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Production of Crude Uricase Enzyme by Novel *Bacillus altitudinis* Strain W.IIISRNs_1.1 From The Hot Spring of Mataumpana, Buton Regency, Southeast Sulawesi

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Abstract
Uricase is an oxidoreductase enzyme that plays a specific role in the process of purine metabolism, especially in degrading uric acid into water-soluble compounds, they are allantoin, CO_2, and H_2O_2. Some sources of uricase include microorganisms, higher plants, and animals, except humans. *Bacillus altitudinis* strain W.IIISRNs_1.1 from the hot spring of Mataumpana is the focus of this work, which works to produce and characterize the uricase enzyme from this novel isolate.The clear zone formed around a colony after being cultured at 45ºC in a solid Glucose Yeast Peptone (GYP) medium containing 0.2 % of uric acid shows bacteria's capacity to digest uric acid.The morphological and physiological identification of strain W.IIISRNs_1.1 is a group of bacteria from *Bacillus* genus,and 16S rRNA analysis identified strainW.IIISRNs_1.1 as *Bacillus altitudinis.* The result of this study shows that the optimum optical density was 1.17 (60 hours of incubation), which was not following the incubation time for the optimum production of the uricase enzyme, which was 36 hours with enzyme activity 0.5198 U/mL and protein concentration of 1.2819 mg/mL. Uricase from *Bacillus altitudinis* strain W.IIISRNs_1.1 best produced with substrate concentration 0.2% of uric acid, worked optimally at 45ºC and pH 8, stable at 45ºC for 2.5 hours, pH 8 and 9 for 1.5 hours. The maximum speed of uricase enzyme work occurred at the substrate concentration (uric acid) 3.5 mM.The uricase enzyme is activated by several metal ions, namely Ca^{2+}, K^{+}, and Ba^{2+} with a concentration of 0.1 M. Enzymes were significantly inhibited by 0.1 M Zn^{2+}, Co^{2+}, and 0.05 mM Na^{+}, Zn^{2+} ions. Uricase from *Bacillus altitudinis*strain W.IIISRNs_1.1 is a uric acid degrading enzyme from a novel source and has not been produced before. This enzyme is potential to be developed in biochemical and clinical applications.
Keywords: Uric acid; Allantoin; Uricase enzyme; *Bacillus altitudinis*; Hotspring

1. Introduction

Uricase (urate oxygen oxidoreductase, EC 1.7.3.3) is an enzyme in the purine degradation pathway which catalyzes the enzymatic oxidation of uric acid to allantoin[1, 2], which is more soluble and easily excreted than uric acid and followed by the formation of carbon dioxide and hydrogen peroxide[3]. Uric acid is one of the primary end products of purine nucleic acid metabolism in humans, which is released into the blood serum or excreted by the kidneys in the urine and intestines[4-8]. The presence of uric acid in the body can be used as an indicator for early warning of various diseases and physiological disorders[9]. The solubility of uric acid in humans is low, and the average blood uric acid level is very close to the uric acid solubility.Normally,concentration of uric acid in urinaryexcretionis the variety of 1.4 to 4.5 mM, and in serum is between 0.13 to 0.46 mM[4, 9, 10]. When the level of uric acid in the blood exceeds its solubility, uric acid crystals form and accumulate in the blood[11]. The buildup of uric acid in the blood plasma results in a hyperuricemia condition, commonly gout[7, 12], hypertension, cardiovascular disease, and renal disease[13]. This excess uric acid will be stored in tissues and joints, causing inflammation[14]. Due to the absence of uricase in the human body, uric acid cannot be further metabolized, and this condition occurs[15]. Therefore, intravenous administration of uricase is an alternative to treat diseases caused by uric acid[12].

Uricase is a good potential enzyme in the medical field[16]. Because it is used as a diagnostic reagentin clinical biochemistry that involves in the detection of

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uric acid concentration ini blood and other biological fluids[17, 18]. Several studies have used uricase as an immobilized-uric acid receptor biosensor, such as Fukuda et al[1], Lokesh Rana et al[9], Natalia Stozhko[19], Zahra Karami et al[10] and SofiiaTvorynska et al [20]. Moreover, Rasburicaseas a protein medicine for treating hyperuricemia. Direct uricase injection is preferable in cases of gout associated with renal complications[2, 17].

Various organisms, including animals, plants, fungi, and bacteria, except humans, generate uricase[2, 3, 17]. Overall, microbial uricase sources are better because of their higher growth rates, simplicity in production, purification, easy optimization of the medium, and it is cost-effective bioprocessing[2, 21]. The uricase enzyme has been widely produced from several microbial sources, including *Bacillus cereus*[2], *Bacillus cereus* SKIII[17], *Bacillus subtilis,* and *Bacillus megaterium*[22], *Comamonas sp*.BT[23], *Pseudomonas aeruginosa*[24, 25], *Pseudomonas otitidis*[26], *Streptomyces rochei* strain AM 32 from soil in Egypt[27], and *Ochrobacterium anthropic* from soil poultry farm[15]. Many sources of the uricase enzyme have been found, especially from microbes, because this enzyme is increasingly important in treatment and diagnosis, new sources are constantly being sought to obtain enzymes with better quality[28].

One of the unique microbes that need to be studied is thermophilic bacteria from hot springs because they contain extracellular thermostable enzymes that make them able to withstand extreme environmental conditions, be it high temperatures, chemical reagents, or extreme pH, in this case including uricase as one of the extracellular enzymes. Research on the isolation and production of uricase enzyme from hot springs from the Malaysian Hot Spring was carried out by Nor Sahslin Irwan Shah Lee et al [26] Based on the ecosystem environment and habitat around the Mataumpana hot spring, Buton Regency, Southeast Sulawesi, it contains thermophilic microorganisms that produceuricase enzymes. In this study, we report for the first time the Production of CrudeUricase Enzyme from Isolates of Thermophilic Bacteria, *Bacillus altitudinis* strain W.IIISRNs_1.1 from Mataumpana Hot Springs.

2. Material and Methods

The materials used in this study include: isolate of thermophilic bacteria W.IIISRNs_1.1from sediment of The hot spring of Mataumpana, Glucose (Merck), Bacto agar (Sigma), yeast extract (Sigma), Bacto peptone (Sigma), $Na_2B_4O_5(OH)_4.8H_2O$(Merck), BH_3O_3 (Merck), $NaH_2PO_4.H_2O$ (Merck), K_2HPO_4, $MgSO_4.7H_2O$, NaCl (Merck), NaOH (Merck), uric acid (Sigma), beef extract (Sigma), Ethanol 75%,Aquadest, folin (Merck), Bovine serum albumin (Sigma).

The instruments usedwere:UV-Visible spectrophotometer (T60, PG Instruments),shaker water bath WB5-18(Labo), autoclave (Hiclave HG-50), incubator (Memmert),oven (Eyela NDO-400, Japan), vortex, Hot plate, micropipette, refrigerator, centrifuge (HERMLE Z 366 K), bunsen, needle ose.

2.1 Microbe Rejuvenation and qualitative test of uricase activity.

Culture stock W.IIISRNs_1.1isolate was re-cultured several times in NA medium to obtain fresh isolates. Furthermore, the fresh isolate W.IVS-3 was put in a solid Glucose Yeast Peptone (GYP) medium with a composition of Glucose 1.0%; yeast extract 0.5%; Meatball peptone 0.5%; beef extract 0.2%; NaCl 0.5%; $MgSO_4$ $7H_2O$ 0.01%; K_2HPO_4 0.01%; uric acid 0.2%; and 2.0% bacto agar [22, 24, 26], as a selective medium for uricase-producing bacteria, then it was incubated at temperatures ranging from 35^0C, 40^0C, 45^0C, 50^0C, 55^0C, 60^0C for 1 - 7 days to produce a clear zone around the colony, which indicated that the isolate was producing uricase because it could decompose the substrate around the colony. Furthermore, the isolates were re-grown on a selective medium under optimal conditions for bacterial growth to determine the uricolytic index (UI) as a qualitative test of uric acid-degrading activity. Then an analysis of its physiological and morphological characteristics and rejuvenation for the enzyme production stage was performed[29].

2.2 Morphological and Physiological Characterization of Bacteria.

The culture stock of thermophilic bacteria, the isolate W.IIISRNs 1.1, was cultivated for 18-24 hours at the temperature of 45°C in the medium of agar slant without uric acid.The morphological identification and the biochemical test were then conducted. Morphological characterization was conducted according to the method of Bergey's Manual of Determinative Bacteriology and by Cappuccino and Sherman[30]. Macroscopic identification was done by comparing colonies from the shape, periphery of the colony, surface texture, and color of the colonies and microscopically observed with a microscope at 1000 times magnification. Cell observations included gram staining, cell shape, and spore staining. The physiological observations included the reactions of catalase, indole, oxidase, citrate, Voges–Proskauer, 6% NaCl, and carbohydrate tests (glucose, arabinose,sucrose, and mannitol)[15].

2.3 Molecular Identification of Bacteria

This stage begins with the isolation of chromosomes from thermophilic bacteria strain

W.IIISRNs_1.1. Isolation of chromosomal DNA was carried out using an organic extraction method. A DNA extraction kit from Geneaid was used for genomic DNA extraction. The 16S rRNA gene was amplified by the PCR (Polymeration Chain Reaction) technique using a chromosomal DNA template. DNA templates purified by 0.4-0.5 were amplified with the Ready-To-Go PCR Beads kit (Pharmacia, Biotech) using universal primers specific for bacteria were forward primer 63f (5'-CGA GCC TAA CAC ATG CAA GTC) and reverse primer 1387r (5'-GGG CGG WGT GTA CAA GGC). Mixture 18.5 L ddH$_2$O; 2.5 L buffer; 1.0 L primary 63f; 1.0 L 1387 r; 0.5 dNTP mix; 1.0 L of DNA polymerase and 0.5 L of template were put into an eppendorf tube, then put into PCR (Gene Amp®PCR System) under the following conditions: pre-PCR (94^{0}C, 2 minutes), denaturation (95^{0}C, 30 seconds) , primer anneling or attachment (55^{0}C, 30 seconds), primer extension (72^{0}C, 1 minute) for 30 cycles, and post-PCR (75^{0}C, 5 minutes).The purified PCR product was sent to Genetika Science Indonesia Laboratories for sequencing. The sequence obtained from sequencing was analyzed using the Basic Local Alignment Search Tool (BLAST) from NCBI (National Center for Biotechnology Information to identify the species[15, 26]. Phylogenetic trees of strain W.IIISRNs_1.1 were then constructed and established using Treecon program to determine the phylogenetic relation.

2.4 Cell Growth and Optimization of Uricase Production

It started with making inoculum medium as a starter, W.IIISRNs_1.1 isolate was cultured in 20 mL uric acid medium with a modified composition: uric acid 0.2% (w/v), beef extract 0.2% (w/v), peptone meatballs 0.5% (w/v), NaCl 0.5% (w/v), yeast extract 0.5% (w/v), K_2HPO_4 0.01% (w/v), $MgSO_4$. 7H_2O 0.01% (w/v), Glucose 1.0% (w/v) [15, 18, 22]and incubated shaking at 45^{0}C for 24 hours at a speed of 180 rpm. It was used as an inoculum for the fermentation medium. The composition of the fermentation medium was the same as that of the inoculum medium. Furthermore, 10 mL of inoculum that had been incubated was inoculated into 100 mL of production medium and incubated with shaking at 45^{0}C for 72 hours. Sampling was carried out every 12 hours to measure microbial growth known as Optical Density (OD) at 600 nm and centrifuged at 3500 rpm, 4°C for 30 minutes. Furthermore, the enzyme activity was measured, and the protein content was measured. Besides optimizing production time, optimization of substrate concentration was also carried out to obtain optimal enzyme production. In this case, it was done by incubating bacterial isolates during the optimal production time with several variations of substrate concentration from 0.1% to 0.3% with an interval of 0.05 for each concentration, and then the activity was measured[14].

2.5 Uricase Enzyme assay

A total of 75 µL of 3.57 mM uric acid was added in 3 mL of 20 mM sodium borate buffer pH 8 followed by 30 µL of crude enzyme and incubated for 10 minutes. For the blank solution, 30 µL of the enzyme was replaced with boric buffer, it was also allowed to stand for 10 min and the reaction was stopped by heating the reaction mixture in a water bath for 5 min. A UV-Visible spectrophotometer measured the decrease in absorbance at a wavelength of 293 nm. One unit (U) of enzyme activity represented the amount of uricase required to convert 1 mol Uric Acid per minute under optimized conditions [2, 22, 31].

2.6 Content Protein assay

The stipulation of protein content was carried out using the Lowry method. Two solutions used in this method were; Lowry A and Lowry B. The first one consisted of 2% Na_2CO_3 in a mixture of 0.1 N NaOH, 1% $CuSO_4$, and sodium potassium tartrate in a ratio of 100:1:1, and the other one was a solution of phospho-tungstic-phospho-molybdic acid (folin) in aquadest (1:1). Protein content was measured by spectrophotometer at maximum wavelength using BSA (Bovin Serum Albumin) as a standard [32].

2.7 The Effect of Temperature on uricase activity and stability

To study the effect of temperature on uricase activity by measuring uricase activity at various incubation temperatures. The optimum temperature was determined by reacting the crude extract of the enzyme with the substrate (uric acid) at 37°C, 40°C, 45°C, 50°C, 55°C and 60°C, which was carried out at the optimum pH [24]. Determination of temperature stability of the uricase activity by performing preincubation crude enzyme uricase at optimum temperature and surrounding areas. Preincubation was conducted for 3 hours and tested uricase activityat each interval of 30 minutes [32].

2.8 The Effect of pH on uricase activity and stability

Uricase activity was tested at several different pHs in which, to determine the optimum pH, it was carried out by reacting the enzyme with a buffer at a pH ranging from 6-10. For pH 6-7, phosphate buffer was used; for the range of pH 8-10, it used sodium borate buffer [23]. The pH stability of the enzyme activity is stipulated by performing pre-incubation in a buffer solution at pH optimum and surrounding areas. Preincubation was conducted for 3 hours and tested uricase activityat each interval of 30 minutes[29].

2.9 The Effect of substrate Concentration

To specify the effect of substrate concentration on crude uricase enzyme activity, the enzyme was reacted with several substrate concentrations (uric acid) from 1.0 mM to 4.5 mM at 0.5 mM intervals. Then, each reaction mixture was incubated at the optimum temperature and pH for 10 minutes, and the activity was measured[29].

2.10 The Effect of Metal Ion on uricase activity

The test was conducted by reacting the enzyme at pH, temperature, and optimum substrate concentration with several metal ions (Ba^{2+}, Co^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, K^{+}, Na^{+}). Each metal was applied at 0.1 M and 0.5 mM concentrations into the enzyme and substrate mixture. At the same time, positive control was made, which was not added with metal ions. After incubation, the reaction results were determined by the value of uricase activity and compared with the control[26, 29].

3. Result and Discussions

3.1 Rejuvenation and qualitative test of uricase activity

The bacterial isolate of W.IIISRNs_1.1is one of the thermophilic bacteria isolated from the sediment of the hot spring of Mataumpana, which has good uricase activity.This can be seen from the optimal bacterial growth in GYP (glucose yeast peptone) agar medium containing 0.2% uric acid after incubation for 7 days at pH 7.0 and temperature 45^{0}C and the presence of a clear zone that appeared around the bacterial colonies during the process of incubation (Figure 1b). Before the W.IIISRNs_1.1 isolate was incubated, the insoluble uric acid was evenly distributed in the GYP agar medium, as shown in Figure 1a. The clear zone indicates that these microbes can degrade uric acid due to the presence of the uricaseenzyme, which can be induced in the presence of uric acid in the growth medium. W.IIISRNs_1.1 isolate was able to grow well starting at 35^{0}C, but at the temperature of 45^{0}C, it only showed uricase activity, so this temperature was the optimum for this bacterium. Furthermore, qualitative uricase activity was tested by observing the clear zone on solid media containing 0.2% uric acid after being incubated for 4 x 24 hours (4 days) at optimum conditions and measuring the Uricolytic index (UI), which is the diameter of the clear zone formed divided by the size of the diameter bacteria colony.

Figure 2 shows the development of bacterial colony size and the clear zone around the colony, where it can be seen that every day the incubation time increases the size of the clear zone around the colony and the magnitude of the uricolytic index. On the day the isolates before inoculated, the medium surface was opaque with insoluble uric acid like condition in Figure 1a, on the 1 x 24 hours (first day) of incubation, bacteria grew and produced a clear zone around the bacterial colony with 9 UI, the second day of incubation the diameter colony size (0.4 cm) and a clear zone (5.2 cm) increased appeared around the colony with 13 UI , and the third day of incubation the diameter colony size (0.5 cm)and the clear zone (7 cm) around the colony was getting more significant with 14 UI, and the last day of incubation (4th day) the diameter of the colony still increased but only 0.05 cm from the previous day and had the largest clear zone diameter (8 cm) around the colony with a uricolytic index of 14.6. This means the uric acid which did not dissolve in the media on the first day was completely degraded by bacteria due to the activity of the uricase enzyme.

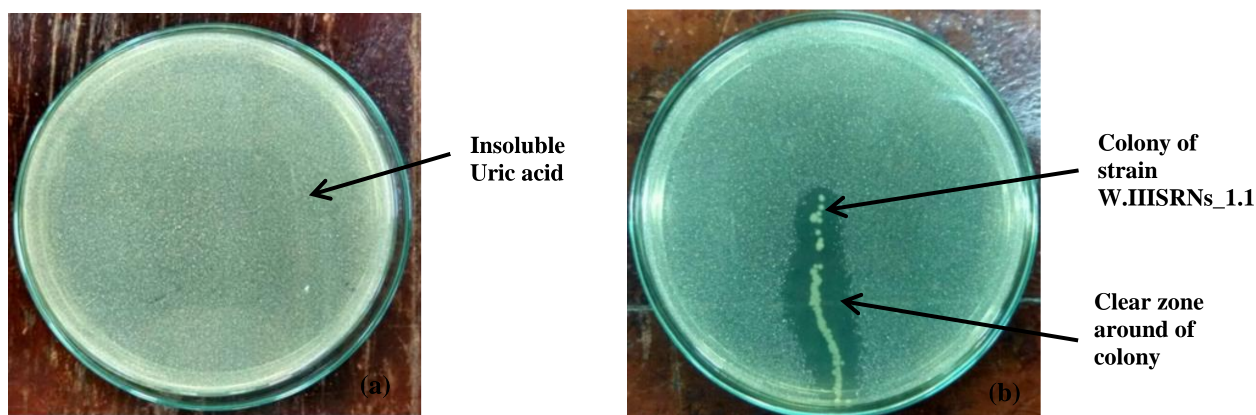


Fig 1.(a). Solid GYP medium conditions on the first day of inoculation before incubation, (b). Colony of Thermophilic Uricase Strain W.IIISRNs_1.1 in solid GYP medium with 0.2% uric acid at 45^{0}C, pH 7

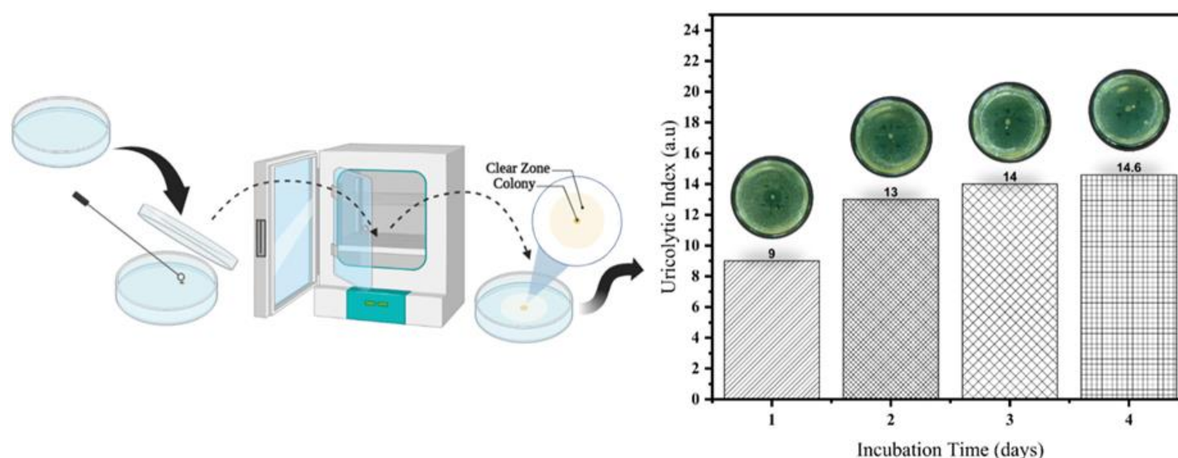


Fig 2. Result of Uricolitic Index test with clear zone around colony isolate W.IIISRNs_1.1 at each incubation time

3.2 Morphological and Physiological Characterization of Bacteria

The result of the morphological test of theW.IIISRNs_1.1 isolatein Table 1 shows that microscopically, it is a type of bacilli gram-positive because its cell wall absorbs the bluish-purple colour of crystal violet during gram and has the cell shape of rods without spore staining as shown in Figure 3.

Macroscopically, the W.IIISRNs_1.1 isolatehad a white colony color. The edges of the colonies were smooth, and the surface of the colonies was seen convex.These results are similar to the characteristics of the strain ZJ 186 isolated from marine soil[33] and strain KA15 isolated from Djudjura Mountains[34], which were identified as *Bacillus altitudinis*, both are rod-shaped, gram-positive, not spore. This strain had white colonies, convex colony surfaces like the strain W.IIISRNs_1.1.

Physiological and biochemical tests of bacteria were undertaken to determine their metabolic activity. Each microorganism had different metabolic characteristics based on the interaction of the metabolites produced with chemical reagents and their ability to utilize certain compounds as carbon sources and energy sources. The biochemical tests mentioned include the IMViC, SIM, TSIA, and sugar fermentation tests[35].Physiological and biochemical tests carried out in this study were the SIM test, MRVP, catalase test, and fermentation test of several types of sugar. The IMViC test consists of indole, Methyl Red, Voges-Proskauer, and Simon's Citrate tests. The indole test aims to see the ability of organisms to degrade the amino acid tryptophan and produce indole. The MR test was carried out to determine the ability of bacteria to produce mixed-acid by adding methyl red reagent. If the test results get a red color (+), then the bacteria can ferment mixed-acid so that it lowers the pH to 4.4, and if the test results get a yellow color (-), then the bacteria ferment other ingredients, causing the pH to be 6[36]. Simon's Citrate test was carried out to see the ability of microorganisms to utilize citrate as a carbon source, describing whether these bacteria produce citrate permease enzymes. The results of the physiological and biochemical tests of the thermophilic bacteria strain W.IIISRNs_1.1, which has uricase activity in this study can be seen in Table 2.

Table 1. Morphological characteristics of uricase-producing thermophilic bacteria strain W.IIISRNs_1.1

Test	Result		
	Strain W.IIISRNs_1.1	**Strain ZJ 186**[33]	**Strain KA15**[34]
Colony shape	Round	Round	Circular
Pigmentation	Milky white	White	White
Colony edge	Smooth	Not clear	Smooth
Colony surface	Convex	Not clear	Convex
Gram color	Violet	Violet	Violet
Gram stain	Positive (+)	Positive (+)	Positive (+)
Spore	Positive (+)	Negative (-)	Negative (-)
Cell shape	Rod	Rod	Rod

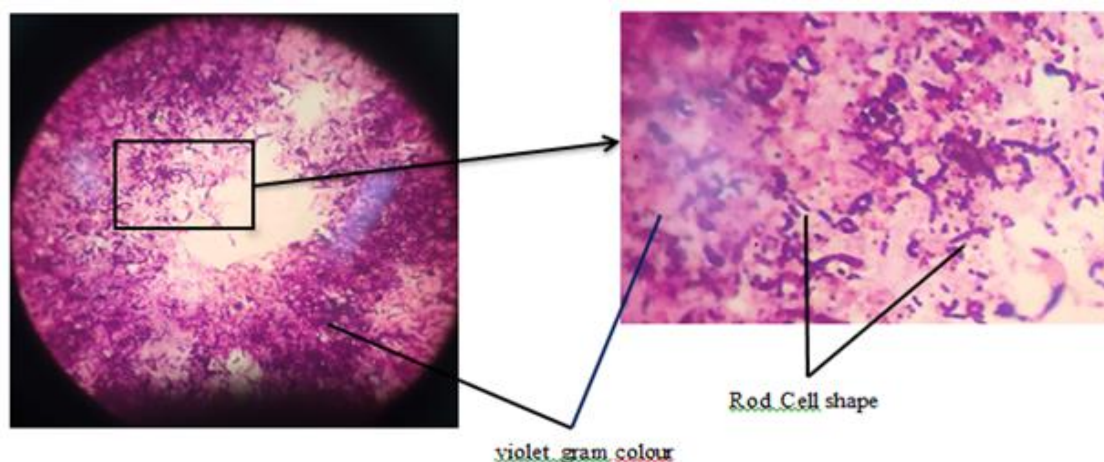


Fig 3. The results of the observation of Gram of Uricase thermophilic Bacteria, isolate W.IIISRNs_1.1 under a microscope with 1000x magnification.

Table 2. Result Physiological and Biochemical Identification of uricase-producing thermophilic bacteria strain W.IIISRNs_1.1

Test		Result
SIM	Indole	Positive
	Motility	Positive
MRVP	H_2S	Negative
	Methyl Red	Positive
	Voger Proskauer	Negative
	Citric	Negative
	Urea	Negative
	Lactose	Positive
	Sucrose	Positive
	Arabinose	Positive
	Glucose	Positive
	Mannitol	Positive
	Catalase	Positive
	Oxidase	Positive
	NaCl 6%	Negative

According to the results of the identification of morphological characteristics, macroscopically and microscopically, the thermophilic bacteria producing the uricase enzyme isolate W.IIISRNs_1.1 (Table 1) showed similar characteristics in general witha genus of*Bacillus*, in which its cells are rod-shaped and gram-positive and have endospores. The bacteria with a stem cell shape, both short rods to single rods and Gram-positive, with endospores, can be bound from the Genus *Bacillus sp*[37]. These results are corroborated by the results of observations of physiological and biochemical tests (Table 2) referred to the "Bergey's Manual of Determinative Bacteriology" explaining the thermophilic bacteria strain W.IIISRNs1.1 positive in the genus Bacillus sp without spores, where the results of the motility test showed the movement of bacteria in the presence of motion aids so that these positive bacteria are motile. *Bacillus sp* can be motile and immotile, the species of Bacillus sp which are motile are *Bacillus altitudinis*, *Bacillus alvei* and *Bacillus thurgenensis*. Therefore, the strain W.IIISRNs1.1 is a bacterium from the genus *Bacillus sp,* similar in characteristics to the type of *Bacillus altitudinis.* Furthermore,to strengthen the morphological and physiological analysis results, it will be continued with identification at the molecular level.

3.3 Molecular Identification of Bacteria

The results of Chromosomal Isolation of bacterial DNA isolate W.IIISRNs1.1 were confirmed by agarose gel electrophoresis and showed a single fragment on the electropherogram. This indicates that the isolation of chromosomal DNA has been carried out well enough to be used as a template for the PCR amplification process. 16S rRNA gene amplification by PCR was carried out for 30 cycles with an annealing temperature of 55°C and elongation temperature of 72°C using a universal primer pair for bacteria. The electropherogram of the 16S rRNA amplicon from isolate W.IIISRNs1.1 is shown in Figure 4a. It can be seen that the obtained amplicon has a fragment size of 1300 bp, a standard size for the length of 16S bacterial rDNA amplified with universal primers. These amplicons were then sequenced using the Dideoxy Sanger method with reverse primer (1386r), and the nucleotide base sequences were blasted to identify the genus and species of bacteria. Sequencing the gene encoding 16S rRNA from thermophilic bacteria isolateW.IIISRNs1.1 obtained complete nucleotide sequence data measuring 1924 bp. The nucleotide sequence data of the 16S rDNA gene were aligned online using the BLAST program via the site https://blast.ncbi.nlm.nih.gov/Blast.cgi.The alignment results with the Gen Bank data base identified the genus and species of bacteria. Strain W.IIISRNs1.1 has 90.13% similarity in identity with *Bacillus altitudinis* Strain GR-8 and *Bacillus altitudinis* strain 3.3. This similarity identity was declared valid for the

identity of the genus and species of bacteria obtained. Statistically, the identification of this method is considered ambiguous or biased if the homology identity of the 16S rDNA sequence is below 70%[38]. The results of the BLAST analysis of 1924 bp can be constructed using the treecon program to study the relationship between bacteria and other bacteria that have been registered in the GenBank database, as shown in Figure 4b. with *Bacillus altitudinis* so that the thermophilic bacteria isolate W.IIISRNs1.1 can be called *Bacillus altitudinis*W.IIISRNs1.1

3.4 Cell growth and the optimization of uricase production

The production of the uricase enzyme was started by growing the bacteria *Bacillus altitudinis* strain W.IIISRNs_1.1in Glucose yeast Peptone broth medium containing 0.2% uric acid as a substrate to be incubated in a shaker incubator at 45°C for 72 hours,

180 rpm with variations in fermentation time. This process was conducted to determine the effect of fermentation time on bacterial growth and the optimum conditions for producing uricase enzymes to produce enzymes with the highest activity and in large quantities. The Fermentation time greatly affects the growth of bacteria or the optical density of *Bacillus altitudinis*strain W.IIISRNs_1.1as shown in Figure 5a, in which the growth curve shows the bacteria encountered optimal cell growth at the 54th hour of incubation with an OD value of 1.036 which measured at a wavelength of 600 nm, after which there was a decrease in biomass production to be precise at 60 hours of incubation. Along with determining the bacterial growth rate or optical density, enzyme activity values were also measured to stipulate the optimum time for uricase production. The activity of the uricase enzyme *Bacillus altitudinis* strain W.IIISRNs_1.1from 0 hours of incubation continued to increase gradually and reached a maximum at 36 hours of incubation with an activity of 0.5198 U/mL. The increasing growth of bacteria caused this so the production of enzymes used to hydrolyze the substrate also increased, but the production of uricase from *Bacillus altitudinis* strain W.IIISRNs_1.1was optimum at the beginning of the exponential phase, not at the peak of biomass production. Therefore, the optimal time to produce uricase enzyme from *Bacillus altitudinis* strain W.IIISRNs_1.1in a medium containing 0.2% uric acid at 45°C was 36 hours, and this condition was used to produce uricase to be characterized.

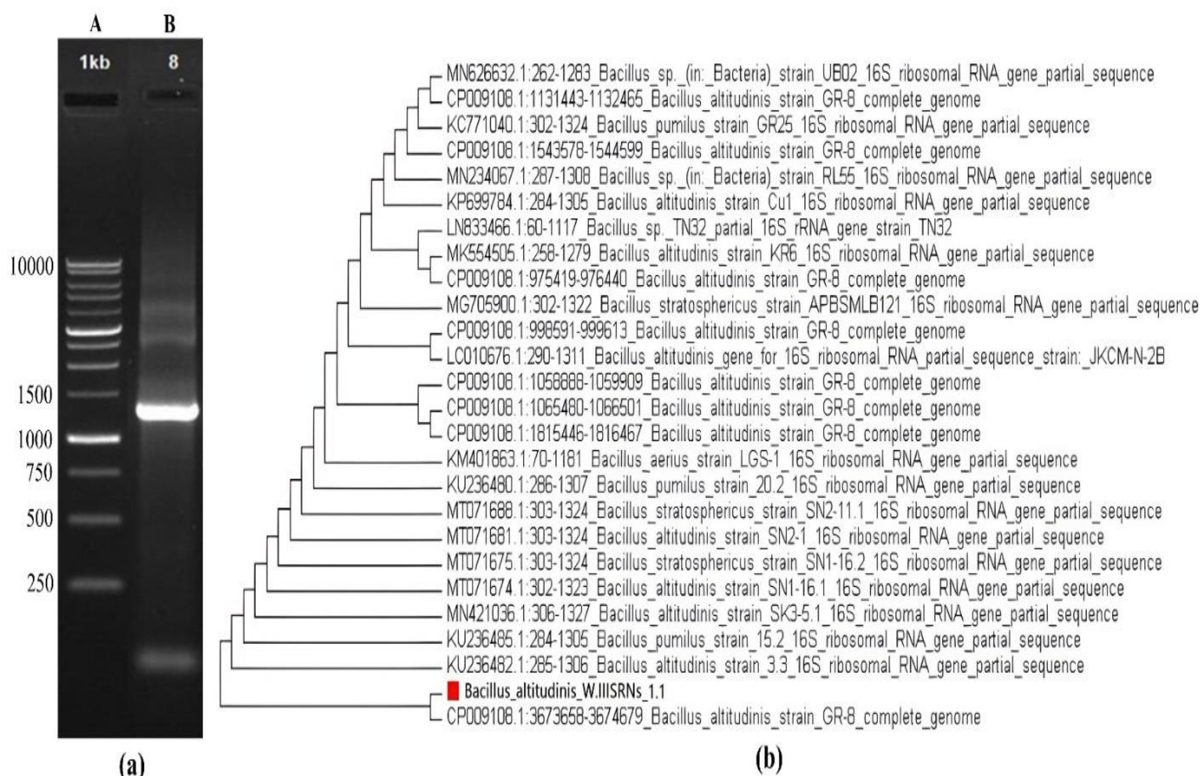


Fig 4. (a) Electroferogram of amplicon 16S rDNA, A. 1 kb DNA Marker Ladder (Gene Ruler 1 kb DNA Ladder) , B. 8= DNA of strain W.IIISRNs_1.1 (b).Phylogenetic position of strain W.IIISRNs_1.1 based on 16s rRNA sequence.

The production time of the uricase enzyme varies depending on the source microorganism,for example, uricase from *Xantomonasfuscans* has a production time of 28 hours with an activity of 149 U/L[14], *Bacillus cereus* strain DL3 has a production time of 20 hours with an activity of 9.55 U/L[31], *Sphingobacterium thalpophilum* (VITPCB5) with a production time of 24 hours with an activity of 3.75 U/mL[21]. However, when viewed from the activity value and production time of the uricase enzyme from *Bacillus altitudinis* strain W.SRNs_1.1 in this study, it was better than uricase from *Xantomonasfuscans* and *Bacillus cereus* strain DL3 because it had higher activity with almost the same production time and the medium used for production has a simpler composition. But the uricase in this study was lower in activity than the uricase produced from *Sphingobacterium thalpophilum* (VITPCB5). However, each enzyme has different characteristics and uniqueness, so it has the same potential to be developed, especially uricase in this study, further purification must be carried out to produce better quality enzymes and be applied in various fields, especially in the medical field.

Measurement of protein content is also needed to find out the value of the specific activity of an enzyme. In this study, the protein content of uricase was determined by the Lowry method. The protein content was determined based on the absorbance value, which was proportional to the protein content at the maximum wavelength (670 nm) against the BSA standard curve (Figure 7b). The protein level of the uricase enzyme at the optimum activity in this study was 1.2819 mg/mL, so the specific activity of the enzyme obtained was 0.4055 U/mg.

Substrate optimization results from several substrate concentrations in the production of uricase enzymes from *Bacillus altitudinis* showed the best performance at 0.2% uric acid substrate concentration with 0.592 U/mL, as shown in Figure 5b.

3.5 The Effect of Temperature on Enzyme Activity and Stability

Figure 6a shows the crude uricase enzyme from *Bacillus altitudinis*strain W.IIISRNs_1.1 with the activity ranging from 37^{0}C to 60^{0}C, but worked optimally at 45^{0}C with an activity value of 0.5198 U/mL. This indicates that the enzyme works slowly at temperatures below 45^{0}C, while at higher temperatures the enzyme works better in accelerating the reaction due to the increase in the kinetic energy of the reacting molecules. However, as the enzymatic reaction progresses, the maximum point will be reached, in this case at the optimum temperature and after that the reaction rate will decrease with increasing temperature[29]. The optimum temperature for the uricase enzyme *Bacillus altitudinis*strain W.IIISRNs_1.1 has similarities with the uricase enzyme from *Pseudomonas aeruginosa*[24], and *Pseudomonas otitidis* strain SN4[26] is also optimum at 45^{0}C.

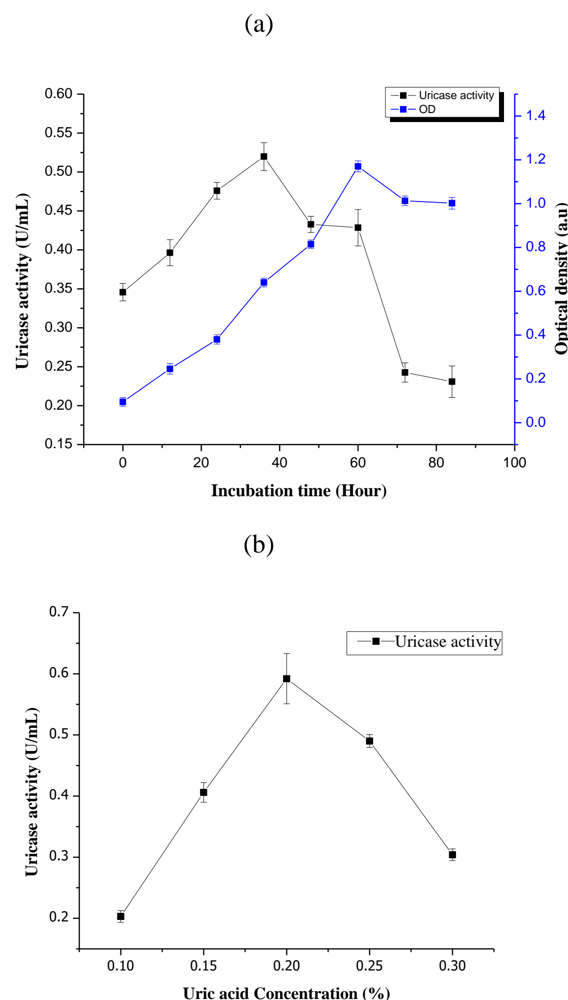


Fig 5. (a) Growth profile of *Bacillus altitudinis* strain W.IIISRNs_1.1 (OD) on λ 600 nm and its uricase production with 0.2% uric acid on 45°C, (b). Effect of uric acid substrate concentration on enzyme activity in producing enzyme uricase from *Bacillus altitudinis* strain W.IIISRNs_1.1.

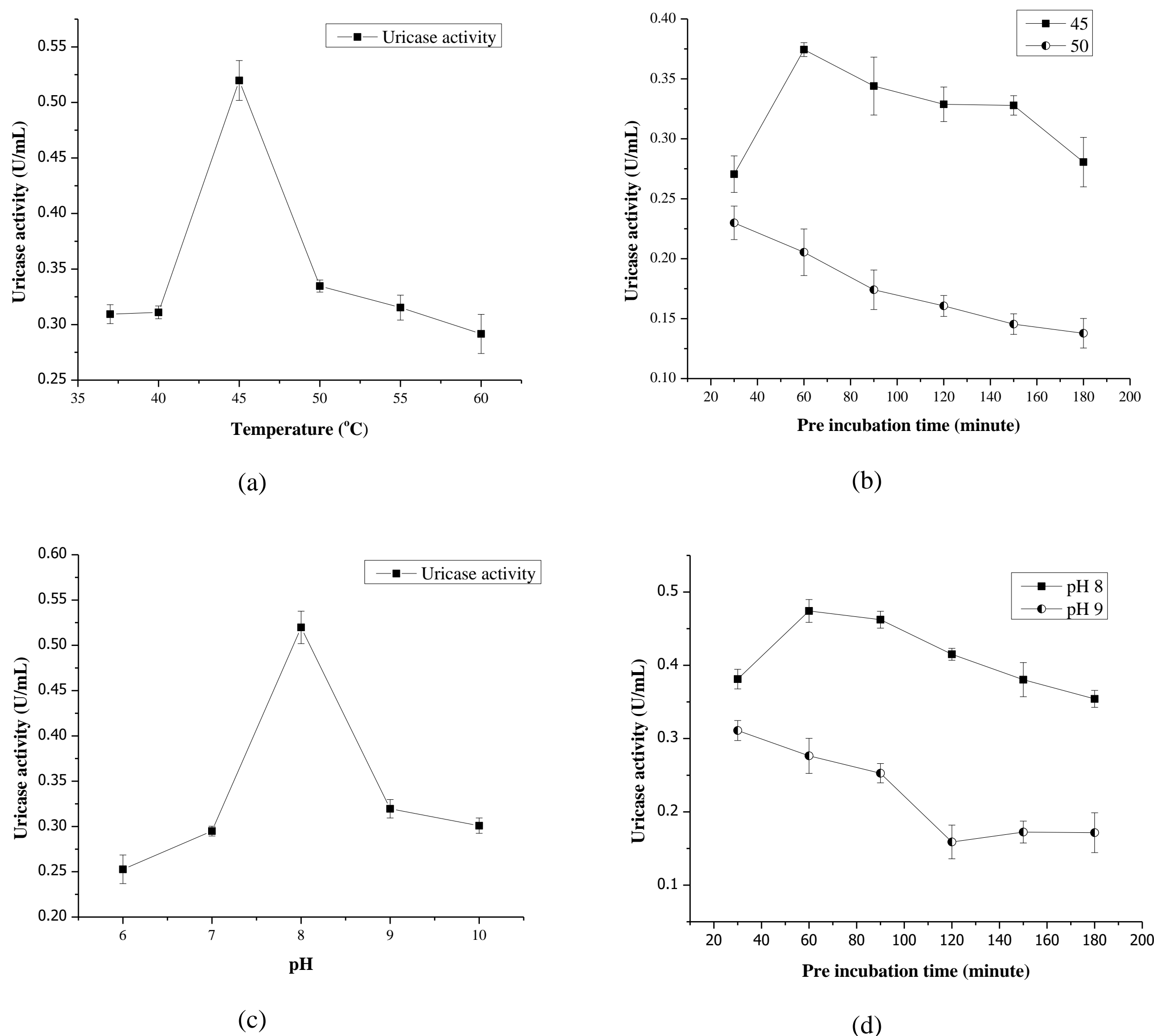


Fig **6.** (a). The effect of temperature on uricase activity *Bacillus altitudinis* strain W.IIISRNs_1.1, (b). The stability of uricase activity *Bacillus altitudinis* strain W.IIISRNs_1.1 on temperature every pre-incubation time, (c). The effect of pH on uricase activity *Bacillus altitudinis* strain W.IIISRNs_1.1, (d). The stability of uricase activity *Bacillusaltitudinis* strain W.IIISRNs_1.1 on pH every pre-incubation time.

The uricase enzyme produced in this study is a thermophilic enzyme because it still showed promising activity up to a temperature of 60°C. Besides that, it is strengthened by the results of the stability test of the uricase enzyme at the incubation temperature at 45^{0}C and 50^{0}C, where the pre-incubation stage showed the enzyme was still stable for 2 hours 30 minutes at 45^{0}C and at 50^{0}C the enzyme was stable for 30 minutes (Figure 6b).

3.6 The Effect of pH on enzyme activity and stability

The effect of pH on the activity of the uricase enzyme can be viewed in Figure 6c, where the enzyme activity increases from pH 6 to pH 8, after which an increase in pH will decrease its activity. Uricase from *Bacillus altitudinis* strain W.IIISRNs_1.1 worked optimally in sodium borate buffer pH 8 (optimum pH) with maximum activity. These results have similarities with Uricase of *Pseudomonas otitidis* strain SN4[26], *Sphingo bacterium thalpophilum*[21], *Bacillus subtilis*, *Bacillus cereus* and *Bacilllus megametrium* [22]. The enzyme's ionic structure depends on its environment's pH, such as proteins in general. The form of enzymes can be positive ions, negative ions or double charged ions (zwitter ions). Thus, changes in environmental pH will affect the effectiveness of the active part of the enzyme in forming an enzyme-substrate complex[29].

The effect of pH on the stability of uricase enzyme activity is represented in Figure 6d, where the results of the stability test show that Uricase from *Bacillus altitudinis* strain W.IIISRNs_1.1was stable at an environment of pH 8 to 90 minutes of pre-incubation,

while at pH 9 the enzyme was only able to maintain its best activity for 60 minutes of pre-incubation. The same results were shown by uricase of *Pseudomonas aeruginosa,* which was stable at pH 7.5 – 9 (45^{0}C, 30 minutes)[24].

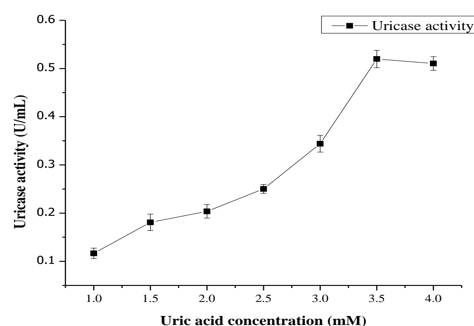
3.7 The Effect of Substrate Concentration

The effect of substrate concentration on crude uricase enzyme activity in this study was an increase in uricase activity from uric acid concentrations of 1.0 mM, 1.5 mM, 2.0 mM to 3.0 mM, and the best enzyme activity performance at a uric acid concentration of 3.5 mM with an activity of 0.5198 U/mL. However, at a concentration of 4.0 mM, the activity of the uricase enzyme decreased but was not significant and even looked constant, as shown in Figure 7a. This shows that the substrate concentration also affected the speed of the reaction catalyzed by the enzyme. When the substrate concentration is increased at a constant enzyme concentration, the reaction rate will increase until it reaches a certain point. In this study, the maximum speed of the enzyme uricase W.IIISRNs_1.1 occurred at a substrate concentration of 3.5 mM, but at a higher concentration, the activity and rate of enzyme action tended to be constant. This is because filled with the substrate or had been saturated with substrate. Therefore, even though the concentration of the substrate was raised, it did not cause an increase in the enzyme activity; this indicates that the substrate had already reached its optimal condition.

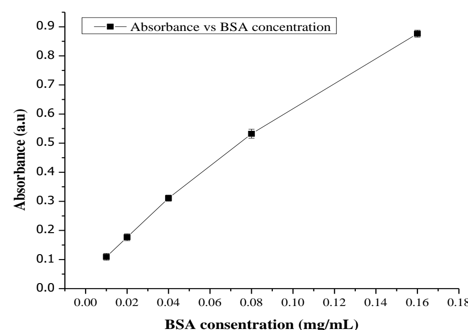
3.8 The Effect of Metal Ions

Most enzymes require a non-protein component to perform their catalytic function, called a cofactor, consisting of an activator and an inhibitor. The substances functions as activators or inhibitors in the enzyme catalysis process are metal ions. At specific concentrations, metal ions can act as components that activate the active site of enzymes when catalyzing substrates or become inhibitors in enzymatic reactions[32]. In this study, the addition of metal ions at concentrations of 0.1 M and 0.05 mM could increase and decrease the activity of the uricase enzyme from *Bacillus altitudinis* strain W.IIISRNs_1.1

Figure 8 shows that crude uricase enzyme without adding metal ions as a control had a relative activity of 100%. It can be seen in the addition of 0.1 M metal ion concentration which acts as an activator were Ba^{2+}, K^+ and Ca^{2+} metals with relative activity of 102%, 103%, and 104%. In comparison, those that slightly inhibited uricase activity were the metal ions of Na^+ and Mg^{2+} with relative activity of 97% and 99%, Co^{2+} and Zn^{2+} which acted as true inhibitors with a relative activity of 78% and 32%. For a concentration of 0.05 mM, none of the metal ions at this concentration can increase the activity of the uricase enzyme, while Zn^{2+}, Ca^{2+}, and Mg^{2+} ionsslightly inhibited uricase activity with relative activity were 96 %, 98%, and 97%, and then Na^+ ion acted as a true inhibitor at this concentration. Something was interesting about the addition of Ba^{2+}, K^+, and Co^{2+} ions at a concentration of 0.5 mM, where all three did not play any role in the uricase enzyme reaction because they had activity relativeto the same as the control 100%.



(a)



(b)

Fig 7. The effect of the substrate concentration on the uricase activity of *Bacillus altitudinis* W.IIISRNs_1.1 at the temperature of 45^{0}C and the sodium borate buffer pH of 8.0, (b). Standard curve showing the absorbance of different concentrations of BSA for each measurement at wavelength 670 nm.

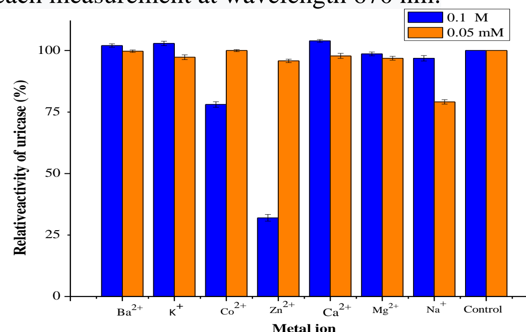


Fig 8. The Effect of more metal ions on uricase activities of *Bacillus altitudinis* W.IIISRNs_1.1 at the uric acid 3.5 mM, the temperature of 45°C, and the sodium borate pH of 8.0.

4. Conclusions

Based on the study's results, we reported that the isolate W.IIISRNs_1.1 from the hot spring of Mataumpana, Buton regency, Southeast Sulawesi produced uricase enzyme was identified as *Bacillus altitudinis* strain W.IIISRNs_1.1. This is the first report on the production of the enzyme uricase from a thermophilic bacterium, *Bacillus atitudinis*. This microbe has an optimum optical density of 1.036 (60 hours of incubation), which is not follow the optimum production incubation time of uricase enzyme, which is 36 hours with enzyme activity of 0.5198 U/mL and protein concentration of 1.2819 mg/mL, at the uric acid 0.2% and 45ºC. Uricase enzyme from *Bacillus altitudinis* strain W.IIISRNs_1.1works optimally at 45ºC and pH 8, is stable at 45ºC and pH 8 for 1.5 hours, and pH 9 for 1 hour, the maximum speed of uricase enzyme work occurs at the substrate concentration (uric acid) 3.5 mM. The enzyme was activated by 0.1 M, Ba^{2+}, K^{+}, and Ca^{2+} ions. The enzyme was inhibited by 0.1 M Co^{2+}, Zn^{2+} ions and 0.05 mM Na^{+}, Zn^{2+} ions.

5. Conflicts of interest

The authors declare no conflict of interest.

6. Acknowledgments

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7. References

[1] T. Fukuda, H. Muguruma, H. Iwasa, T. Tanaka, A. Hiratsuka, T. Shimizu, K. Tsuji, T. Kishimoto, Electrochemical determination of uric acid in urine and serum with uricase/carbon nanotube /carboxymethylcellulose electrode, Anal Biochem 590 (2020) 113533.

[2] S.M. Khade, S.K. Srivastava, K. Kumar, K. Sharma, A. Goyal, A.D. Tripathi, Optimization of clinical uricase production by Bacillus cereus under submerged fermentation, its purification and structure characterization, Process Biochemistry 75 (2018) 49-58.

[3] S.O. Pustake, P.K. Bhagwat, P.B. Dandge, Statistical media optimization for the production of clinical uricase from Bacillus subtilis strain SP6, Heliyon 5(5) (2019) e01756.

[4] F. Foroughi, M. Rahsepar, H. Kim, A highly sensitive and selective biosensor based on nitrogen-doped graphene for non-enzymatic detection of uric acid and dopamine at biological pH value, Journal of Electroanalytical Chemistry 827 (2018) 34-41.

[5] T. Dhanasekaran, A. Padmanaban, G. Gnanamoorthy, S. Praveen Kumar, A. Stephen, V. Narayanan, Electrocatalytic activity of C-dots for highly sensitive detection of Uric acid, Materials Today: Proceedings 14 (2019) 545-552.

[6] S. Verma, J. Choudhary, K.P. Singh, P. Chandra, S.P. Singh, Uricase grafted nanoconducting matrix based electrochemical biosensor for ultrafast uric acid detection in human serum samples, Int J Biol Macromol 130 (2019) 333-341.

[7] X. Wang, Q. Yao, X. Tang, H. Zhong, P. Qiu, X. Wang, A highly selective and sensitive colorimetric detection of uric acid in human serum based on MoS2-catalyzed oxidation TMB, Anal Bioanal Chem 411(4) (2019) 943-952.

[8] Verdiansah, Pemeriksaan Fungsi Ginjal, praktis 43(2) (2016) 149-154.

[9] L. Rana, R. Gupta, M. Tomar, V. Gupta, Highly sensitive Love wave acoustic biosensor for uric acid, Sensors and Actuators B: Chemical 261 (2018).

[10] Z. Karami, N. Sohrabi, A. Badoei-dalfard, A specific, rapid and high-throughput cascade catalytic method for determination of plasma uric acid by using uricase and trivalent peroxidase-mimicking DNAzyme, Biocatalysis and Agricultural Biotechnology 24 (2020).

[11] X. Hu, Y. Wang, C. Liu, Z. Jin, Y. Tian, Dextrin-uricase conjugate: Preparation, characterization, and enzymatic properties, Int J Biol Macromol 111 (2018) 28-32.

[12] S. Mirzaeinia, M. Pazhang, M. Imani, N. Chaparzadeh, A.R. Amani-Ghadim, Improving the stability of uricase from Aspergillus flavus by osmolytes: Use of response surface methodology for optimization of the enzyme stability, Process Biochemistry 94 (2020) 86-98.

[13] Y. Zhou, B. Ling, H. Chen, L. Wang, Mn2+-doped NaYF4:Yb,Er upconversion nanoparticles for detection of uric acid based on the Fenton reaction, Talanta 180 (2018) 120-126.

[14] S.K. Ram, K. Raval, P.E. JagadeeshBabu, Enhancement of a novel extracellular uricase production by media optimization and partial purification by aqueous three-phase system, Prep Biochem Biotechnol 45(8) (2015) 810-24.

[15] F. Atty, J. JosepH, Isolation and Identification of Uric Acid Degrading Bacteria, Optimization of Uricase Production and Purification of Uricase Enzyme, International Journal of Advanced Research 4(12) (2016) 2732-2742.

[16] M.N. Omar, A.B. Salleh, H.N. Lim, A. Ahmad Tajudin, Electrochemical detection of uric acid via uricase-immobilized graphene oxide, Anal Biochem 509 (2016) 135-141.

[17] S.E. Tork, M.M. Aly, S.Q. Al-Fattani, A new uricase from Bacillus cereus SKIII: Characterization, gene identification and genetic improvement, Int J Biol Macromol 165(Pt B) (2020) 3135-3144.

[18] I. Handayani, T. Utami, C. Hidayat, E. Rahayu, Screening of lactic acid bacteria producing uricase and stability assessment in simulated gastrointestinal conditions, International Food Research Journal 25(4) (2018) 1661-1667

[19] N. Stozhko, M. Bukharinova, L. Galperin, K. Brainina, A Nanostructured Sensor Based on Gold Nanoparticles and Nafion for Determination of Uric Acid, Biosensors (Basel) 8(1) (2018).

[20] S. Tvorynska, J. Barek, B. Josypčuk, Flow amperometric uric acid biosensors based on different enzymatic mini-reactors: A comparative study of uricase immobilization, Sensors and Actuators B: Chemical 344 (2021).

[21] R. Ravichandran, S. Hemaasri, S.S. Cameotra, N.S. Jayaprakash, Purification and characterization of an extracellular uricase from a new isolate of Sphingobacterium thalpophilum (VITPCB5), Protein Expr Purif 114 (2015) 136-42.

[22] D. Iswantini, N. Nuhidayat, Trivadila, A. Nurjayati, Penentuan KinetikaUrikase dari Sel *Bacillus subtilis*, *B. megaterium*, dan *B. cereus* Jurnal Ilmu Pertanian Indonesia 16(2) (2011) 112-118.

[23] T. Ghosh, P. Sarkar, Isolation of a novel uric-acid-degrading microbe Comamonas sp. BT UA and rapid biosensing of uric acid from extracted uricase enzyme, J Biosci 39(5) (2014) 805-19.

[24] S. Abdullah, M. Flayyih, Production, Purification and Characterization of Uricase Produced by Pseudomonas aeruginosa, Iraqi Journal of Science 56 (2015) 2253-2263.

[25] S. Khade, S.K. Srivastava, A.D. Tripathi, Production of clinically efficient uricase enzyme induced from different strains of Pseudomonas aeruginosa under submerged fermentations and their kinetic properties, Biocatalysis and Agricultural Biotechnology 8 (2016) 139-145.

[26] S. Shahir, N. Ibrahim, H. Mansouri Khosravi, N.S. Irwan Shah Lee, Isolation, partial purification and characterization of thermophilic uricase from Pseudomonas otitidis strain SN4, Malaysian Journal of Microbiology (2015).

[27] N.E.-A. El-Naggar, Isolation, Screening and Identification of Actinobacteria with Uricase Activity: Statistical Optimization of Fermentation Conditions for Improved Production of Uricase by Streptomyces rochei NEAE-25, International Journal of Pharmacology 11(7) (2015) 644-658.

[28] A. Badoei-dalfard, M. Shaban, Z. Karami, Characterization, antimicrobial, and antioxidant activities of silver nanoparticles synthesized by uricase from Alcaligenes faecalis GH3, Biocatalysis and Agricultural Biotechnology 20 (2019).

[29] S. Sarni, H. Natsir, S. Dali, Production and Characterization Chitosanase of Sponge Symbiont Bacteria Klepsiella sp to Hydrolyze Chitosan Be Chitoologosaccarides, Marina Chim. Acta international Journal 17(1) (2016).

[30] J.G. Cappuccino, N. Sherman, Microbiology: A Laboratory Manual, Fifth Edition, Publishing as Benjamin Cummings, San fransisco: Pearson Education, Inc, 1999.

[31] P. Nanda, P.E. Babu, Isolation, screening and production studies of uricase producing bacteria from poultry sources, Prep Biochem Biotechnol 44(8) (2014) 811-21.

[32] H. Natsir, A.R. Patong, M.T. Suhartono, A. Ahmad, Production and Characterization of Chitinase Enzymes From Sulili Hot Spring ini South Sulawesi ,Bacillus sp. HSA,3-1a, Indo. J.Chem 10(2) (2010),263 - 267.

[33] X.B. Jin, R.j. Sun, J.-q. Zhu, Z. Liu, Q. Wang, S.-Y. Ye, Isolation and Identification of Bacillus altitudinis ZJ 186 from Marine Soil Samples and its Antifungal Activity Against Magnaporthe oryzae, Current research in Bacteriology 5(1) (2012).

[34] K.-L. Asmani, K. Boaucem, S. Mechri, B. Jaouadi, Identification and characterization of Bacillus altitudinis strain KA15 newly isolated from the highest summit of the Djurdjura Mountains in Kabylia, Algeria, Mol2net, International Conference Seris on Multidisciplinary Sciennces (6) (2020) 1-6.

[35] T.S.M. Ginting, Isolasi dan identifikasi bakteri Gram negatif pada kambing peranakan etawa (PE), J. Ilm. Mhs. Vet. 2(3) (2018) 351–360.

[36] V. Hemraj, Diksha, S. Sharma, G. Avneet, A Review On Commonly Usd Biochemical Test for Bacteria Innovare Journal of Life Science 1(1) (2013).

[37] F. Puspita, M. Ali, R. Pratama, Isolation and Characterization of Morphology and Physiology of Endophytic Bacillus sp. from Oil Palm Plants (Elaeis guineensis Jacq.) J. Agrotek. Trop 6(2) (2017) 44-49

[38] B.G. Hall, Building phylogenetic trees from molecular data with MEGA, Mol Biol Evol 30(5) (2013) 1229-35.