increase unsaturated fatty acid content in palm oil through conventional breeding is time consuming due to the long generation time. Theabove limitation make oil palm an idealcrop for the use of genetic engineering tools. Genetic engineering is basically identification of the SAD as target gene.

The first step total RNA was extracted from mesocarp*Elaeisguineensis*Jacq.var. Tenera. First strand cDNA synthesis of the upstream (5'region) and downstream (3'region) using *reverse transcriptase*with specific primer and oligo-dT as the primer. Then,dChomopolymeric tailing on the 3'-end of the 5'region cDNA.cDNA amplification through Rapid Amplification of cDNA Ends (RACE) PCRtechniques using gene specific primer. Based on the RACE PCR amplification of the upstream (5'RACE) and downstream (3'RACE) segment of SAD gene, a866 bp and 915 bp fragments were obtained respectively. The cDNA used as the insert for cloning SAD gene including ligation to pGEM-T *easy* (Promega) and transformation in host *E.coli* strain DH5α. Finally, the SAD gene sequencing using SP6 and T7 as the primers.Sequence analysis showed that the 5'region cDNA and 3' region cDNA of SAD obtained is 525 bp and 327 pb.Sequence analysis using Geneiousprogram showed 99,6% (5'region cDNA) and 92,2% (3'region cDNA) similarity with the sequence of gene encoding fromSAD *Elaeisguineensis*var. Tenera (Accsesion No U68756) deposited at the GenBank.

Keyword: *Elaeisguineensis*Jacq.,*stearoyl-ACP desaturase*, Mesocarp, RACE PCR, Cloning

ISSN: 2088-9771

TAPS and TTE Buffer as Mobile Phase in DNA Sequencing Using ABI Prism 310 Genetic Analyzer

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ABSTRACT

Running buffer is one of the important components in the process of DNA separation in capillary electrophoresis system. In addition to stabilize and dissolve the DNA, running buffer also serves as conductor in electrophoresis and accelerates the process of injection. This study aims to determine the composition of running buffer that can produce high resolution and optimum performance of DNA separation by capillary electrophoresis system, with lower cost per run.

The running buffer used in the ABI Prism 310 instrument consists of 100mM TAPS (N-tris-(hydroxymethyl) methyl-3-aminoprophane-sulfonic acid) and 1 mM EDTA, pH 8 (pH is adjusted by addition of NaOH). Basically, the running buffer for capillary electrophoresis can be formulated in various compositions, including Bis-Tris, TAPS and / or TAPSO as main components, with the addition of chelating agents, such as EDTA (Hacker, 2007) and urea (Albarghouthi & Barron, 2000). In this study, two running buffer formulations were evaluated by using the ABI Prism 310 Genetic Analyzer and its performance were compared with standard running buffer. The two alternative buffers are TAPS buffer (100mM TAPS with the addition of 1mM EDTA) and TTE Buffer (50mM Tris, 50mM TAPS, with the addition of 2mM EDTA). We use pGEM-3Zf(+) template and M13 primer in cycle sequencing, and POP6 as sieving matrix in 36cm thin capillary.

We evaluate the buffer effectiveness from performance and quality of sequencing results, based on raw data, electropherogram, S/N ratio, length of sequence reading, and its conformity with pGEM sequence. The results showed that there are no significant differences either in raw data or in S/N ratio between the three buffers. On average, TAPS buffer resulted the longest sequence reading compared with the rest buffers, and also showed the highest average signal intensity. TTE buffer results approximately equivalent length of reading and signal intensity with the standard buffer. From conformity analysis, high conformity with pGEM sequence was showed from all of buffers used. This indicates that TAPS buffer has the best performance, and both TAPS buffer and TTE buffer can be used as alternative buffers in DNA sequencing by using the ABI Prism 310 Genetic Analyzer.

Keywords: DNA sequencing, Capillary electrophoresis, running buffer, TAPS buffer.

ISSN: 2088-9771

The Quality of *Spermatozoa* and Histological Figure of *Tubulus Seminiferus* of White Rat (*Rattus norvegicus*, L.) Testis After Supplemented by Green Pea Sprout Juice (*Phaseolus radiatus*, L.)

Imam Fuad Zamzami

Abstract

The myth that green peanut may increase fertility lead to carry out the present research in proving its validity empirically. The research is aimed to find effect of green pea supplement on quality of *spermatozoa* (observed parameters: concentration, motility, viability and *spermatozoa* morphology) of white rat (*Rattus norvegicus,* L.), and to find the effect on ability of *tubulus seminiferus* in supporting processes of spermatogenesis (observed parameters: diameter of *tubulus seminiferus,* solidity of prospective *spermatozoa* cells, and histological figure of *tubulus seminiferus* microanatomy of white rat (*Rattus norvegicus,* L.) testis.

Green pea (*Phaseolus radiatus,* L.) sprouts used as juice supplement are derived from small-sized green peanut seeds that people commonly consume. The supplement is divided into 4 classes treating with concentration variations of 0%, 30%, 60%, and 100%). The experiment uses 16 white rats deriving from *strain Wistar,* three months olds with approximately weight of 200-300 grams. The supplements are feed orally 2 ml/1 rat/day for 49 days (one cycle of spermatogenesis on rat).

Generally, the results show that the green pea sprout juice supplement has positive effects on the increase of *spermatozoa* quality, viewed from concentration (less significant), motility (significant), viability (less significant), and morphology of white rat (*Rattus norvegicus,* L.) *spermatozoa*. The supplement has also positive effects on the increase of *tubulus seminiferus* ability in supporting processes of spermatogenesis, viewed from diameter of *tubulus seminiferus* (less significant), solidity of prospective *spermatozoa* cells (significant), and histological figure of *tubulus seminiferus* microanatomy of white rat (*Rattus norvegicus,* L.) testis. The supplement with concentration of 100% is recommended to be consumed routinely to enhance quality of *spermatozoa* and *tubulus seminiferus* ability in supporting processes of spermatogenesis.

Keywords: Fertility, sprout, green peanut (*Phaseolus radiatus,* L.), *spermatozoa,* spermatogenesis.

ISSN: 2088-9771

Application of ISSR Molecular Marker for Detecting Polimorphisme among 21 Accessions of Sambiloto (*Andrographis paniculata* (Burm.f.) Wallich Ex Ness) from North Sumatera, Jambi and West Nusa Tenggara

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Abstract

Inter-simple Sequence Repeat (ISSR) is a molecular marker system which allows detecting polymorphisms in inter-microsatellite loci without previous knowledge of a DNA sequence. ISSR is informative regarding numerous loci and are suitable to discriminate closely related genotype variants with simple and quick PCR-based method. The aim of this research was to detect polimorphisme among 21 accessions of sambiloto (*Andrographis paniculata* (Burm.f.) Wallich Ex Ness) collected from North Sumatra, Jambi and West Nusa Tenggara provinces. DNA amplification of sambiloto accession performed using five ISSR primers, namely: SBLT2, SBLT3, SBLT5, SBLT13 and SBLT15. The result obtained that among 21 accesion collected from North Sumatera and West of Nusa Tenggara had polimorphisme when amplification with SBLT2, SBLT3 dan SBLT15 primers whereas the accession of Jambi province have the same dna profile.

Keywords: ISSR, sambiloto, *Andrographis paniculata*, accesion, polimorphisme

ISSN: 2088-9771

Cytochrome c Oxidase 1 (COI) Mitochondria Gene Haplotype Variations of *Scirpophaga incertulas*

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One of the main insects caused damage yields in rice in Asia, *Scirpophaga incertulas* or yellow rice stem borer (Lepidoptera: Crambidae). Hence, we aimed to explore further the haplotype variations of the *S. incertulas* of COI gene to fulfill the haplotypes variation data in Indonesia, i.e. Central Java, Special Region of Yogyakarta (DIY), East Java, and Bali. Previous study found six haplotypes of *cytochrome c oxidase* 1 (COI) of *S. incertulas* from Java. Hence, we aimed to explore further the haplotype variations of the *S. incertulas* of COI gene to fulfill the haplotypes variation data in Indonesia, i.e. Central Java, Special Region of Yogyakarta (DIY), East Java, and Bali, This study revealed three new haplotypes (haplotype 7, 8, and 9) of *S. incertulas* COI sequences. Those new haplotypes of *S. incertulas* COI gene were found in Lumajang, Sleman, and Pemalang region. Database of COI haplotype variations of yellow rice stem borer can be used as marker to differentiate *S. incertulas* with other species of rice stem borers during the first larva instar for their early detection.

Keywords: mitochondrial genome, molecular detection, moth, Crambidae

ISSN: 2088-9771

Plant Parameters that Contribute to Reduce of Noise Levels

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ABSTRACT

Special Region of Yogyakarta is now very crowded with a motor vehicle. The number of vehicles is increase every year and not accompanied by improved facilities and infrastructure that can minimize the negative effects of the presence of motor vehicles such as sound pollution. Noise pollution is actually happening on the street highway and it can be reduced by using natural sound absorbers in the form of plants. However, it is very necessary that proper plant selection, plant-related parameters that most contribute to muffle the sound.
The research was done by creating a plot with the size of 10x10m. The laying of the plot was chosen at a location representative, that is all there is on the right side and left the jail the same sound source. Then the plants that exist in the plot measured stem height off the branches, canopy height, trunk circumference and density. Collection of statistical data were analyzed with multiple regression and Backward elimination.
The results of this study showed the indicate that with sound source distance of 10 meters from tree, plant parameters whose role is lepa branch stem height on average, at a distance of 20 meters of variables that play a role is the average width of the canopy, and at a distance of 30 meters whose role is high branch off the trunk. From these results the factor most responsible for the plant parameters are separated caban trunk and canopy width.

Key word: plant, noise levels, Backward elimination

ISSN: 2088-9771

Developing Locally-Made Laboratory Equipment by Modification Using TRIZ Method

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Most universities in developing countries are unable to supply equipment for science laboratory work. This is due to the lack of sufficient funds to purchase imported equipment and maintenance fee. To overcome the problem, we should produce locally made laboratory equipment. One of possibilities is we modify existing marketed products to fulfil labpratory work needs.

This work elaborate how to develop locally made laboratory equipment by modifying such common existingproductsusing TRIZ. The product being developed is radial chromatoghraphy which is still beyond the reach of many laboratories in developing country.TRIZ is used to develop modification ideas of the product and assure the product perform better while having reasonable development cost.

Keywords: locally made equipment, laboratory work, TRIZ, development cost

ISSN: 2088-9771

Antibacterial Activity on Singawalang (*Petiveria alliace*) Leaf to Mycobacterium Tuberculosis Sensitive Strain H37Rv and Multi-Resistant Strains Labkes Eh & Sr

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ABSTRACT

Antituberculosis activity was tested against leaf singawalang (*Petiveria alliacea*). Antituberculosis activity of the extract were tested against M. tuberculosis H37Rv sensitive and resistant to isoniazid, rifampin, ethambutol and streptomycin.

Leaves singawalang (*Petiveria alliacea*) extracted by maceration and reflux using 96% ethanol and water. Against these two extracts tested antituberculosis activity to know the difference with the extract preparation activities. Viscous extract is then tested its effect on the activity of M. Sensitive tuberculosis (H37Rv) and resistant to four primary antituberculosis drugs with comparator drugs rifampin, ethambutol, isoniazid, streptomycin and the control medium, followed by testing fractions hexane, ethyl acetate and water. The parameters tested is to compare the amount of microbial growth from week 4 to week 8.

The test results showed that ethanol extract of leaves singawalang (*Petiveria alliacea*) active against M. tuberculosis sensitive and resistant in 1280 and 2560 ppm concentration against LJ medium used, while in the water extract of leaves singawalang (*Petiveria alliacea*) showed no activity at concentrations of anti-tuberculosis used. Antibacterial activity on M. Tuberculosis H37Rv strains sensitive and resistant to water and ethyl acetate fraction gave the antibacterial activity with MIC value of each fraction is 1280 ppm.

Comparator drug rifampicin (40 mg / ml) and streptomycin (4.0 mg / ml) are active against M. tuberculosis H37Rv and were resistant to ethambutol and isoniazid, INH (0.2 microg / mL) and ethambutol (2.0 ug / mL) active against M. tuberculosis H37Rv and were resistant to rifampicin and streptomycin.

Key words: singawalang, anti-tuberculosis

ISSN: 2088-9771

Effect the Variation Kascing Fertilizer on Growth Plant of Green Mustard (*Brassica juncea* L.)

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This study aimed to determine the effect of variations in dose of kascing fertilizer on plant growth green mustard (*Brassica juncea* L.). The study was conducted in a greenhouse integrated laboratory UIN Sunan Kalijaga Yogyakarta in January to February 2011. The design used the Completely Randomized Design (RAL) with 5 treatments 3 times the replications. The treatment given is K0 (0 g / plant), K1 (30 g / plant), K2 (60 g / plant), K3 (90 g / plant), and K4 (120 g / plant). The treatment dose of kascing fertilizer variations affect plant height, number of leaves, wet weight and dry weight of green mustard plants. From the results showed that administration of fertilizer kascing significant effect on the development of the green mustard plant. Kascing fertilizer than as a nutrient source for plants can also improve the texture of the soil which can eventually increase the production of green mustard plants. Can be seen from the results of the study, found that the provision of fertilizer kascing significant effect on almost every developmental parameters were observed. The highest yield on the 90 g dose variation/ plant that is on the parameters of plant height and plant dry weight. While the number of leaves of the highest dose of 120 g / plant and wet weight at doses of 60 g/plant. Can be seen from the results of the study, found that the provision of fertilizer kascing significant effect on almost every developmental parameters were observed. The highest yield on the 90 g dose variation / plant that is on the parameters of plant height and plant dry weight. While the number of leaves of the highest dose of 120 g / plant and wet weight at doses of 60 g / plant. This can occur because of dosing possible are able to provide the nutrients needed by the mustard plants. So the plants can grow well in comparison with a lower dose. From the results of ANOVA analysis showed significant results with 38 003 F count on the development of plant height, H0 is rejected because the F count more> than the F table. While the development of counting the number of leaves F 27.15.

Keywords : Dose Kascing Fertilizer, Plant Growth green mustard.

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