PRODUCTION OF NUTRIENT SOURCES FOR *RHIZOBIUM*

Mr. Chardchai Burom

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ไรโซเบียมเป็นแบคทีเรียที่พบอยู่ร่วมกับรากพืชตระกูลถั่วและช่วยตรึงไนโตรเจนจากบรรยากาศ ซึ่งเป็นประโยชน์มากด้านธุาตอาหารไนโตรเจนกับพืชอาศัย ปัจจุบันมีการผลิตหัวเชื้อไรโซเบียมเพื่อการปลูกพืชตระกูลถั่วที่มีความสำคัญทางเศรษฐกิจ ซึ่งไรโซเบียมหลายสายพันธุ์ต้องการแมนนิทอลและกลีเซอรอล เป็นแหล่งอาหารหลักและไม่สามารถใช้ทั้งกลูโคสซึ่งเป็นน้ำตาลที่แบคทีเรียโดยทั่วไปนำไปใช้ได้ง่าย และซูโครส ซึ่งแมนนิทอลและกลีเซอรอลมีมูลค่าสูงกว่ากลูโคสประมาณ 12-20 เท่า ทำให้มีต้นทุนสูงในการผลิตหัวเชื้อ การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อผลิตกลีเซอรอลและแมนนิทอลจากวัตถุดิบมูลค่าต่ำ ได้แก่ แป้ง และซูโครส โดยจุลินทรีย์ในกลุ่มยีสต์ เพื่อนำไปเลี้ยงไรโซเบียมกลุ่มที่เจริญช้า ซึ่งอาจช่วยลดต้นทุนในการผลิตหัวเชื้อได้ จากการคัดเลือกยีสต์จำนวน 147 ไอโซเลทที่แยกได้จากแหล่งธรรมชาติและ 15 สายพันธุ์จากแหล่งเชื้อพันธุ์จุลินทรีย์ ด้านความสามารถในการผลิตกลีเซอรอลและแมนนิทอลจากแป้งและซูโครส พบว่ายีสต์ที่ให้ชื่อว่าไอโซเลท KAY1 ซึ่งแยกได้จากผลกระเจี๊ยบสด สามารถย่อยแป้งได้ดีและสามารถผลิตแมนนิทอลสะสมภายในเซลล์ในปริมาณสูงสุดคือ 1.2-1.5 กรัมต่อลิตรของอาหารเลี้ยงเชื้อ เมื่อเลี้ยงยีสต์นั้นในอาหารที่มีแป้งชนิดใดชนิดหนึ่งคือ แป้งมันสำปะหลัง แป้งข้าวเจ้า หรือแป้งข้าวกล้อง ในปริมาณ 2 เปอร์เซนต์เป็นส่วนประกอบ การให้ความร้อนต่อเซลล์ยีสต์ที่อุณหภูมิ 45 องศาเซลเซียสเป็นเวลา 20 นาที ก่อนนำไปเลี้ยงในอาหารแป้ง ช่วยส่งเสริมความสามารถในการผลิตแมนนิทอล ให้ได้ปริมาณเพิ่มขึ้นอีกประมาณ 1.2 เท่า ยีสต์ไอโซเลท PUY4 ซึ่งแยกได้จากผลพุทราสุก มีความสามารถในการผลิตกลีเซอรอลที่ตรวจพบในอาหารเลี้ยงเชื้อในปริมาณ 14 กรัมต่อลิตร เมื่อเลี้ยงในอาหารที่มีซูโครส 5 เปอร์เซนต์เป็นส่วนประกอบ และเมื่อนำไรโซเบียมกลุ่มที่เจริญช้า คือ *Bradyrhizobium japonicum* USDA 11O และ *Bradyrhizobium* spp. THA 5 มาเลี้ยงในอาหารที่มีแมนนิทอลซึ่งเตรียมได้จากเซลล์ของยีสต์ไอโซเลท KAY1 พบว่าเชื้อไรโซเบียมทั้ง 2 สายพันธุ์สามารถเจริญได้จำนวนเซลล์ถึง 10^8 เซลล์ต่อมิลลิลิตร สำหรับอาหารเลี้ยงไรโซเบียมที่มีกลีเซอรอลเป็นส่วนประกอบ พบว่ากลีเซอรอลที่เตรียมจากอาหารเหลวที่ผ่านการเลี้ยงยีสต์ไอโซเลท PUY4 สามารถส่งเสริมการเจริญของไรโซเบียมทั้งสองสายพันธุ์ได้ดีเช่นเดียวกัน และให้ผลทำนองเดียวกับอาหารเลี้ยงไรโซเบียมที่เตรียมโดยใช้กลีเซอรอลและแมนนิทอลที่ผลิตเป็นการค้า และจากการศึกษาเพื่อจัดจำแนกชนิดของยีสต์ พบว่ายีสต์ไอโซเลท KAY1 และ PUY4 จัดอยู่ในสกุล *Rhodotorula* และ *Pichia* ตามลำดับ

สาขาวิชาเทคโนโลยีชีวภาพ

ปีการศึกษา 2544

ลายมือชื่อนักศึกษา.....

ลายมือชื่ออาจารย์ที่ปรึกษา.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

CHARDCHAI BUROM : PRODUCTION OF NUTRIENT SOURCES FOR *RHIZOBIUM*

THESIS ADVISOR : ASST. PROF. SUREELAK RODTONG, PH.D., 202 PP.

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Rhizobia are the effective nitrogen fixation bacteria in symbiosis with legumes. Rhizobial inoculants are currently produced for growing several economic legumes. The utilization of carbon compounds by rhizobia varied with their species and strains. Most slow-growing rhizobia cannot use the simple sugar, glucose, as well as sucrose. Mannitol and glycerol are mainly used for the cultivation of these slow-growing rhizobia. Prices of the carbohydrate compounds are about 12-20 times more expensive than glucose. Consequently, the production of these legume inoculants is costly. This study was aimed to produce glycerol and mannitol for *Rhizobium* cultivation by microbial conversion of cheap substrates such as starch and sucrose. The production cost of rhizobial inoculants might be reduced. A total of 147 yeasts isolated from natural sources and 15 yeast type strains were screened for their glycerol and mannitol production capabilities. It was found that the yeast isolate KAY1 isolated from rozelle fruit could utilized starch, and was proved to be the highest accumulation of mannitol in its cell when cultured in the medium containing 2% of either cassava starch, rice starch, or Khao-klong starch. The maximum yield of mannitol was 1.2-1.5 grams of mannitol per litre of cutured medium of yeast. When the yeast cells were heated at 45^{o}C for 20 min, the production of mannitol in yeast cells was increased 1.2 times higher than untreated cells. The yeast isolate PUY4 isolated from Phut-sa (*Zizyphus jujuba* Lamk) fruit could produce glycerol detected in its broth culture at the concentration of 14 grams of glycerol per litre of cutured medium. When cultured two slow-growing rhizobium strains, *Bradyrhizobium japonicum* USDA 110 and *Bradyrhizobium* spp. THA 5, in the medium containing mannitol prepared from KAY1 cells. The good growth of about 10^{8} cells/ml of the two strains of *Bradyrhizobium* was obtained. When cultured the two Bradyrhizobial strains in the medium containing glycerol prepared from cultured medium of isolate PUY4, their growth was also enhanced. The similar results were achieved when the same compositions of rhizobial media were prepared using either commercial glycerol and mannitol. Yeast isolates KAY1 and PUY4 were identified as belonging to genera *Rhodotorula* and *Pichia*, respectively.

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา.....

ปีการศึกษา 2544 ลายมือชื่ออาจารย์ที่ปรึกษา.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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LIST OF ABBREVIATIONS

A_w	water available
ADP	adenosine 5'- diphosphate
AMP	adenosine 5'- monophosphate
ATP	adenosine 5'-triphosphate
°C	degree celcius
cm	centimetre
e.g.	for example
et al.	et alia (and other)
etc.	et cetera, and others
g	gram
GPY	Glucose-peptone-yeast extract
h	hour
HPLC	High-Performance Liquid Chromatography
IUB	Indiana University Bloomington
L	litre
µl	microlitre
µg	microgram
min	minute
ml	millilitre
mg	milligram
MY	Malt-yeast extract
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide+ H, symbol for hydrogen, the reduced from of NAD
NADP	nicotinamide adenine dinucleotide phosphate

NADPH	nicotinamide adenine dinucleotide+ H, symbol for hydrogen, the reduced from of NADP
OD	optical density
pp.	page
rpm	round per minute
TLC	Thin-Layer Chromatography
w/v	weight by volume
YEPD	Yeast extract-peptone-dextrose
YM	Yeast-mannitol

CHAPTER I

INTRODUCTION

For mass cultivation of root-nodule bacteria used as legume inoculants, it would be great benefit to have low cost and locally available substrates. One of these substrates is starchy material, which is generally available in several countries including Thailand. Bradyrhizobia, the symbionts of some economic legumes, such as soybean, are able to use only glycerol and mannitol to support their growth, but unable to use organic acids, disaccharides, trisaccharides, and polysaccharides or starch for growth (Stowers, 1985). They lack invertase (Martinez-de Drets and Arias, 1972) or amylase, required for hydrolysing the substrates. Mannitol is universally used for bacterial cultivation, both for fast- and slow-growing *Rhizobium,* and glycerol can support a very high bacterial density (Balatti *et al*., 1987). To produce glycerol and mannitol, carbohydrates from agricultural products can be converted into high valued nutrient sources by microorganisms, especially yeasts. Both culture medium, and yeast cell extracts containing glycerol and mannitol, can then be used directly for the cultivation of Bradyrhizobia.

1.1 Rhizobial characteristics

Rhizobia or root nodule bacteria are medium-sized, rod shaped cells, gram-negative, 0.5-0.9 μm in width and 1.2-3.0 μm in length. They do not form endospore and mobile by single polar flagellum or two to six peritrichous flagella. Uneven gram is frequenly encountered with rhizobia, depending on the age of the culture. Cells from a young culture and nodule bacteroids usually show even gram staining while older and longer cells give a banded appearance with unstained areas. These unstained areas have been indentified to be large granules of polymeric betahydroxybutyric acid (PHBA). The PHBA is refractile under phase-contrast microscopy. Rhizobia are predominantly

aerobic chemoorganotrophs and are relatively easy to culture (Allen, 1980). They grow well in the presonce of O_2 and utilize relatively simple carbohydrates and amino acid compounds. With the exception of a few strains, they have not been found to fix atmospheric nitrogen in the free-living form except under special conditions. Optimal growth conditions are of most strains at a temperature range of 25°-30° C and pH of 6.0-7.0. Despite their usual aerobic metabolism, many strains are able to grow well under microaerophilic conditions at O_2 tensions of less than 0.01 atm (Graham and Parker, 1964). Generally, most rhizobia produce white colonies, but those that nodulate *Lotononis bainesii* produce a characteristic red nonheme carotenoid pigment when cultured in yeast-mannitol (YM) medium. Most rhizobia only absorb congo-red (diphenyldiazo-bis-α-nephthylaminesulfonate) dye, which is included in culture media for isolating rhizobia. However, if the culture medium is not buffered, acid-producing rhizobia cause the dye to turn purple. Other interesting and useful characteristics of rhizobia are other growth reactions in the standard YM mediam containing bromthymol blue (BTB) as the pH indicator. Fast growing rhizobia produce an acid reaction in the YM medium containing bromthymol blue (pH 6.8) while slow growers produce an alkaline reaction (Date, 1979).

The basic knowledge on the rhizobial identification by different standards such as 1) some morphological and physiological aspects 2) serological marker 3) intrinsic antibiotic resistant profile 4) nitrogen fixation potential 5) host-dependent cross-inoculation group 6) molecolar biology techniques, etc. For instant of some morphological and physiological aspects as growth rate that *Rhizobium* was fast-grower and *Bradyrhizobium* was slow-grower, colony type, acid-base reaction on YM medium containing bromthymol blue. This technique could segregate only 2 groups were fast-grower that could produce acid while slow-grower that could produce base, and IAA production which could use for separate only soybean nodulation rhizobia (Somasegaran *et al*., 1965; Norris, 1964). The serology, indirect procedures for identifying rhizobia by antigen-antiboby reaction are highly reacts only with the antigen that elicited its formation. Bacterial strains contain naturally occurring mutants which are resistant to certain antibiotics. This resistant may be used for the recognition of rhizobial strains. The cross inoculation group system of classifying rhizobia has been subjected to much criticism, because it is not a taxonomic one, it is the best practical system currently

available (Burton, 1965). The molecular biology techniques, the new technique for study genotypic and phylogeny of rhizobia also has been developing from time to time.

Eventually, with the development of molecular sequencing technique especially of the 16S rRNA gene, the situation has changed. The most prominent person in initiating this revolution study of microbial phylogeny and evolution was Carl Woese who from the sixties on has relentlessly worked towards defining the natural order of microbial life (Woese 1987; Woese 1994; Morell 1997).

The current status of rhizobial taxonomy as shown in table 1, a few years after the work of Young *et al.* (1996), the first phylogenetic trees based on close to full-length 16S sequences were published for rhizobia and related organishs (Willems and Collins, 1993; Yanagi and Yamasato, 1993). These trees showed that rhizobia can be devided into three clusters: 1) the fast-or moderate-growing rhizobia in the genera *Rhizobium, Sinorhizobium, Mesorhizobium,* and *R.galegae*, which is located in the branch containing serveral *Agrobacterium* species, 2) the slow-growing *Bradyrhizobium* and 3) the stem nodulating *Azorhizobium*. Sequencing of the full length of the 16S rRNA gene is now required for the description of new species. The correlation between full-length sequences and various shorter fragments has in general proved to be good and very useful when used for initial strain recognition and screening of large numbers of isolates (Oyaizu *et al*., 1993; Laguerre *et al*., 1993; So *et al*., 1994; van Rossum *et al*., 1995; Hernandez-Lucas *et al*., 1995; Urtz and Elkan, 1996).

Table 1. Currently described species of rhizobia.

***Rhizobium* species**	**Representative strains**
Rhizobium	
Rhizobium leguminosarum	ATCC 10004
bv. *viciae*, *trifolii* and *phaseoli*	
Rhizobium tropici type B	CIAT 899
type A	CFN 299

Table 1. (Continued).

***Rhizobium* pecies**	**Representative strains**
Rhizobium etli	CFN 42, CE-3
Rhizobium hainanense	CCBAU 57015
Sinorhizobium	
Sinorhizobium meliloti	ATCC 9930
Sinorhizobium fredii	USDA 205
Sinorhizobium terange	ORS 1009
Sinorhizobium saheli	ORS 609
Sinorhizobium medicae	A 321, CC 169
Mesorhizobium	
Mesorhizobium loti	NZP 2213
Mesorhizobium huakuii	CCBAU 2609
Mesorhizobium ciceri	UPM-Ca7
Mosorhizobium mediterraneueum	UPM-Ca36
Mosorhizobium tianshanense	CCBAU 3306
[Rhizobium]	
[Rhizobium] galegae	HAMBI 540
Bradyrhizobium	
Bradyrhizobium japonicum	ATCC 10324
Bradyrhizobium elkanii	USDA 76
Bradyrhizobium liaoningense	2281
Azorhizobium	
Azorhizobium caulinodans	ORS 571

Source: Young and Haukka (1996).

1.1.1 Carbon metabolism in *Rhizobium* species

A) Carbon nutrition in free living rhizobia

Many investigations have examined the nutritional diversity of carbon utilization by rhizobia. Mannitol, glucose, sucrose and maltose were established early as useful carbon sources for rhizobia (Fred, 1912; Ziefel, 1911, quoted in Stowers, 1985). One of the earliest delineations of fast- and slow-growing rhizobia was based on carbon nutrition. Some evidences summarized in Table 2, suggests that fast-growing rhizobia are capable of growing on a variety of carbon substrates, whereas slow-growing rhizobia are more limited in their ability to use diverse carbon sources.

Fast-growing rhizobia are able to use a broad range of hexoses, pentoses, disaccharides, trisaccharides, and organic acids (Table 2). On the other hand, slow-growing rhizobia are unable to use disaccharides, trisaccharides, and organic acids for growth (Table 2). Mannitol, the traditional carbon source for rhizobia, gave highly variable growth responses with all rhizobia, but in particular the slow growers (Elkan *et al*., 1968). Gluconate has been recommended for slow-growing rhizobia, but has given poor growth responses with cowpea rhizobia. Glycerol is the most universally used carbon sources among rhizobia (Arias *et al.*, 1976, quoted in Stowers, 1985).

As stated in the previous section, rhizobia are able to use a broad range of sugars, sugar alcohols, and organic acids (Table 2) as well as aromatic compounds for growth. A broad distinction has been made between fast-and slow-growing rhizobia in their ability to use disaccharides. Fast-growing rhizobia were able to use sucrose and other disaccharides, whereas slow growers were unable to metabolize sucrose. Sucrose uptake by *R. leguminosarum* and *R. trifolii* has been determined to be constitutive. Uptakes of sucrose by *R. meliloti* and a fast-growing cowpea rhizobia isolate were determined to be inducible. Identical observations were reported for maltose uptake. Lactose uptake was inducible with all fast-growing species. Evidence suggest that fast-growing rhizobia possess at least two transport mechanisms for disaccharides-one for sucrose, maltose, and trehalose and another for lactose (Stower, 1985).

Table 2. Summary of carbon utilization by *Rhizobium* spp[a].

Carbon source	Fast-growing species			Intermediate	Slow-growing species	
	Group A	Group B	Group C	Group D	Group E	Group F
Glucose	+[b]	+	+	+	+	+
Fructose	+	+	+	+	+	+
Galactose	+	+	+	+	+	+
Gluconate	*N.D.*[c]	*N.D.*	+	+	+	+
Mannose	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+
Rhamnose	+	+	+	-[d]	*N.D.*	*N.D.*
Dulcitol	+	±[e]	-	-	*N.D.*	*N.D.*
Arabinose	+	+	+	+	+	+
Xylose	+	+	+	+	±	+
Raffinose	+	+	+	-	*N.D.*	*N.D.*
Sucrose	+	+	+	+	-	±
Lactose	+	+	+	+	±	±
Trehalose	+	+	+	-	*N.D.*	*N.D.*
Glycerol	*N.D.*	*N.D.*	+	+	+	+
Pyruvate	+	*N.D.*	+	-	±	-
Citrate	+	+	-	-	-	-
Succinate	+	*N.D.*	+	+	±	+
Fumarate	+	*N.D.*	+	-	*N.D.*	-
Malate	+	*N.D.*	+	-	-	±

[a]Group A = *Rhizobium meliloti*, *R. leguminosarum*, *R. trifolii*, *R. phaseoli*; Group B = fast-growing *Rhizobium* spp.; Group C = fast-growing *Rhizobium japonicum*; Group D = intermediate group rhizobia; Group E = *Rhizobium japonicum*; Group F = cowpea miscellany rhizobia. [b] + indicates strains able to use the carbon source for growth; *N.D.* [c] not determined; [d] – indicates

strains unable to use the carbon source for growth; [e] ± indicates more than one strain able to use the carbon source for growth.

Source: Stowers (1985).

1.1.2 Carbon metabolism in free-living rhizobia

It is well established that rhizobia possess the Entner-Doudoroff (ED) pathway or the ketogluconate aldolase (Figure 1) and most hexose metabolism proceeds through the ED pathway (Glenn, *et al*., 1984; Katznelson, 1955; Katznelson, *et al*., 1957, quoted in Stowers, 1985). A hexose cycle has been shown to operate simultaneously in some slow-growing rhizobia, allowing for the conservation of hexoses for biosynthesis from glyceraldehyde-3-phosphate(G3P). The hexose cycle recycles G3P to glucose-6-phosphate via triose phosphate isomerase, Fructose bisphosphate aldolase, hexose diphosphatase and phosphoglucose isomerase (Moat, 1995).

The operation of the Embden-Meyerhof-Parnas (EMP) pathway (Figure 2) in rhizobia appears to be strain-dependent, representing only low levels of activity when observed. Phosphofructokinase activity was not detected in *R. trifolii*, indicating the lack of the EMP pathway in this species. Phosphofructokinase activity was detected in glucose-grown *Rhizobium* sp. 32H1, but not with succinate-, gluconate-, mannitol-, or fructose-grown cells (Stowers, 1985). Fructose bisphosphate aldolase, another key enzyme of the EMP pathway, has been demonstrated in rhizobia. This enzyme appears to be involved in the hexose cycle as described above rather than with the EMP pathway.

The tricarboxylic acid (TCA) cycle is operational in rhizobia. The operation of the TCA cycle has been confirmed using radiorespirometry (Stowers *et al*., 1983, quoted in Stowers, 1985). Specifically labeled pyruvate gave the following $^{14}CO_2$ evolution patterns: C-1 > C-2 > C-3. The preferential release of the C-1 (carboxyl carbon) of pyruvate followed by the C-2 (carbonyl carbon) and C-3 (methyl carbon) is indicative of an operational TCA cycle. Central carbon catabolic pathways are outlined for fast- and slow-growing rhizobia in Figures 3 and 4, respectively.

Mannitol, the most common carbon source for *Rhizobium* cultivation, is metabolized via a mannitol dehydrogenase, producing fructose. In *R. meliloti*, an NAD-linked polyol

dehydrogenase is used for the metabolism of mannitol or arabitol, whereas sorbitol is metabolized via a specific sorbitol dehydrogenase. Another fast grower, *R. trifolii*, possesses five distinct polyol dehydrogenases; inositol dehydrogenase for inositol; ribitol dehydrogenase for ribitol; D-arabitol dehydrogenase for D-arabitol, D-mannitol, and D-sorbitol; xylitol dehydrogenase for xylitol; and D-sorbitol and dulcitol dehydrogenase for dulcitol, ribitol, xylitol, and D-sorbitol. All polyols tested, except xylitol and inositol, induced more than one polyol dehydrogenase. Glycerol supports the growth of both fast and slow growers (Table 2). The metabolism of glycerol proceeds via glycerol kinase and glycerolphosphate dehydrogenase, producing G3P that is further metabolized to pyruvate. Both enzymes of glycerol metabolism are inducible, showing maximal activity with glycerol-grown cells (Stowers, 1985).

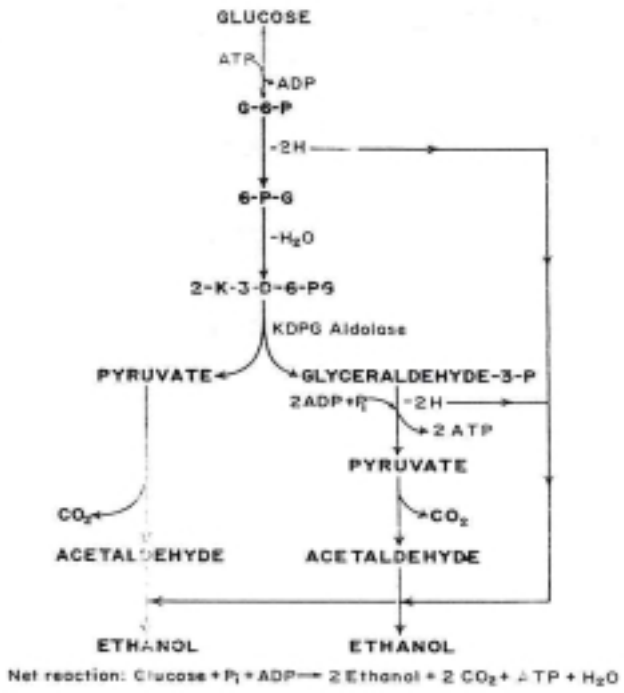


Figure 1. The ketogluconate aldolase or Entner-Doudoroff pathway. G-6-P = glucose-6 phosphate; 6-P-G = 6-phosphogluconate; 2-K-3-D-6-PG or KDPG= 2-keto-3-deoxy-6 phosphogluconate.
Source: Moat (1995).

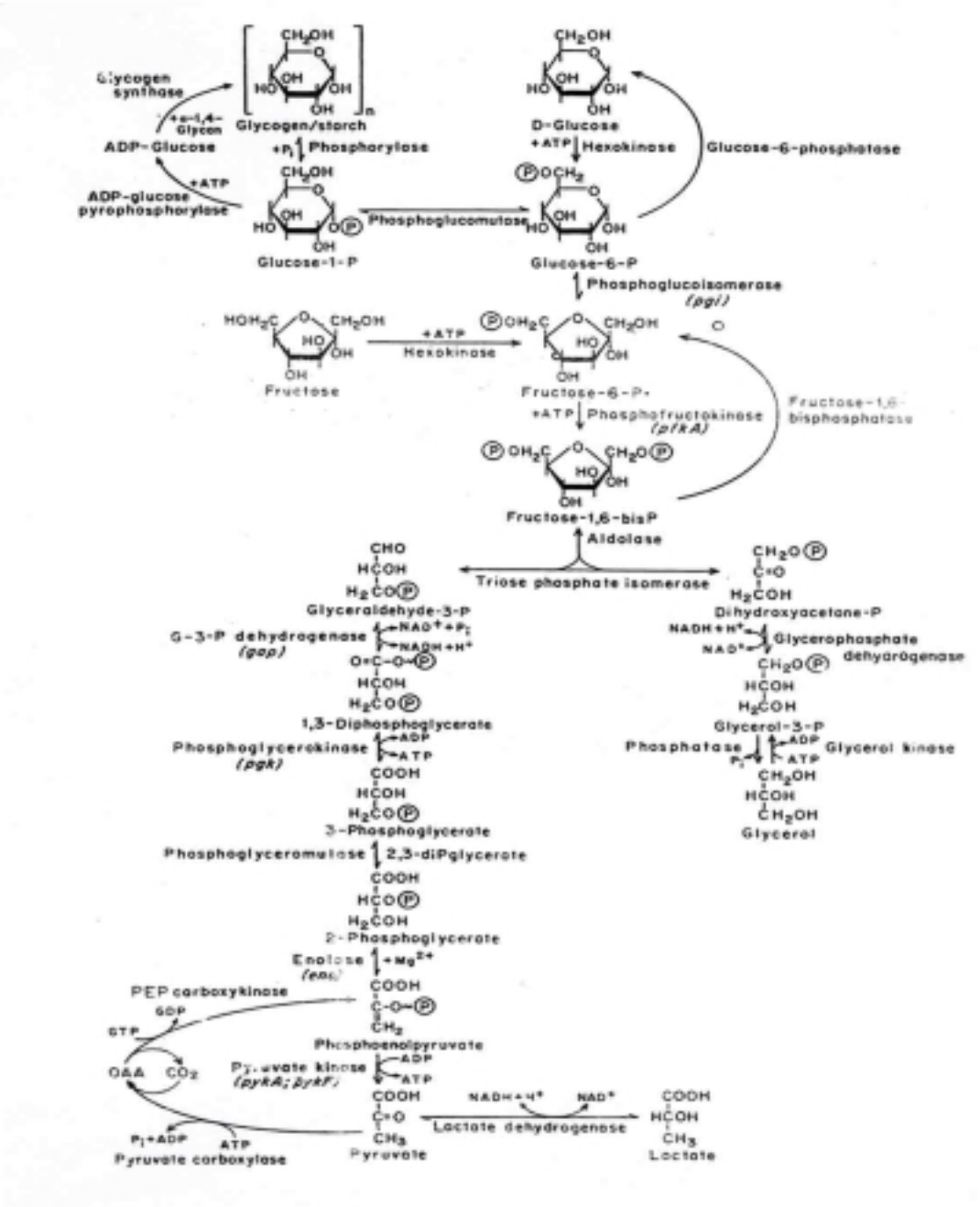


Figure 2. The fructose bisphosphate (FBP) aldolase or Embden-Meyerhof-Parnas (EMP) pathway of glycolysis.

Source: Moat (1995).

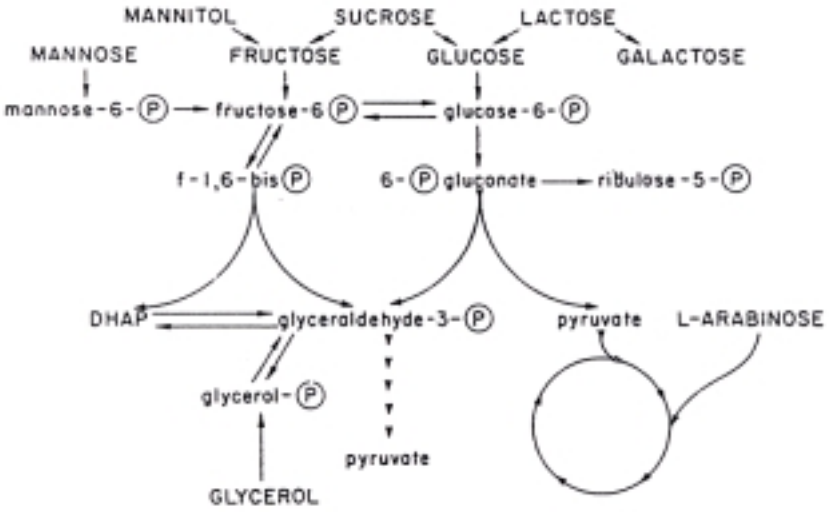


Figure 3. Metabolic pathways for carbon catabolism in fast-growing rhizobia. Source: Stowers (1985).

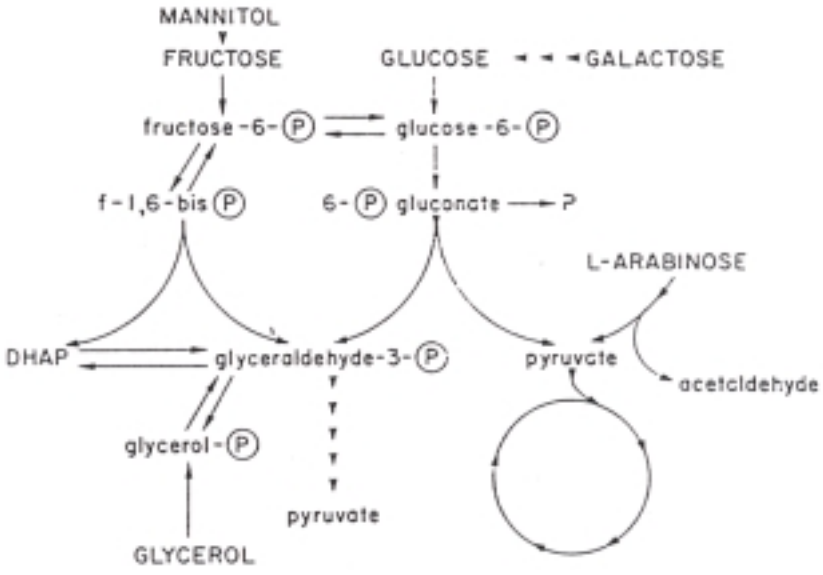


Figure 4. Metabolic pathways for carbon catabolism in slow-growing rhizobia. Source: Stowers (1985).

1.2 Classification of yeasts

Mankind has been concerned with fermentations for at least 8,000 years. The ancient people of Sumaria, Babylon, and Ur documented beer making in their artwork, and somewhat later (~ 4,000 B. C.), the Egyptians depicted the baking of leavened bread. Although these processes were eventually used world wide, it was not until 1837 that independent studies by Cagniard de la Tour, Schwann, and Kutzing show yeasts to be responsible for fermentation. *S. cerevisiae* was the species generally used in baking and brewing, but there were about 600 known yeast species and certain others are also important commercially.

The yeasts are a phylogenetically diverse group of fungi whose teleomorphs or sexual states are found among two major taxonomic classes, the Ascomycotina and the Basidiomycotina. Consequently, the term "yeast" is one of convenience that describes predominantly unicellular organisms that undergo vegetative cell division either by budding or by fission. Furthermore, in contrast to higher fungi, the sexual state of a yeast is not enclosed in a fruiting body (Verachtert *et al*., 1990).

1.2.1 Ascomycetous yeasts

Historically, ascomycetes have been placed in two taxonomic classes, or as subclasses in some treatments, the Hemiascomycetes and the Euascomycetes. Hemiascomycetes are characterized by asci that are not enclosed within a fruiting body (ascocarp), whereas Euascomycetes usually form asci within or upon fruiting bodies. Budding yeasts, fission yeasts and yeastlike genera such as *Ascoidea, Cephaloascus,* and *Taphrina* are assigned to the Hemiascomycetes, but taxonomic treatment of yeasts typically exclude the yeastlike taxa.

Earlier proposals for the ascomycetous yeast have been summarized by Lodder (1970) and Kreger-van Rij (1984). Those systems were mostly based on type of vegetative cell division, ploidy and morphology of ascospores. Another aspect of classification is whether the ascomycetous yeasts represent primitive organisms, as is suggested by their appearance, or whether they are reduced forms derived from various more advanced fungal lineages. Cain (1972) proposed that yeasts represent reduced forms, citing the hat–shaped (galeate) ascospores common to *Pichia (Hansenula), Cephaloascus*, and *Ceratocystis* as evidence of an adaptation that would have occurred just once in

evolutionary history. Redhead and Malloch (1977) accepted this proposal and further argued for the derived status of various yeastlike ascomycetes, which they assigned to existing to existing families of mycelial fungi. Von Arx and Van der Walt (1987) also agree with the concept that ascospore shape was of phylogenetic significance and suggested additional relationships between yeasts and euascomycete families.

In the last few years, the system of classification for yeasts, as well as for other fungi, has been revolutionized by the introduction of phylogenetic analysis of molecular sequences. A majority of the studies had focused on rRNA gene sequences, but comparisons of other molecules have given similar results (Kurtzman, 1994). Some studies have demonstrated the following: (1) budding yeasts and yeastlike taxa such as *Ascoidea and Cephaloascus* are members of the same clade and this clade is a sister groups to the euascomycetes, some of which are dimorphic and have a yeast phase; (2) with few exceptions (e.g., *Eremascus*), euascomycetes form asci within or upon a fruiting body, whereas members of the yeast clade do not; (3) *Schizosaccharomyces*, *Taphrina*, *Protomyces*, *Saitoella* and *Pneumocystis* form a divergent group of taxa basal to the other ascomycetes; (4) ascospore shape, presence or absence of hyphae and pseudohyphae, presence or absence of budding, and most assimilation reactions are not predictive of phylogenetic of phylogenetic relationships (Barns *et al*., 1991; Berbee and Taylor, 1993; Hausner *et al*., 1992; Hendrinks *et. al*., 1992; Nishida and Sugiyama, 1993; Walker, 1985). The classification system used for ascomycetous taxa, is given in Table 3.

Table 3. Classification of the ascomycetous yeasts.[a]

Class **Order** **Family**[b] **Genus**	**Family**[b] **Genus**
Phylum: Ascomycota	Metschnikowiaceae T. Kamienski
"Archiascomycetes"	*Clavispora*
Schizosaccharomycetales Prillinger, Dorfler, Laaser, Eckerlein &	*Metschnikowia*
Lehle ex Kurtzman	Saccharomycetaceae G. Winter

Table 3. (continued).

Class **Order** **Family**[b] **Genus**	**Family**[b] **Genus**
Schizosaccharomycetaceae Beijerinck ex Klocker	*Arxiozyma*
Taphrinales Gaumann & C.W. Dodge	? *Cyniclomyces*
Taphrinaceae Gaumann	? *Debaryomyces*
Taphrina	? *Dekkera*
Lalaria (Anamorph of *Taphrina*)	? *Issatchenkia*
Pneumocyxtidaceae O.E. Eriksson	? *Saturnispora*
Pneumocystis	*Torulaspora*
Euascomycetes	? *Williopsis*
? *Endomyces* [c,d] (*E. scopularum*)	*Zygosaccharomyces*
Oosporidium	Saccharomycodaceae Kudryavtsev
Hemiascomycetes	? *Hanseniaspora*
Saccharomycetales Kudryavtsev	? *Nadsonia*
(synonym Endomycetales Gaumann)	*Saccharomycodes*
Ascoideaceae J. Schroter	? *Wickerhamia*
Ascoidea	Saccharomycodaceae von Arx & van der Walt
Cephaloascaceae L.R. Batra	? *Ambrosiozyma*
Cephaloascus	*Saccharomycopsis*
Dipodascaceae Engler & E. Gilg	Candidaceae Windisch ex van der Walt (Anamorphic)
Dipodascus	*Aciculoconidium*
Galactomyces	*Arxula*
? *Sporopachydermia*	*Blastobotrys*
? *Stephanoascus*	*Botryozyma*
? *Wickerhamiella*	*Brettanomyces*
? *Yarrowia*	*Candida*
? *Zygoascus*	*Geotrichum*
Endomycetaceae J. Schroter	*Kloeckera*
? *Endomyces*[c,d] (*E. decipiens*)	*Myxozyma*
? *Helicogonium*[c]	*Schizoblastosporion*
? *Myriogonium*[c]	*Sympodiomyces*
? *Phialoascus*[c]	*Trigonopsis*
? *Trichomonascus*[c]	
Eremotheciaceae Kurtzman	
Eremothecium	
? *Coccidiascus*	
Lipomycetaceae E.K. Novak & Zsolt	
Babjevia	
Dipodascopsis	
Lipomyces	
Zygozyma	

[a] Authority names for genera are given in the individual chapters.

[b] A question mark preceding the genus name indicates that family assignment is uncertain.

[c] Placement in the class Hemiascomycetes is uncertain. [d] The genus *Endomyces* and the family Endomycetaceae are uncertain.

Source: Kurtzman and Fell (1998).

1.2.2 Heterobasidiomycetous yeasts

Many heterobasidiomycetes have a unicellular budding haploid state alternating with a dikaryotic hyphal state, i.e., they are dimorphic and possess a yeast state. In some species, diploid, aneuploid or dikaryotic yeast states occur, and many anamorphic taxa are known only as yeasts. For teleomorphic yeasts, the hyphal state may be limited in culture, e.g. in some species of *Cystofilobasidium* Oberwinkler & Bandoni, but it can be indefinite and perhaps unlimited in many others, e.g. most species of *Rhodosporidium* Banno, *Tremella* Persoon and *Sirobasidium* de Lagerheim & Patouillard. With few exceptions, our knowledge of development in nature, ecology, and general biology of these fungi is fragmentary or nonexistent. Species parasitizing economically important annual plants (e.g., Ustilaginales) have hyphal phases of known duration and extent, but even in these species, little is known of the frequency of occurrence or distribution of the free-living yeast states. For some taxa, e.g. the sporobolomycetaceous taxa, the abundant and widespread occurrence of the yeast states is known, but occurrence of dikaryotic mycelia in nature is essentially unknown.

The terms “yeast” or “yeast state” are applied to the budding unicellular phase or phase in heterobasidiomycetous life histories. These yeast states are readily obtained from basidiospores in species with macroscopically visible basidiomata, e.g., many species of *Tremalla*, *Sirobasidium*, *Holtermannia* Saccardo & Traverso, *Cystobasidium* (de Lagerein) Neuhoff, *Mycogloea* Olive, and from those causing visible disease symptoms in plants, e.g., species of *Ustilago* (Persoon) Roussel, *Itersonilia* Derx, and others. The yeast states provide one approach to studies of relationships of budding fungi, including determining relationships of anamorphic yeasts of uncertain affinity. Yeasts or yeast states are presently known from Agaricostibales, Atractiellales, Cryptobasidiales. Exobasidiales, Filobasidiales, Graphiolales, Platygloeales, Septobasidiales, Tremellales, and

Ustilaginales (Oberwinkler, 1987). However, yeast states of many of these groups of organisms rarely isolated in nature, i.e., other than from the basidiocarps.

Fungi that form short hyphal fragments or cellular aggregates are frequently referred to as yeastlike, e.g., species of *Trichosporon* Behrend. It should be stressed, however, that the distinctions among the various morphs (unicellular, yeastlike and pseudohyphal) are no always clear. Yeast/hyphal dimorphism which occurs in many groups of Heterobasidiomycetes, seems to be an important texonomic feature. This monograph includes only a small number of those taxa in which obligatory and stable yeast states occur in the normal course of the life history. The classification system used for basidiomycetous taxa is given in Table 4.

Table 4. Summary of basidiomycetous yeast groups.

Characteristic **Order** **Family** **Genus**	**Characteristic** **Order** **Family** **Genus**
Telemorphic taxa	Microstromaceae
I. With "simple" septal pores	Microstroma
A. Basidia cylindric, transversely septate	Exobasidiales
Ustilaginales	Exobasidiaceae
Ustilaginaceae	*Brachybasidium*
Microbotryum	*Dicellomyces*
Schizonella	*Exobasidiellum*
Sorosporium	*Exobasidium*
Sphacelotheca	*Laurobasidium*
Sporisorium	II. With dolipore septa, parenthesomes cupulate
Ustilago	A. Basidia "cruciate-septate
Ustilentyloma and probably with *Ustilago*-type basidia[a]	Tremellales
Sporidiales	Sirobasidiaceae
Sporidiobolaceae	*Fibulibasidium*
Leacosporidium	*Sirobasidium*
Rhodosporidium	Tremellaceae
Sporidiobolus	*Bulleromyces*
?Erythrobasidium	*Itersonilia*[e]
?Kondoa	*Holtermannia*
?Sakaguchia	*Phyllogloea*

Table 4. (continued).

Characteristic	**Characteristic**
Order	**Order**
Family	**Family**
Genus	**Genus**
Platygloeales	*Sirotrema*
Cystobasidiaceae	*Tremella*
Colacogloea	*Trimorphomyces*
Cystobasidium	B. Basidia aseptate
Kriegeria[b]	Filobasidiales
Mycogloea	Filobasidiaceae
Occultifer	*Cystofilobasidium*[f]
Septobasidium	*Syzygospora*
Atractiellales[d]	Anamorphic taxa
Chionosphaeraceae	Sporobolomycetaceae
Chionosphaera	*Bensingtonia pro parte*
Stilbum	*Kurtzmanomyces*
Agaricostillbales	*Rhodotorula pro parte*
Atractogloeaceae	*Sporobolomyces pro parte*
Agaricostilbum	*Sterigmatomyces*
B. Basidia globose, nonseptate	Cryptococcaeae
Graphiolales	*Bullera*
Graphiolaceae	*Cryptococcus*
Graphiola	*Fellomyces*
C. Basidia cylindric, nonseptate	*Kockovaella*
Cryptobasidiales	*Phaffia*
Cryptobasidiaceae	*Trichosporon*
Conyodyctum	*Tsuchiyaea*
Cryptobasidium	*Udeniomyces*
	?Hyalodendron
	?Moniliella

[a] Although economically important smuts have studied in detial, type of teliospore germination is unknown in many species. Direct conjugation of basidial cells occurs in some taxa with *Ustilago*-like basidia and this can result in the complete absence or infrequent occurrence of a yeast state.

[b] *Kriegeria (Xenogloea) eriophori* (monotypic) parasitizes monocots; its relationship to the mycoparasitic taxa placed in the Cystobasidiacese and to most other Platygloeales, may by distant.

[c] Yeast states probably occur in these two genera.

[d] Basidia can be cylindric, transversely septate (e.g. in *Stilbum*), or clavete holobasidia (e.g. as in *Chionosphaera*).

[e] *Itersonilia perplexans* appears to belong in this group (but see also under f), but basidia have not been found. Because of the known features and the rather isolated position among anamorphic yeast groups, it is classified with the Tremellales here.

[f] *Cystofilobasidium* has thick-walled teliospores which germinate with holobasidia. Because of biochemical traits such as cell wall composition, dolipores without parenthesomes, and apparently unique molecular characteristics, Thus, the genus was tentatively placed here. Recent partial 26S rDNA sequences (Fell *et al*., 1992) suggest a more distant relationship between *Cystofilobasidium*, *Mrakia*, *Xanthopyllomyces*, and *ltersonilia* on one side with the Tremellales and the genera *Filobasidium*, and *Filobasidiella* on the other.

Source: Kurtzman and Fell (1998).

1.3 Sugar alcohols in eukaryotic microorganisms

1.3.1 Sugar alcohols, polyhydroxy alcohols or polyols

In sugar alcohols, the carbonyl oxygen of the parent monosaccharide has been reduced, resulting in polyhydroxy alcohols. Table 5 shows examples of sugar alcohols. Both glycerol and *myo*-inositol are important components of lipids. Ribitol is a component of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), and of the teichoic acids, complex polymers found in the cell wall of certain Gram-positive bacteria. Xylitol is derived from xylose and is a common constituent of sugarless chewing gum. D-Sorbitol is an intermediate in a metabolic pathway from glucose to fructose the occurs in certain tissues. In general, sugar alcohols are named by replacing the *-ose* suffix of the parent monosaccharides with *-itol* (Voet, 1995).

1.3.2 Roles of sugar alcohols in fungi

Sugar alcohols (polyols) are ubiquitous soluble carbohydrates in fungi and their major roles are as reserve compounds and osmoregulatory molecules (Brown, 1978). They are often found together with other reserve carbohydrates like glycogen and trehalose. Fungi that grow in high sugar or salt concentrations synthesize polyols like glycerol, arabitol, erythritol and mannitol and such compounds are the basis of osmotolerance, functioning as compatible solutes. Compatible solutes are generally defined as being low molecular weight, neutral compounds with can be accumulated to high intracellular concentrations without causing inhibition of enzymes (Brown, 1978; Moat *et al*., 1995)

Accumulated intracellular polyols have at least three major physiological functions: (i) osmoregulators, (ii) food reserves, and (iii) protectors of enzyme activity at low levels of a_w. Substances with the third function have been called “compatible solutes” (Brown and Simpson, 1972).

Compatible solutes must always behave as osmoregulators, however, and the definition can logically be extended to include solutes which combine functions (i) and (iii). Potassium chloride (and K^+) has this role in halophilic bacteria (Aitken and Brown, 1972, quoted in Brown, 1978). Brown (1978) reported that if a cell was subjected to a water stress by exposing it to a concentrated solution (low a_w) it will either (a) equilibrate thermodynamically with the solution by solution (low a_w) it will either

Table 5. Structure and characteristic of sugar alcohols.

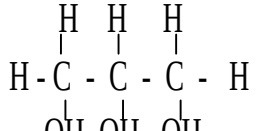
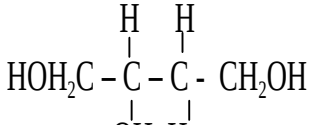
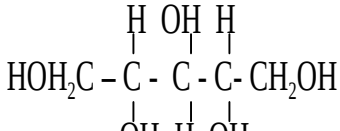
Sugar alcohols	Structure	Description
Glycerol		Glycerol is a crytalline substance with a very low melting point (18°C). It is a constituent of oils and fats, and is obtained from them by fermentation. In that sense, glycerol is naturally occurring.
Erythritol (1,2,3,4-Butanetetrol, D, L-Erythritol, Erythrite)		This four-carbon polyol occurs in lichens, algae and grasses. Its melting point is 121.5° C, and it is very soluble in water.
Xylitol		Xylitol is a five-carbon sugar alcohol found in fruits and vegetables, but the concentration is usually less than 1%. Its parent sugar, xylose, is a component of hemicellulose in wood and corn cobs. The crystalline xylitol melts at 94°C.

Table 5. (continued).

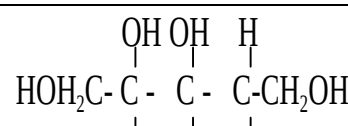
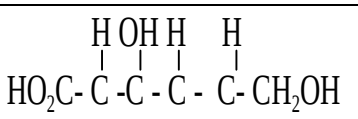
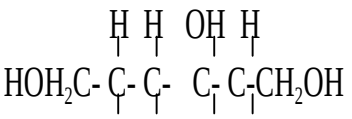
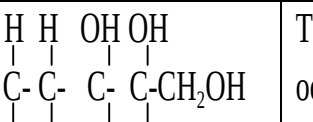
Sugar alcohols	Structure	Description
Arabitol (D-Arabitol, Arabinitol)		Arabitol is not often found in nature, but is present in some lichens and mushrooms. *Fistulina hepatica* is reported to contain 9.5% on a dry basis. Arabitol can also be obtained by the reduction of either D-arabinose or D-xylose. Its crystals melt at 102°C and the specific rotation in borax solution is + 7.8°. L-Arabitol does not occur in nature, but has been synthesized.
Galactitol (Dulcitol, Dulcite)		Galactitol is rather widely distributed in plants, and occurs in high concentration in the manna of *Melanpyrum nemorosum*. It may also be synthesized by the reduction of galactose. Its crystals melt at 188-189°C.
Sorbitol (D-Sorbitol, D-Glucitol)		Sorbitol is widely distributed in plant, particularly in fruits, e.g. apples, cherries, peaches, berries, etc. It may be synthesized by the reduction of D-glucose. The crystals melt at 90°C. The L-form is known, but does not occur naturally.

Table 5. (continued).

Sugar alcohols	Structure	Description
Mannitol (D-mannitol, Mammite)		The sugar alcohol, mannitol, is naturally occuring and is found in exudates of olive and plane trees, and in seaweed. It may be prepared by the reduction of mannose. The crystals melt at 166-168°C. The L-form is known, but does not occur in nature.

Source : Shallenberger (1993); Christain (1994); Voet (1995).

(a) equilibrate thermodynamically with the solution by losing water and perhaps by admitting some of the extracellular solute(s), in which case it had died or become dormant, or (b) suffered a temporary loss of water but used energy to accumulated a solute(s) to a concentration thermodynamically commensurated with the extracellular a_w. The solute can be a metabolite which was retained or an extracellular substance which was accumulated. The effect of solution accumulation was to lower intracellular a_w values to the extended that water again entered the cell to achieved thermodynamic parity with the outside. Thermodynamic parity normally included a factor for an appropriated level of turgor pressure.

1.4 Physiology of xerotolerant microorganisms

The term “osmophilic” was introduced in 1912 (Brown, 1978). It has been useful but it is misleading, firstly because of the connotations which osmotic pressure can have, and secondly because its suffix, “-philic”, implies a requirement for, rather than a tolerance of, a concentrated growth medium (Brown, 1976, quoted in Brown, 1978). Sugar- and salt-tolerance can be used in

specific cases, but Brown (1978) proposed the term “xerotolerant” to cover this entire group of microorganisms.

The natural habitats of the xerotolerant yeasts include floral nectaries; the yeasts are commonly associated with bees and honey. If honey is fermented, xerotolerant yeasts are usually responsible. Their commercial significance lies primarily in the food industry since they can cause spoilage of wine must, syrups and conserves, fruit juices, dessert wines, dried fruits, molasses and malt extract. They are also used in the preparation of various oriental fermented foods including soy sauce and miso paste. Their habitats therefore normally place them in contact with high concentrations of non-electrolytes and sometimes, in special circumstances, with moderately high concentrations of salt (Brown, 1978). The majority of yeasts display a moderate tolerance to growth at low water potentials, but some of those with the capacity to grow well at very low water potentials (i.e. the osmotolerants) are very important economically as food spoilage yeasts. Examples of osmotolerant yeasts included *Candida mogii*, *Debaryomyces hansenii*, *Metschnikowia bicuspidata*, *Schizosaccharomyces octospoms* and *Zygosaccharomyces rouxii* (Walker, 1998).

1.5 Production of sugar alcohols by yeasts

Different strains of *S. cerevisiae* were found to form amounts of glycerol varying between 4.2 to 10.4 g/l (Radler and Schutz, 1981), and two diluted honeys (napunyah, *Eucalyptus ochrophloia*, and medium amber) were fermented at 20°C and 30°C using a wine yeast *S. cerevisiae* (AWRI 729) and mead yeast *S. bisporus* (AWRI 366). Fermentations with wine yeast proceeded faster to give higher alcohol and lower residual sugar products than those with mead yeast which utilised glucose more slowly than fructose. The fermentation ceased after similar times at both temperatures although alcohol production and sugar utilization were more rapid during early stages of fermentation at 30°C than at 20°C (Wootton *et al*., 1983).

The production of glycerol by *Hansenula anomala* in molasses corn steep liquor based media was studied. The accumulation and yield of glycerol was dependent on the medium composition and aeration rate. The control of pH did not affect the yield, but the intermittent addition

of sugar during fermentation resulted in significant increase in production of glycerol (Parekh and Pandey, 1985).

Glycerol production from glucose using an osmophilic yeast, *Pichia farinosa* (ATCC 20210), in an alkaline medium has been investigated in shake flasks. The amount, form, and mode of sodium carbonate addition have been found to affect the yield of glycerol, ethanol, and biomass. These effects are explained in terms of the critical parameters of pH and dissolved oxygen levels in the medium. Relatively high glycerol yields and concentrations coupled with rapid fermentations have been obtained (Vijaikishore and Karanth, 1987). Cells of the osmophilic yeast, *P. farinosa*, were immobilized in sintered glass rings for the production of glycerol (Bisping *et al*., 1990). The kinetics of production were observed under different conditions in batch, fed-batch and semicontinuous fermentations in fixed-bed column reactors and compared with those of free cells. Cells at the concentration of $2.6x10^9$ cells/g sintered glass were adsorbed. The glycerol productivity was up to 8.1 g/l per day. The highest concentration reached in batch culture was 86 g/l with immobilized cells (Bisping *et al*., 1990).

Yeast strains were examined for their ability to produce glycerol. *Kluyveromyces marxianus* NRRLY-665 was chosen for optimization due to its comparatively high growth rate (0.5 h^{-1}) and glycerol yield (9.5% by weight on lactose) at 37°C and pH 7. Optimal conditions for glycerol formation were 30-37°C and pH 6-7 (Rapin *et al*., 1994).

Apiculate wine yeasts were studied for their ability to produce glycerol, acetaldehyde, ethyl acetate, sulphur dioxide and hydrogen sulfide in synthetic medium. *Hanseniaspora guilliermondii* produced smaller quantities of glycerol, acetaldehyde and hydrogen sulphide than *Kloeckera apiculata*. Selected strains of apiculate yeasts might favour an enhanced flavour formation and yield desirable characteristics to the final product (Romano *et al*., 1997).

The production of L-iditol (iditol) from L-sorbose with D-sorbitol dehydrogenase coupled with NADH regeneration under methanol oxidation was studied with resting cell system of a methanol yeast, *C. boidinii* (*Klockera* sp.) No. 2201. The highest amount of iditol, 142-148 g/l (94-98% conversion rate), was obtained from 150 g/l of sorbose in the presence of 0.5 M methanol at pH 6.5 (Vongsuvanlert and Tani, 1988). The production of erythritol by n-alkane-grown. *C. zeylanoides*

KY 6166 was also studied using large scale fermentors. The medium pH was kept below 4.0 during fermentation in either 5-liter or 300-liter fermentor. This strain could produce about 180 mg/ml meso-erythritol and a small amount of mannitol. The yield corresponded to 90% of n-alkane consumed (Hattori and Suzuki, 1973). Onishi and Suzuki (1969) reported that *C. guilliermandii* var. *soya* (ATCC 20216) consumed 5.1 g of D-xylulose, and produced 2.8 g of xylitol per 100 ml of xylulose.

Torulopsis versatilis and *T. anomala* were found to be the potential mannitol producers. Under optimal conditions, the yeasts produced mannitol at the yield of 30% of the sugar consumed (Onishi and Suzuki, 1968). Erythritol, D-arabitol, D-mannitol, and a heptitol-like compound were produced from glycerol by aerobic fermentation of yeasts. *Trigonopsis variabilis* produced erythritol, *C. polymorpha* ATCC 20213 produced D-arabitol, and *T. manitolfaciens* CBS 5981 produced D-mannitol at high yields of 12.6%, 28.4% and 31.0% of the glycerol consumed, respectively (Onishi and Suzuki, 1970).

The yeast, *Z. rouxii* ATCC 12572, was selected for its ability to produce appreciable levels of ethanol and of various polyols from concentrated glucose media (20%, w/v) (Grolean *et al*., 1995). *Z. rouxii* was shown to yield large quantities of glycerol and of the mixture of arabitol and mannitol. The appropriate agitation and aeration (1 vvm) allowed *Z. rouxii* to utilize glucose readily leading to high polyol production. Depending on the fermentation conditions used, *Z. rouxii* ATCC 12572 give either ethanol or various polyols as main fermentation products.

1.6 Sugar alcohols metabolism in yeasts

Osmotolerant yeasts capable of growing in high sugar or salt environments synthesize polyols (sugar alcohols) such as glycerol which function as compatible solutes in osmoregulation (Walker, 1998). Glycerol is involved in carbon metabolism of *S. cerevisiae* in different ways (Figure 5). It can be utilized as a sole carbon source under aerobic condition (Barnett, 1990). However, glycerol is a by-product when glucose or other easily fermentable sugars are converted to ethanol. The production of ethanol from glucose is a redox-neutral process. The role of NADH-consuming glycerol formation is thought to be that it maintains cytosolic redox balance, compensating for cellular reactions which produce NADH (reduced nicotinamide adenine dinucleotide) (Dijken *et al*., 1986, quoted in Nevoigt,

1997). Glycerol is synthesized by reducing dihydroxyacetone phosphate to glycerol-3-phosphate which is catalyzed by a NAD dependent cytosolic G3P dehydrogenase (ctGPD), followed by dephosphorylation of glycerol-3-phosphate by a specific phosphatase (GPP) (Gancedo, 1968). Dihydroxyacetone phosphate, the substrate for the glycerol formation pathway, can be provided either by the glycolytic degradation of sugars or by the gluconeogenic pathway when non-fermentable carbon sourecs are used (Nevoigt, 1997).

Metabolism of mannitol and other hexitols like D-glucitol (sorbitol) and D-galactitol involves specific hexitol phosphate dehydrogenase activity (Figure 6) (Walker, 1998). If mannitol were produced by the direct reduction of fructose by $NADPH_2$, the reaction would be freely reversible. No adenosine triphosphate (ATP) would be an irreversible reaction, and, for each molecule of mannitol formed, one molecule each of ATP and $NADH_2$ would be utilized (Lee, 1967).

Pathways for the biosynthesis and utilization of mannitol have been described for many organisms. Basically, there are two mechanisms. In some organisms fructose in reduced directly by an NADPH (reduced nicotinamide adenine dinucleotide phosphate)-linked mannitol dehydrogenase. In a second mechanism, fructose-6-phosphate (F6P) is reduced to mannitol-1- phosphate (M1P) via and NADH (reduced nicotinamide adenine dinucleotide)-linked M1P dehydrogenase. M1P is acted upon by a specific phosphate (mannitol-1-phosphatase) that releases inorganic phosphate and mannitol (Strandberg, 1968). Boonsaeng *et al.* (1976) reported that two pathways for mannitol formation in fungi. One pathway involves the direct reduction of fructose to mannitol in the presence of reduced nicotinamide adenine dinuclcotide (NADH) or reduced NAD phosphate (NADPH) by D-mannitol: NAD^+_2-oxidoreductase while the other pathway involves the reduction of fructose-6-phosphate to mannitol-1-phosphate in the presense of NADH by D-mannitol-1-phosphate: NAD^+_2-oxidoreductase followed by the hydrolysis of mannitol-1-phosphate to mannitol by D-mannitol-1-phosphatase.

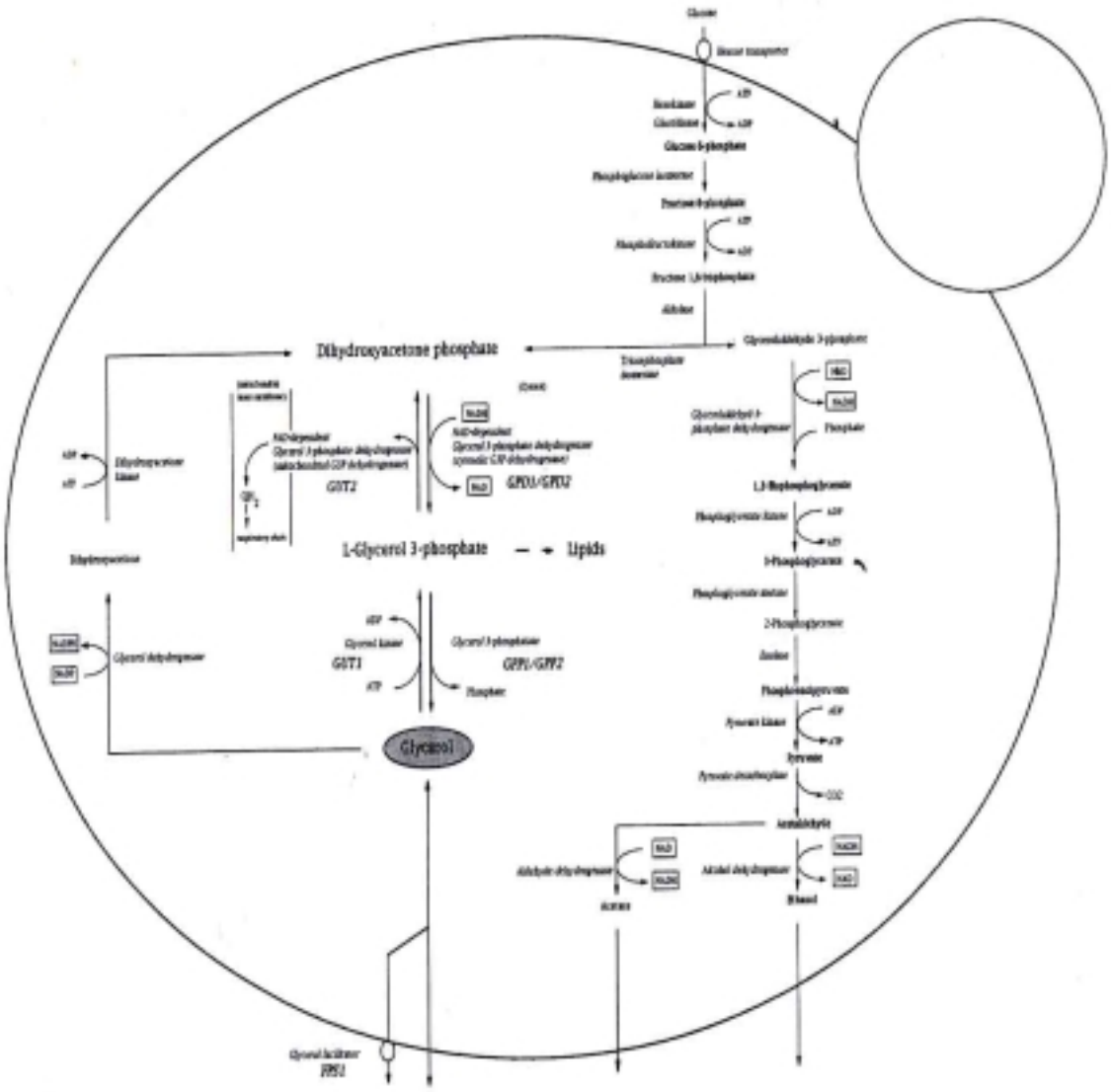


Figure 5. Important pathways of glycerol metabolism in *Saccharomyces cerevisiae***.** Source: Nevoigt (1997).

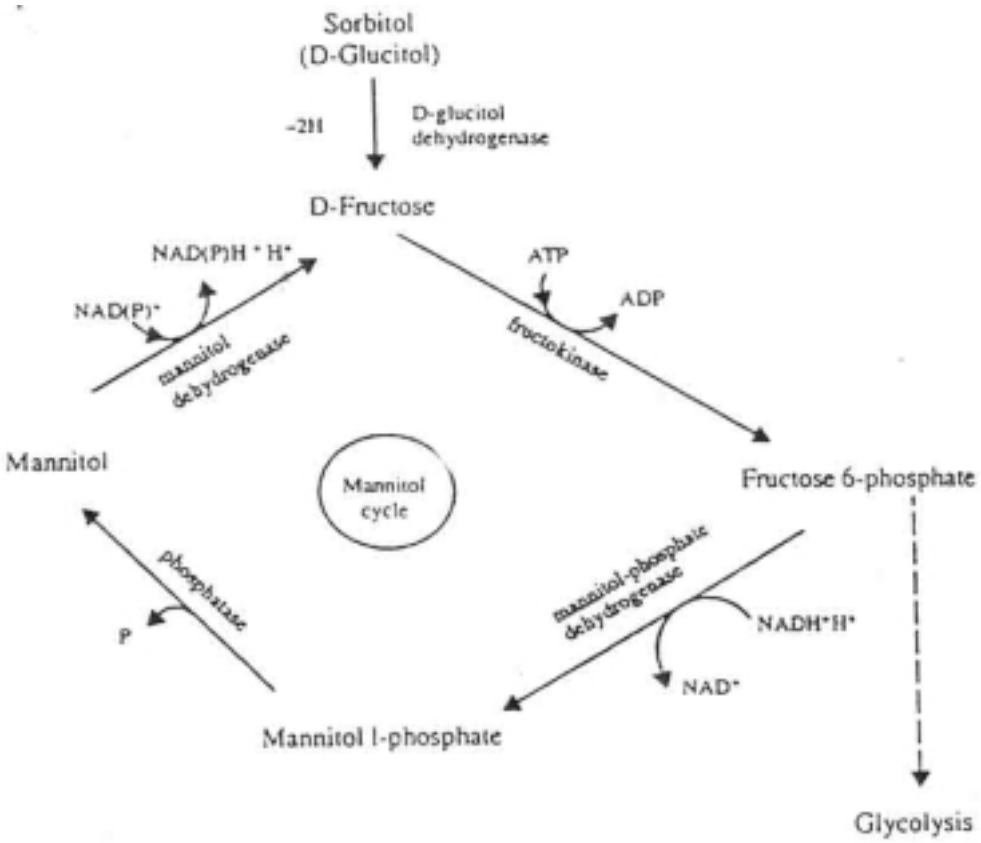


Figure 6. Pathways of mannitol metabolism in yeasts.
Source: Walker (1998); Moat (1995).

1.7 Starch as a sole carbon source

1.7.1 Composition and nutritonal aspects of starch

In superficial terms, starch is a mixture of the predominantly linear α-(1 $\rightarrow$ 4)-glucan, amylose, and the highly branched, high molecular weight amylopectin-and $\propto$-glucan, based on 1-4 glycosidic linkages with $\propto$-(1 $\rightarrow$ 6) branch points (Galliard, 1987). Starch occurs in the form of water-insoluble granules as a major carbohydrates in all higher plants. It is a heterogeneous polysaccharide composed of amylose and amylopectin, two high-molecular weight components that

may be present in different ratios. Usually, amylopectin is the major constituent (75-80%), but waxy (100% amylopectin) and high-amylose (70% amylose) starches are equally know. Both polymers exhibit quite distinct characteristics, such as physicochemical properties and susceptibility to enzymes, which are closely related to structural differences. Amylose is an essentially linear molecule with the degree of polymerization of 200-2000. It composes of α–1,4-linked D-glucose residues with only a very limited number of branch points (e.g., α–1,6-linked D-glucose), producing a few relatively long side chain. Amylopectin has a much higher degree of polymerization (~60,000) and is characterized by multiple branching (Whistler, 1984). The minor components that are associated with starch and which may play significant roles in the functional behavior of starch, the importance of starch is clear from the analysis of dry matter in the raw materials that form the staple foods of various populations (Table 6).

1.7.2 Starch-degrading enzymes

Some characteristics of various important enzymes catalyzing degradation of starch and related amylaceous megalo- and oligo-saccharides are summarized in Table 7. Compared to O-glycoside hydrolases, hexosyltransferases are of minor importance, though not negligible. Additional but less common enzymes and not produced by yeasts, include oligo-1, 6-glucosidase (EC 3.2.1.10), amylo-1, 6-glucosidase (EC 3.2.1.33), isopullulanase (EC 3.2.1.57), exo-maltotetrahydrolase (EC 3.2.1.60), exo-maltotetraohydrolase (EC 3.2.1.98), and cyclomaltodextrin glucanotransferase (EC 2.4.1.19). Vast amounts of data have accumulated on the amylolytic enzymes of plants, animals, bacteria, and fungi, as evidenced by numerous reviews. Microbial amylases have acquired an established position as industrial catalysts. Although the early studies on the amylolytic yeast *Saccharomycopsis fibuligera* were carried out some decades ago. Yeasts have been thoroughly investigated essentially during the past decade as their biotechnological potentials for the production of single-cell protein (SCP) or ethanol from starchy biomass (Verachtert, 1990; Kearsley *et al*., 1995).

1.7.3 Extracellular yeast amylases

The simple starch assimilation test used in yeast classification indicates that a large number of yeast species can grow in the medium containing some types of soluble starch as sole carbon sources (Barnett, *et al*., 1983; Kreger, 1984, Quoted in Verachtert, 1990). When assessing

Table 6. Composition of raw materials for staple foods of different populations**.**

Raw material	Moisture Content (%)	Major components (% dry weight basis)		
		Starch	Protein	Fat
Wheat, grain	14	67	15	2
Wheat, white flour	14	77	13	2
Rice, polished	12	89	8	1
Maize, kernel	65	57	12	7
Potato	76	75	8	<1
Yam	73	90	7	<1
Cassava (manioc, tapioca)	12	90	<1	<1

Source: Galliard (1987).

starch assimilation, different types of soluble starch have been used. One strain may differ in ability to utilize various types of soluble starch, which emphasizes the need for adequate standardization when performing such tests. Differences in ability to assimilate various soluble starch preparations found between species are likely to reflect differences in type and/or location of the amylase(s). Amylaceous poly-, megalo-, and higher oligo-saccharides could not enter the yeast cell. Consequently, the enzymes involved in the dissimilation and assimilation of starch and related polysaccharides were found outside yeast cell membrane, that is, located in the external cell environment (periplasmic space) or secreted in the culture medium (extracellular).

Several comparative studies on starch assimilation and amylase production by yeasts have been conducted. The ability to utilize starch is widely distributed among yeast genera (Verachtert *et al.*, 1990). Species displaying some degrees of starch degradation are found in about 25 yeast genera (MgCann *et al*., 1986, Quoted in Verachtert *et al*., 1990). Unfortunately, numerous species exhibit only a limited amylolytic potential. The nature and location of their amylolytic enzymes have scarcely been studied. Only a preliminary characterization has been carried out for the

amylase systems of the yeast species (Table 8). Of special interest are the species secreting amylase systems that enable extensive starch hydrolysis. This is usually accomplished by the concerted action

Table 7. Classification of major enzymes involved in starch breakdown.

EC Number	Recommended name[a]	Systematic name	Origin[b]	Reactions catalyzed
3.2.1.1	α-Amylase	1, 4-α-D-Glucan glucanohydrolase	A, B, F, P, Y	Endohydrolysis of 1, 4-α-D glucosidic linkages in starch, glycogen, and related polysaccharides and oligosaccharides, liberating reducing groups in α-configuration.
3.2.1.2	β-Amylase	1, 4-α-D-Glucan maltohydrolase	B, P	Hydrolysis of 1-4-α-D-Glucosidic linkages, successively, from the nonreducing ends of starch, glycogen, and related polysaccharides and oligosaccharides, producing β-D-maltose.
3.2.1.54	Cyclomaltodextrinase (cyclodextrinase)	Cyclomaltodextrin dextrinhydrolase	B, Y	Hydrolysis of cyclomaltodextrins to linear maltodextrin, which can be further hydrolyzed.

Table 7. (continued).

EC Number	Recommended name[a]	Systematic name	Origin[b]	Reactions catalyzed
3.2.1.3	Exo-1,4-α-D-Glucosidase (glucoamylase, amyloglucosidase)	1, 4-α-D-Glucan glucohydrolase	A, B, F, Y	Hydrolysis of terminal 1, 4-linked α-D-glucose residues, successively, from nonreducing ends producing β-D-glucose. Most forms of the enzyme also hydrolyze 1, 6-α-D-Glucosidic bonds next to an α-1, 4 linkage. More rapid action on poly- than on oligosaccharides.
3.2.1.20	α-D-Glucosidase (maltase)	α-D-Glucoside glucohydrolase	A, B, F, P, Y	Hydrolysis of terminal, Nonreducing 1, 4-linked α-D-glucose residues with release of, α-D-glucose. Action on polysaccharides, if any, is very slow compared with oligosaccharides.
3.2.1.41	Pullulanase (debranching enzyme, limit dextrinase, R-enzyme)	Pullulan 6-glucanohydrolase	B, P	Hydrolysis of 1, 6-α-D-Glucosidic linkages in pullulan, amylopectin, and in α- and β- amylase limit dextrins of amylopectin. Limited action on glycogen.

Table 7. (continued).

EC Number	Recommended name[a]	Systematic name	Origin[b]	Reactions catalyzed
3.2.1.68	Isoamylase (de-branching enzyme)	Glycogen 6-glu-canohydrolase	B, Y	Hydrolysis of 1, 6-α-D-Glucosidic branch linkages in glycogen, amylopectin, and their β-limit dextrins; 1, 6 linkage hydrolyzed only if at a branch point. Unable to attack pullulan, and limited action on α-limited dextrins.
3.2.1.24	1, 4-α-D-Glucan 6-α-D-Glucosyl-transferase (trans-glucosidase)	1, 4-α-D-Glucan 1, 4-α-D-Glucan (D-glucose) 6-α-D-glucosyl- transferase	F, P, Y	Transfers an α-D-glucosyl residue in a 1, 4-α-D-glucan to the primary hydroxyl group of glucose, free or combined in a 1,4-α-D-glucan.

[a]Name other than those recommended by IUB are given in parentheses.

[b]A = animals; B = bacteria; F = filamentous fungi; P = plants; Y = yeasts.

Source: Verachtert *et al*. (1990).

of extracellular α-amylase and glucoamylase, and both activities have been reported for some species (Table 8). The amylase systems of these yeasts deserve further investigation. The enzymes of several strains producing both α–amylase and glucoamylase have been purified and characterized. These strain are *C. antarctica*, *Filobasidium capsuligenum*, *Lipomyces kononenkoae*, *Saccharomycopsis capsularis*, *S. fibuligera*, and *Schwanniomyces occidentalis* (Verachtert *et al*., 1990). In addition, an isoamylase and a cyclodextrinase from *L. kononenkoae*, an α-gluco-sidase and an α–amylase of *L. starkeyi*, and a transglucosidase from *S. capsularis* have been studied. The properties of

glucoamylases secreted by *Ambrosiozyma monospora*, *C. tsukubaensis*, and *S. cerevisiae* var. *diastaticus* have also been determined (Verachtert *et al*., 1990; Sukhumavasi *et al*., 1975; Wang *et al*., 1984, and Laluce *et al*., 1988).

Table 8. Some properties of partially characterized amylolytic yeast.

Species[a]	**Amylase(s)**	**Location**[b]	**General characteristics**
C. albicans *(C. claussenii, C. langeronii, C. stellatoidae)*	-	E	Glucose and malto-oligosaccharides as hydrolysis products; glucoamylase with relatively high activity on malto-oligosaccharides partially characterized.
C. fennica (Tr. fennicum)	α - Amylase, Glucoamylase[c]	E	Phadebas substrate hydrolyzed.
C. homilentoma	α - Amylase, Glucoamylase[c]	E	Phadebas substrate hydrolyzed.
C. silvanorum	α - Amylase, Glucoamylase[c]	E	Phadebas substrate hydrolyzed.
C. tropicalis	Glucoamylase	E	Intracellular amylase with glycosyltransferase activity also characterized.
Cr. flavus	α-Amylase	E	Phadebas substrate hydrolyzed.
Cr. luteolus	α-Amylase	E	Crosslinked amylose liquefied.

Table 8. (continued).

Species[a]	Amylase(s)	Location[b]	General characteristics
D.polymorphus (D.phaffii, P. polymorpha)	-	C	Two activities (α-Amylase, glucoamylase) reported, but not separated.
P. anomala (C. pelliculosa)	-	C	Two activities tentatively designated as glucoamylase.
P. burtonii	α-Amylase	E	Extracellular glucoamylase also reported but not separated; α-amylase partially cell-associated.
Rh. ingeniosa (T. ingeniosa)	α-Amylase	E	Major portion associated with cell; Phadebas substrate hydrolyzed.
Rhodosporidium sp.	Glucoamylase	E	Hydrolyzes raw starch.
Sp. holsaticus		E	Probably a glucoamylase.
Tr. pullulans	α - A m y l a s e , glucoamylases	E	α-Amylase hydrolyzes β-cyclodextrin; gluco-amylase are glycoproteins with debranching activity.

[a]Name other than those currently recommerded are shown in parentheses. Abbreviations for genera: *C.= Candida*; *Cr.= Cryptococcus*; *D.= Debaryomyces*; *P.= Pichia*; *Rh. = Rhodotorula*; *Sp.= Sporobolomyces*; *Tr.= Trichosporon*; *T.= Torulopsis*.

[b]C = cell-associated periplasmic; E = extracellular (secreted in the medium).

[c]Preliminary identification based on starch hydrolysis pattern.

Source: Varachtert *et al*. (1990).

1.8 Extracted yeast cell products

Yeasts are rich sources of proteins, nucleic acids, vitamins and minerals but with negligible levels of triglycerides. When extracted by either acids (to produce hydrolysates) or enzymes (autolysates) or salt (plasmolysates), yeasts develop characteristic savoury flavours and aromas which are utilized in numerous processed and convenience foods where a ‘meaty flavour’ is sought, e.g.

dried soups, gravy granules, flavoured potato snacks, “Marnite” and “Vegemite” (Walker, 1998). Yeast extracts are normally produced from spent (and debittered) brewer’s yeast, but increasingly on a larger scale from baker’s yeast propagated by the fed-batch process (Reed and Nagodawithana, 1991). Other uses of yeast extracts are in the preparation of microbiological growth media, (e.g. YEPD (Yeast-Extract-Peptone-Dextrose)). Other applications of yeast cells and yeast cell extracts have emerged, and these are summarized in Table 9.

Table 9. Industrial uses of yeast biomass.

Cell product	**Type of yeast product**	**Example of use**
Whole-cell products	Compressed baker’s yeast/ active dried yeast	Baking, brewing, winemaking and distilling
	Yeast cream	Baking and distilling
	Fodder yeast/single-cell protein	Animal feed
	Biotherapeutic/growth factor	Human/animal probiotics
	Reagent yeasts	Biocatalysts in organic chemistry
	Biosorbent yeasts	Heavy metal sequestration
	Mineral yeasts	Nutritional trace elements (Cr, Se) source
	Cosmetic/pharmaceutical yeasts	Skin respiratory factor
	Pigmented yeasts	Feed colorants
	Biological control yeasts	Antifungal agents in agriculture
	Pollution control yeasts	BOD reductions

Table 9. (continued).

Cell product	Type of yeast product	Example of use
Extracted-cell products	Yeast extracts	Food use and microbiological growth media
	Yeast RNA derivatives	Flavour enhancers and pharmaceutical use
	Yeast cell walls	Food and pharmaceutical use
	Yeast B-complex vitamins	Capsules/tablets for dietary supplements
	Yeast enzymes	Invertase and lactase for food use
	Recombinant yeasts	Therapeutic proteins

Source: Walker (1998).

1.9 Objectives

The purpose of this research was

1) To screen effective strains of microorganisms, particularly yeasts, to be used for the conversion of starch to glycerol and mannitol for the cultivation of *Rhizobium*, particularly, *Bradyrhizobium*.

2) To obtain low cost nutrient sources for *Rhizobium* cultivation.

Bradyrhizobia, the synbiont of many tropical legumes, including soybean, are able to use only glycerol and mannitol which are costlier than other carbon sources such as glucose. It will be of great benefit if a cheap and locally available substrate such as starch could be used for the glycerol and mannitol production.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

All chemicals used were of analytical grades, and were purchased from Sigma Chemical Co. (St. Louis, MO, USA), Difco (Detroit, MI, USA), Carlo Erba Reagenti, Fluka Chemika, Merck (Darmstadf, Germany), Pronadisa (Alcobendas, Madrid, Spain), Unilab (Auburn, Australia)**,** and Boehringer Mannheim (Mannheim, Germany).

2.1.2 Microorganisms

Microorganisms screened for their abilities of sugar alcohol production were as follows: type cultures (*Saccharomycopsis fibuligera* TISTR 5033, *Rhodotorula rubra* TISTR 5067, *Hansenula anomala* TISTR 5082, *H. anomala* TISTR 5113, and *Kluyveromyces maxianus* TISTR 5270) obtained from the Thailand Institute of Scientific and Technological Research (TISTR); *Candida utilis*, *C. famata*, *C. krusei*, *C. tropicalis*, *Geotricum candidum*, *Saccharomyces bayanus*, *S. cerevisiae* var. *burgandi*, *S. cerevisiae* var. *champaign*, and *S. cerevisiae* obtained from stock cultures of the School of Microbiology, Suranaree University of Technology, Thailand; yeast isolates Y24, Y60, Y64, and Y69 were obtained from Chumkhunthod (2000); and yeast strains isolated from sample sources (section 2.2). Stock cultures were maintained using Malt-yeast extract (MY) agar (Appendix B2) and stored at 4°C.

Two strains of *Bradyrhizobium* which were *Bradyrhizobium japonicum* USDA 110 (U.S. Department of Agricultural, Beltsville, Md, USA), and *Bradyrhizobium* spp. THA 5 (Ministry of Agriculture and Co-operation, Thailand), were used for the evaluation of media prepared from nutrient sources produced by yeasts.

2.1.3 Equipment and other materials

Equipment used was as follows: High-Performance Liquid Chromatography (HPLC) (Thermo Separation Products Inc., USA), Thin-Layer Chromatography (TLC), UV-visible spectrophotometer (Ultrospec 2000, Phamacia Biotech Inc., England) Hot air oven, Incubator, Autoclave, Refrigerator (4°C), Deep freezer (-20°C and -70°C) (Heto Ultrafreeze, USA), Larminar flow hood, Rotary shaker (New Brunswick Scientific Inc., Germany), Water bath, Microwave oven, Centrifuge, Analytical balance, Pan balance, pH meter, Compound microscope, Haemocytometer, Micropipette sets, and Vortex mixer. Other materials were petridish, test tubes, plastic bag, ice box, basic microbiological equipment, glycerol combination test kit, and liquid nitrogen (Thai Industrial Gas Co. Ltd., Thailand).

2.2 Methods

2.2.1 Isolation and collection of yeasts

Yeast strains were isolated from fresh fruits, fermented fruits, beverages, Khao-mak, solid waste from black soy sauce factories (Nguan Chiang Food Industry Co., Ltd., Thailand), fresh fruits (e.g. pine apple, grape, rambutan etc.), and various agricultural sources such as cassava root, yam bean etc. One gram fresh weight of each sample was suspended in 1 ml of sterile water. The appropriate dilution of the sample was plated on Malt-yeast extract agar medium. The plates were incubated at 30°C for 2 days. Yeast colonies having different morphology were collected and further purified. Yeast isolates were then maintained using Malt-yeast extract agar.

2.2.2 Screening and selecting of glycerol- and mannitol-producing yeast strains

For screening of glycerol- and mannitol-producing yeast strains, one loop of the 3-day yeast culture growing on Yeast extract-peptone-dextrose (YEPD) agar containing 2% glucose (Appendix B4) was inoculated into 8 ml of Yeast extract-peptone-dextrose medium containing 5% glucose (Appendix B3). The culture was incubated at 30°C for 4 days, then analysed for glycerol and mannitol, production. Yeast cells were removed by centrifugation at 8,000 rpm for 10 min at 4°C (Figure 7). Cells were disrupted by using 4 cycles of freezing in liquid nitrogen (-196°C), and thaw at 70°C. Glycerol and mannitol concentrations were primarily determined from both

culture medium and cell lysate using Thin-layer chromatography (TLC) analysis (Lajunen *et al*., 1980; Hellmut *et al*., 1990) (Figure 8). The method described in the glycerol test combination kit (Boehringer Mannheim) was also applied for the detection of glycerol.

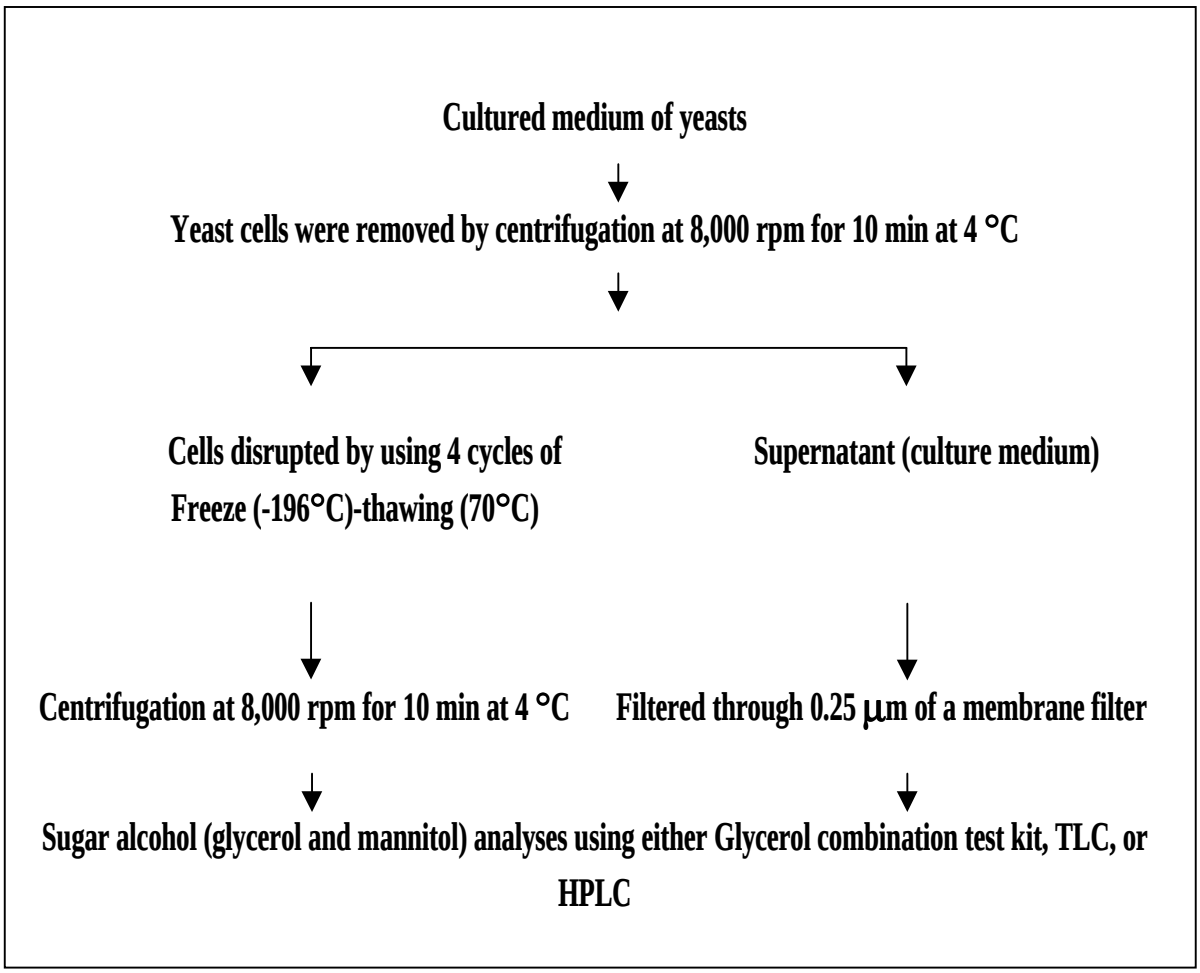


Figure 7. Outline of glycerol and mannitol determination from cultured medium of yeasts.

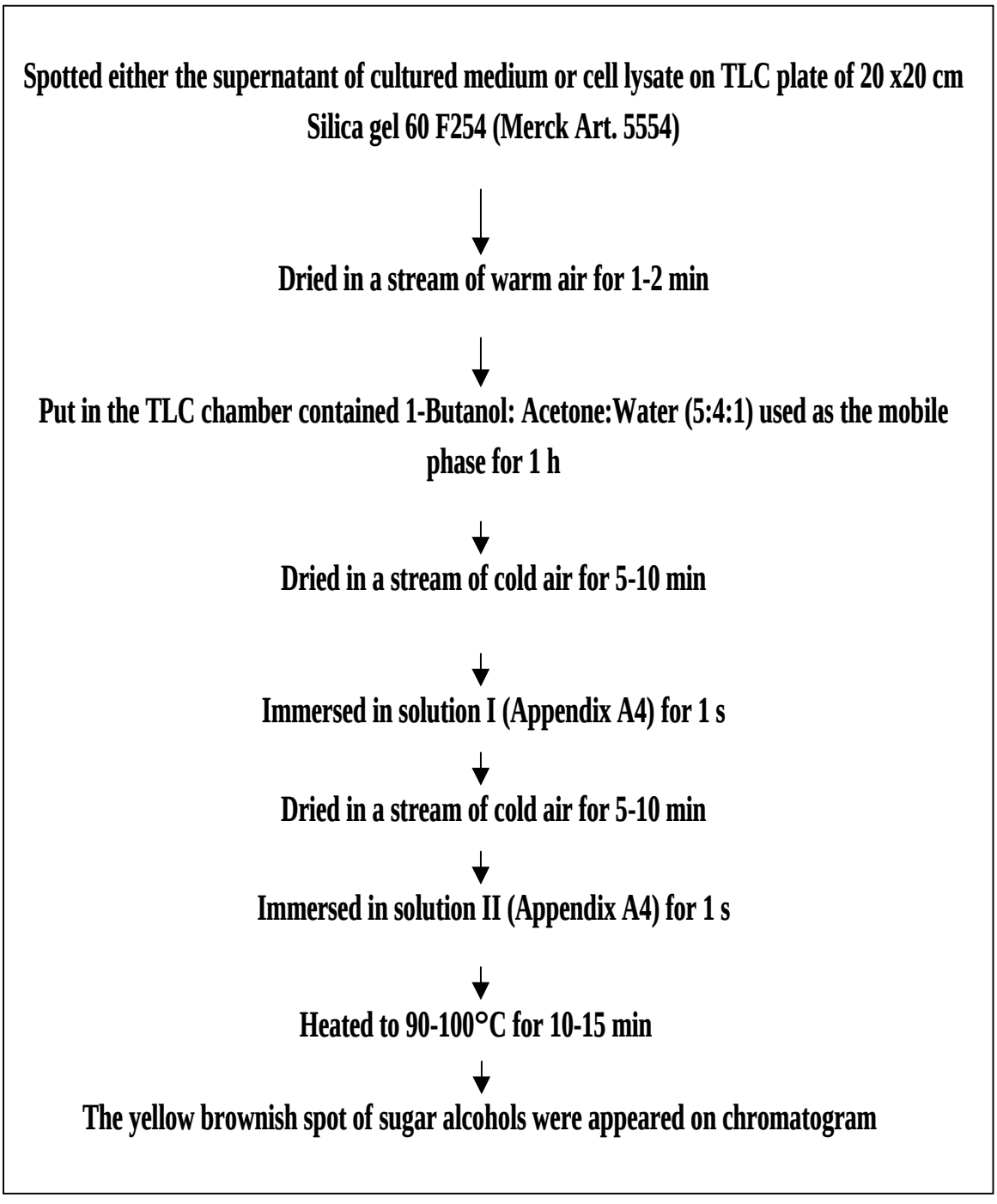


Figure 8. Outline of the Thin-Layer Chromatography (TLC) analysis of sugar alcohols.

2.2.3 Screening and selecting of starch-utilizing yeast strains

Yeast strains (Section 2.2.1) were screened for their amylase production capabilities. The yeast isolates were cultured by patching cells on starch agar (Appendix B5) plate surface (two replicates), and allowed to grow for 3 days at 30°C. Amylolytic zones corresponding to enzymatic degradation of starch were detected by staining the plates with iodine solution (Hesseltine *et al*., 1963, Appendix A5). Colonies exhibiting large clear zones comparing to each other were selected, and maintained using soluble starch agar medium (Appendix B6).

The starch hydrolysis and sugar alcohol production of by selected yeast strains was also determined in liquid medium. Yeast isolates were cultivated in 50 ml of starch broth (Appendix B7) containing 2% (w/v) of either cassava starch (Korat Cassava Industrial, Nakhon Ratchasima, Thailand), or rice starch (Rongsenmechoheng Co., Ltd., Nakhon Ratchasima, Thailand), or Khao-klong starch (Rongngan Pochanahanchainsiri Co., Ltd. Bangkok, Thailand) as their carbon sources with shaking at 200 rpm and incubating at 30°C for 72 hours (two replicates). Samples were collected. Three millilitres of culture medium were drawn aseptically from the fermentation flask and centrifuged for 10 min at 4,500 rpm. The supernatant was analyzed for reducing sugars by the DNS method (Miller, 1959) (Appendix A6). Cells were disrupted by using 4 cycles of freezing in liquid nitrogen (-196°C) and thaw at 70°C, then assayed for sugar and sugar alcohols by High-Performance Liquid Chromatography (HPLC). Growth as cell numbers was determined using haemocytometer (Appendix C1). Starch hydrolysis enzymes were determined.

The measurement of α-amylase activity was performed in test tubes by adding 0.5 ml enzyme solution (diluted if necessary) to a 0.5 ml substrate solution containing 0.0067 M NaCl and 1% soluble starch in 100 ml of 0.02 M phosphate buffer (pH 6.9) (two replicates). After 10 mins incubation at 30°C, the reaction was terminated by adding 0.5 ml 3,5-dinitrosalicylic acid solution and quickly placing the reaction tubes into a boiling water bath for 5 min. The reaction tubes were then cooled in running tap water and diluted with 4 ml doubled-distilled water. The extent of product formation by enzyme reaction was monitored at 540 nm absorbance (A_{540}). Glucose was used as a standard. One unit of α-amylase activity was defined as the amount of enzyme required to liberate one μM of glucose/min or in terms of milligram of maltose liberated in 1 minute at 30°C by 1 ml of the enzyme solution (Berfeld, 1951), even though, in the case of

amylase action, the actual reaction products are dextrin rather than maltose. Specific activity was expressed as amylase activity per milligram of protein. Protein is determined according to Lowry *et al*., (1951) (Appendix A8). For β-amylase 0.016 M acetate buffer, pH 4.8 was investigated. For glucoamylase activity, standard was assayed in the same manner as α-amylase except that the reaction was carried out in 0.05 M sodium acetate buffer, pH 4.5. One glucoamylase unit was defined as the amount of enzyme that released one μM of glucose/min (Tan *et al*., 1984).

2.2.4 Comparison of sugar alcohol production capabilities of selected starch-utilizing yeast strains

Selected yeast isolates were tested for their abilities to convert starch into glycerol and mannitol.

A) Preparation of inocula

Two loopfuls of selected yeast isolates grown for 2 days at 30°C on soluble starch agar medium were inoculated into 100 ml of starch broth. The inoculated starch broth was incubated on a rotary shaker with operating at 200 rpm and incubating at 30°C for 24-48 hours until the culture grow approximate 10^7 cells/ml.

B) Sugar alcohol production from starch

One millilitre of inocula (Section 2.2.4 A) was inoculated into 50 ml of starch broth. Three types of starch: cassava starch, rice starch, and Khao-klong starch were tested as microbial carbon sources. The inoculated starch media were incubated on a rotary shaker operating at 200 rpm and incubating at 30°C for 3 days.

C) Analysis of yeast products

The progress of yeast product formation was determined by their reducing sugars, and glycerol and mannitol production of some starch. Three millilitres of each culture medium (Section 2.2.4 B) were drawn aseptically from the fermentation flask and centrifuged for 10 min at 4,500 rpm. The supernatant was analyzed for reducing sugars by the DNS method. Cell number was determined with a haemocytometer. And the cell viability was examined by using the

standard plate count method using Malt-yeast extract medium. Reducing sugars, glycerol, and mannitol concentration were analysed as follows:

1) Reducing sugars

The method of DNS colorimetric determination of reducing sugars were used. Alkaline 3,5-dinitrosalicylic acid (DNS) forms a red-brown reduction product, 3-amino-5-nitrosalicylic acid, when heated in the presence of reducing sugars. The intensity of the color developed at 540 nm was used to determine reducing sugars from the available carbohydrate content of the medium following hydrolysis of carbohydrate to reducing sugars. One ml of sample was added into 3 ml of the DNS reagent, then mixed well. The mixture was heated at 100°C for 10 min. After rapid cooling to room temperature, the reaction mixture absorbance was determined at 540 nm. Reducing sugars contents were calculated along with the standard curve of glucose.

2) Glycerol, mannitol and other sugar alcohols

A) Primarily qualitative Thin-Layer Chromatography analysis was performed with silica gel 60 F254 (MERCK Art. 5554) and elution with 1-Butanol-Acetone-Water (5:4:1) (Lajunen *et al*., 1980) for the determination of sugar alcohols. The plates were sprayed with $AgNO_3$-NaOH (Hellmut *et al*., 1990) followed by heating to 90-100°C for 10-15 min. Standard solution was 0.5% solutions of glucose, glycerol, mannitol, sorbitol and xylitol.

B) Quantitative High-Performance Liquid Chromatography was equiped with a refractometer RI-1530 (Jasco, Japan), and Rezex RCU-USP Sugar alcohols column (ϕ 250x4.0 mm, Phenomenex). The column temperature was kept constant at 30°C. The mobile phase was deionized water with a flow rate of 0.2 ml/min. Before analysis, culture samples were centrifuged at 8,000 rpm for 10 min at 4°C. The sample was filtered through a Whatman membrane filters.

2.2.5 Optimization of some glycerol and mannitol production conditions

To obtain the efficient glycerol and mannitol production, some optimal conditions for the cultivation of yeast were investigated.

A) Determination of the suitable and optimum concentration of carbon source

Three types of starch: cassava starch, rice starch, and Khao-klong starch were used as carbon sources with various concentrations: 0%, 1%, 2%, 3%, 4%, and 5% (w/v) for yeast cultivation (three replicates). One millilitre of 2% inoculum size ($1.0x10^7$ cells/ml) was inoculated into 250 ml Erlenmeyer flask contained 50 ml of starch medium. The inoculated starch media were then incubated on a rotary shaker operating at 200 rpm and incubating at 30°C. Changes in glycerol, mannitol, and reducing sugar concentrations were measured each day during cultivation for 5 days. Three millilitres of cultured medium were drawn aseptically from the fermentation flask and centrifuged for 10 min at 4,500 rpm. The supernatant was analyzed for reducing sugars by the DNS method. Cells were disrupted by using 4 cycles of freezing in liquid nitrogen (-196°C) and thaw at 70°C, then assayed for sugar and sugar alcohol by High-Performance Liquid Chromatography (HPLC). Cell number was determined using heamocytometer.

B) Determination of the suitable and optimum concentration of nitrogen source

Two types of nitrogen source (ammonium sulfate and urea) were used with various concentrations: 0%, 0.1%, 0.3%, 0.5%, and 0.7% (w/v) (three replicates). One millilitre of 2% inoculum size was inoculated into 250 ml Erlenmeyer flask contained 50 ml of starch medium composted the optimal concentration of either cassava starch**,** rice starch, or Khao-klong starch, and shaken on a rotary shaker operating at 200 rpm/min and incubating at 30°C. Changes in glycerol, mannitol, and reducing sugars concentrations were measured each day during fermentation for 7 days. The determination of growth and product analysis was mentioned in Section 2.2.5 A.

C) Determination of the suitable inoculum size

Two loopfuls of selected yeast isolate grown for 2 days at 30°C on starch agar medium were inoculated into 100 ml of starch broth. The inoculated starch broth was incubated on a rotary shaker with operating at 200 rpm and 30°C for 24-48 hours until

approximate $1.0x10^7$ cells/ ml obtained. Various concentrations: 1%, 2%, 3%, 4%, 6%, 8%, and 10% (v/v) of each inoculum size were tested using 250 ml Erlenmeyer flask contained 50 ml of starch medium containing 2% (w/v) of either cassava starch**,** rice starch, or Khao-klong starch (three replicates) as carbon sources, and shaken on a rotary shaker operating at 200 rpm/min and incubating at 30°C. Changes in glycerol, mannitol, and reducing sugars concentration were measured each day during fermentation for 7 days. The determination of growth and product analysis was mentioned in Section 2.2.5 A.

2.2.6 Sugar alcohol overproduction using physical and chemical stress

To obtain the overproduction of sugar alcohols, stress conditions were applied to select yeast isolates. Various conditions which were heat shock treatment, salt-stress, and pH regulation by the addition of $CaCO_3$ were investigated.

A) Heat shock treatment of yeast cells

Heat shock treatment was performed at 45°C for 0, 20, 40, and 60 min (Figure 9). Yeast cells were grown in 50 ml of pre-culture medium (starch medium containing the optimal concentration of either cassava starch**,** or rice starch, or Khao-klong starch) (three replicates), then shaken on a rotary shaker operating at 200 rpm, and incubating at 30°C for 24-48 hours. Ten millilitres of each pre-culture approximate 10^8 cells per ml were drawn aseptically from culture flask and pipette into the test tubes, heat shocked by raising the temperature from 30°C to 45°C in water bath for the desirable periods. After heat shock treatment, the starch medium were shaken on a rotary shaker operating at 200 rpm, and incubating at 30°C for 6 days. Changes in glycerol, mannitol, and reducing sugars were measured at the maximum yield period during fermentation. Three millilitres of culture medium were drawn aseptically from the fermentation flask and centrifuged for 10 min at 4,500 rpm. The supernatant was analyzed for reducing sugars by the DNS method. Cells were disrupted by using 4 cycles of freezing in liquid nitrogen (-196° C) and thaw at 70°C, then assayed for sugar and sugar alcohol by High-Performance Liquid Chromatography (HPLC). Total cell number was determined using heamocytometer. Viable cell number were determined from the number of colonies that appear on plates of Malt-yeast extract agar.

B) Salt-stress conditions

Fifty millilitres of starch medium containing optimal concentration of either cassava starch**,** rice starch, or Khao-klong starch containing 0%, 3%, 5%, and 7% (w/v) of NaCl (three replicates). The appropriate concentration of inocula were inoculated into the starch medium, shaken on a rotary shaker operating at 200 rpm, and incubating at 30°C for 6 days. Changes in glycerol, mannitol, and reducing sugars were measured at the maximum yield period during fermentation. The determination of growth and product analysis was mentioned in Section 2.2.6 A.

C) Calcium carbonate ($CaCO_3$) addition

Fifty millilitres of starch medium containing optimal concentration of either cassava starch**,** rice starch, or Khao-klong starch containing 0%, 0.25%, 0.5%, and 0.75% (w/v) of $CaCO_3$ (three replicates). The appropriate concentration of inocula which was 24-48 hour old was inoculated into the starch medium, shaken on a rotary shaker operating at 200 rpm and incubating at 30°C for 6 days. Changes in glycerol, mannitol, and reducing sugars were measured at the maximum yield period during fermentation. The determination of growth and product analysis was mentioned in Section 2.2.6 A.

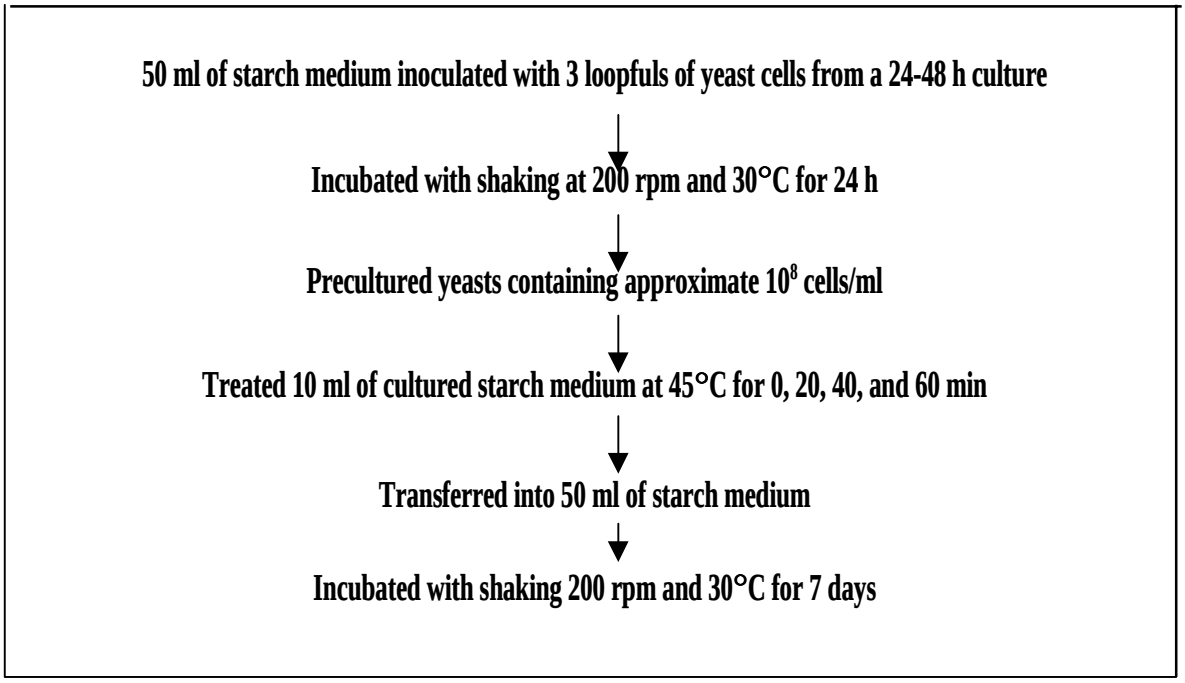


Figure 9. Outline of heat shock treatment for yeast cells.

2.2.7 Production of glycerol and mannitol for *Bradyrhizobium* cultivation

A) Production of glycerol

The glycerol-producing yeast isolates were selected for this experiment. Fermentation experiments were carried in 500 ml of YEPD medium containing 5% sucrose (three replicates). A starter was prepared by using 50 ml of YEPD medium containing 2% sucrose in a 250 ml Erlenmeyer flask inoculated with a loopful of a 2 day-slant culture. The starter culture was incubated at 30°C for 24 hours on a rotary shaker operating at 200 rpm. Then, two percent inoculum size of the starter culture was inoculated into 500 ml of YEPD medium containing 5% sucrose, with shaking at 200 rpm, and for 30°C. Glycerol concentration was measured each day during fermentation for 6 days. Three millilitres of culture medium were drawn aseptically from the fermentation flask and centrifuged for 10 min at 4,500 rpm. The supernatant was analyzed for glycerol by High-Performance Liquid Chromatography (HPLC). Cells were disrupted by using 4 cycles of freezing in liquid nitrogen (-196°C), and thaw at 70°C, then assayed for sugar alcohol by High-Performance Liquid Chromatography (HPLC). Cell number was determined with a heamocytometer. Viable cell number were determined from the number of colonies that appear on plates of Malt-yeast extract agar.

B) Production of mannitol

The selected sugar alcohol-producing yeast isolate was maintained on Malt-yeast extract agar slant. Fermentation experiments were carried in 500 ml of 2% starch medium (cassava starch, rice starch, or Khao-klong starch) (three replicates). A starter was prepared by using 100 ml of 2% starch medium in a 250 ml Erlenmeyer flask inoculated with a loopful of a 2 day slant culture. The starter culture was incubated at 30°C for 24 hours on a rotary shaker operating at 200 rpm, then the starter was heated by heat shock treatment mentioned in section 2.2.6 A. The appropriate inoculum size of the starter culture was inoculated in the 500 ml of 2% starch medium (cassava starch, rice starch, or Khao-klong starch), with shaking at 200 rpm, and for 30°C. Changes in glycerol, and mannitol were measured at the maximum yield period during fermentation. The determination of growth and product analysis were mentioned in Section 2.2.7.A.

2.2.8 Cultivation of *Bradyrhizobium* using nutrient sources produced by yeasts

The slow-growing *Bradyrhizobium japonicum* USDA110 (U.S. Department of Agriculture, Beltsville, Md, USA), and *Bradyrhizobium* spp.THA5 (Ministry of Agriculture and Co-operation, Thailand) were used in this study. The rhizobia were maintained on Yeast-mannitol (YM) agar (Appendix B14). Selected glycerol and mannitol-producing yeast strains were applied for the preparation of nutrient sources for *Bradyrhizobium*.

A) Cultivation of *Bradyrhizobium* using medium containing glycerol produced by yeasts

The cultured medium (section 2.2.7A) containing glycerol was used to prepare *Bradyrhizobium* culture medium according to the composition of the standard G5 medium (Appendix B15) (two replicates). The G5 medium was used for the comparison of rhizobial growth. Five percent inoculum size of *Bradyrhizobium* grown for 6 days at 28°C on Yeast-mannitol broth were inoculated into 100 ml of the two culture media as mentioned above. The cultures were incubated at 28°C on a rotary shaker at 185 rpm for 6 days. The viable cell number of bacteria was estimated by counting the number of colony-forming units on Yeast-mannitol agar plates.

B) Cultivation of *Bradyrhizobium* using medium containing mannitol produced by yeasts

The cultured medium (section 2.2.7 B containing mannitol was used to prepare *Bradyrhizobium* culture medium according to the composition of Yeast-mannitol medium (two replicates). Yeast-mannitol medium was used for the comparison of rhizobial growth. Five percent inoculum sizes of *Bradyrhizobium* grown for 6 days at 28°C on Yeast-mannitol broth was inoculated into 100 ml of the two culture media as mentioned above. The cultures were incubated at 28°C on a rotary shaker at 185 rpm for 6 days. The viable cell number of bacteria was estimated by counting the number of colony-forming units on Yeast-mannitol agar plates.

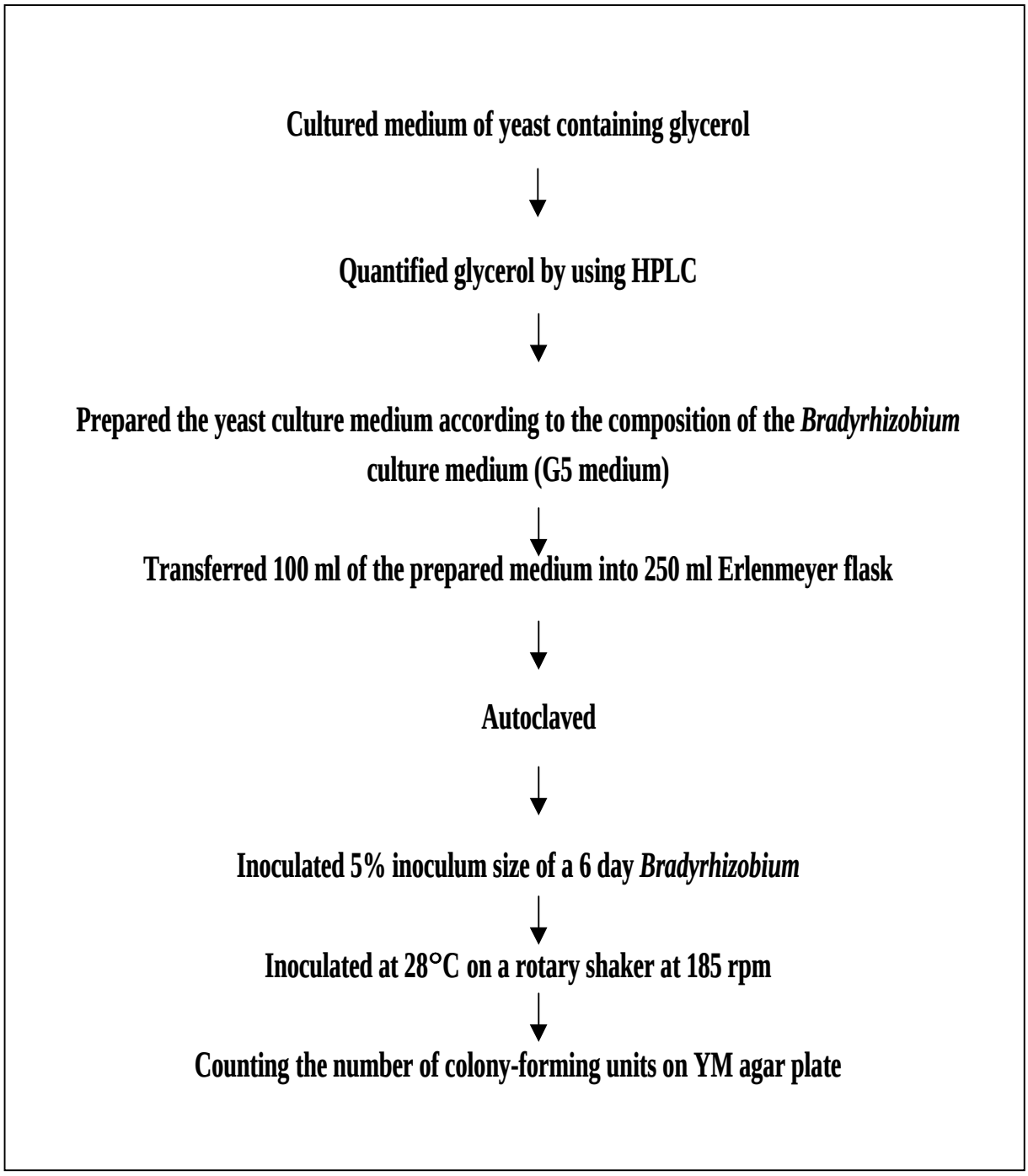


Figure 10. Outline of *Bradyrhizobium* cultivation on media containing glycerol produced by yeasts.

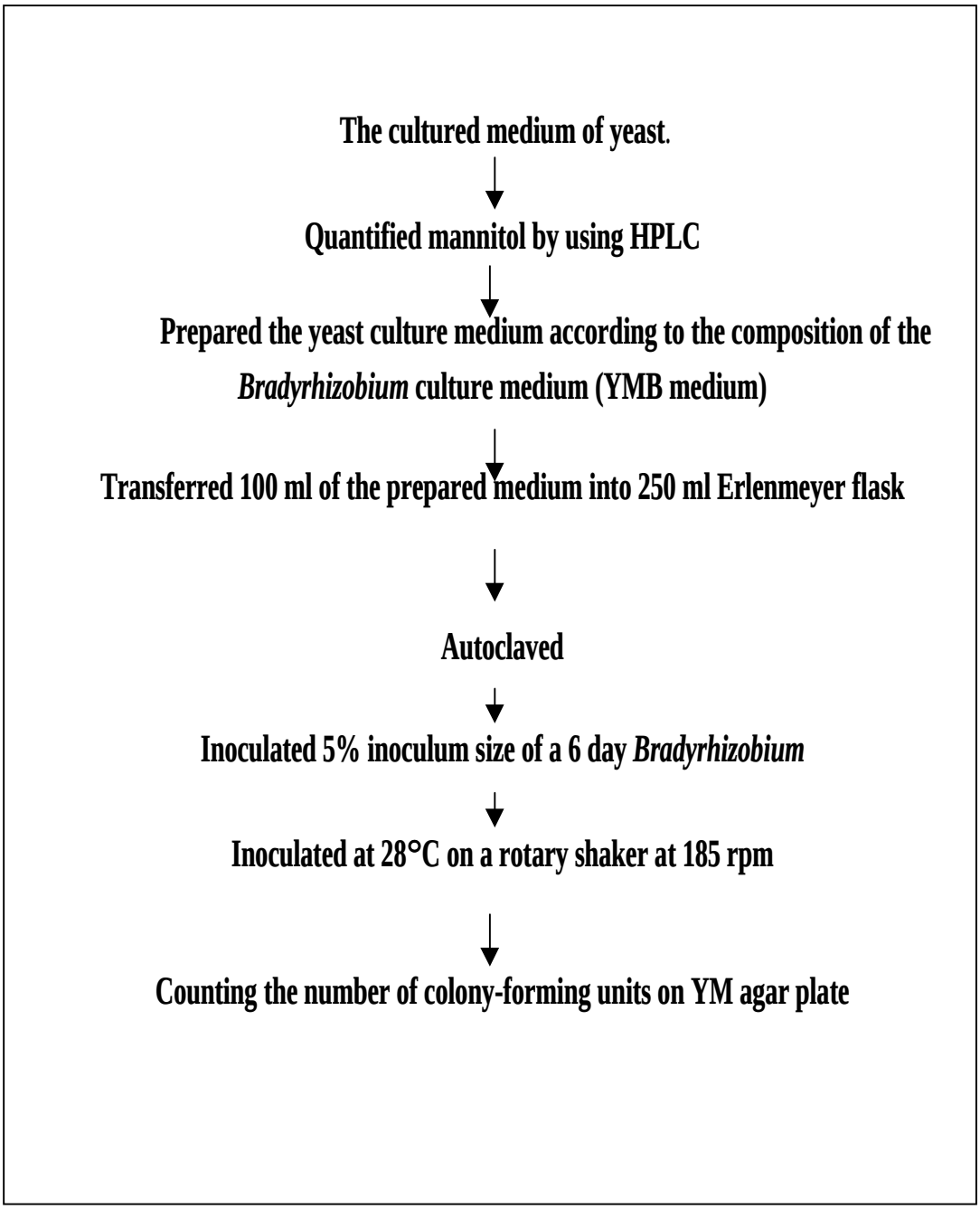


Figure 11. Outline of cultivation of *Bradyrhizobium* on media containing mannitol produced by yeasts.

2.2.9 Identification of selected yeast isolates

The identification of selected yeasts was performed as the methods described by Barnett *et al.* (1990), and Kurtzman and Fell (1998). Yeast cell morphology was observed including spore formation, budding cells, vegetative cells as well as pseudomycelia or pseudohyphae. The physiological and biochemical characterization i.e., fermentation and assimilation of carbon sources, urea hydrolysis and nitrate assimilation was studied. Microbial growth were also investigated at 25, 30, 35, 37 and 40°C.

A) Ascospore formation

The strain to be examined was brought to active growth by cultivating on Malt-Yeast extract agar at 30°C for 24-48 hours. Special pre-sporulation media applied were Acetate agar 2 (McClary *et al.*, 1959) (Appendix B1), Glucose-peptone-yeast extract (GPY) agar (Appendix B11), and Malt-yeast extract agar (Appendix B2). Tubes of sporulation media were slightly inoculated by yeast culture and incubated at suitable temperatures as mentioned above. Preparations of material from the culture were examined under the microscope after 2 or 3 days, 1 week, and then at weekly intervals for at least 6 weeks. Either lactophenol cotton blue mounted or stained heat-fixed preparation was used for the observation of ascospore formation. A heat-fixed preparation was flooded with a solution of 0.5% malachite green and 0.05% basic fuchsin and heated to stain for 1 min, then washed throughly in flowing water and blotted dry.

B) Physiological and biochemical characterictics

1) Fermentation of carbohydrates

In this experiment, eleven carbon compounds were used, namely: D-glucose, D-galactose, maltose, sucrose, α,α-trehalose, melibiose, lactose, cellobiose, melezitose, raffinose, D-xylose. The ability was tested in Fermentation basal medium (Appendix B9) containing 2% (w/v) solutions of sugar. Two ml of basal medium was added into tubes 150x12 mm in size, which containing Durham tubes (approximately 50x6 mm in size). One ml of concentrated, filter-sterilized sugar solution was aseptically added to give a final sugar concentration of 2% (w/v). The tubes of test media were inoculated with cells from a 24-hour culture, and incubated at 25°C for up to 28 days. The tubes were shaken and inspected at frequent

intervals for accumulation of gas in the insert and, if used, a change of color in the indicator. The results were scored depending on the time taken to fill the insert with gas and the amount accumulating, and compared with the descriptions according to Kurtzman and Fell (1998).

2) Assimilation of carbon compounds

Eighteen carbon compounds namely: D-glucose, D-galactose, maltose, sucrose, α,α-trehalose, melibiose, lactose, cellobiose, melezitose, raffinose, D-xylose, L-arabinose, fructose, D-gluconate, D-mannitol, L-rhamnose, D-ribose, and salicin were used in this study. The tests were carried out in ELISA plates. Each well containing 50 μl of 6.8% (w/v) of one test substrate (dupplicated), except those for negative controls, which had no carbon source added, and those for positive controls, which contained glucose. Suspensions of cells from young actively growing cultures were used to inoculate into wells of test media. The young yeast culture was grown on Malt-yeast extract medium for 24 hours at 30°C, 200 μl of the suspension of yeast in Malt-yeast extract medium was pipetted aseptically into 6 ml of fermentation basal medium and each well was then inoculated with 120 μl of the resulting suspension, to give a final sugar concentration of 2% (w/v). The tests were incubated at 25°C for 24-48 hours. The acid production was examined by changing of indicator color. Results were compared to the descriptions according to Kurtzman and Fell (1998).

3) Assimilation of nitrogen compounds

The medium used for testing the ability to utilize nitrogen source was Nitrate broth (Appendix B13). Six ml of medium was transferred into tubes (150x12 mm in size), which contained Durham tubes (approximately 50x6 mm in size). The tubes of test media were inoculated with 0.1 ml of cell suspension from a 24-hour culture, and then incubated at 30°C for 3-7 days. The tubes were shaken and inspected at frequent intervals for the accumulation of gas in Durham tubes. The method of testing for the utilization of nitrate in liquid medium was the chemical detection of nitrate. One of tubes gave a positive reaction for nitrate if the yeast utilizes nitrate. A few drops of reagent 1, and reagent 2 (Appendix A7) were added to the culture. The development of a distinct pink or red color indicates the presence of nitrite produced as a result of the partial utilization of nitrate. The absence of color might indicated that the nitrate had been

completely consumed. Therefore, to test for nitrate in the medium, a small pinch of zinc powder was added to the tube in which the previously described test had just been done (Kurtzman and Fell, 1998). Any nitrate still present was reduced to nitrite in the presence of the zinc and the characteristic pink color will develop after a few minutes.

4) Hydrolysis of urea

A tube containing 0.5 ml of Urea R broth (Appendix B8) was inoculated by a loopful of cells from a one- or two-day-old culture, and incubated at 37°C (irrespective of whether the yeast could grow at this temperature). The tube was examined every half hour for a change of color to red, most do so within two hours and all within four hours, which indicated hydrolysis of urea.

5) Effect of temperature on cell growth

When testing in liquid medium, a tube of Glucose-peptone-yeast extract broth was inoculated with 0.1 ml of a suspension cells from a culture of 24-48 hours old, and then incubated at 25, 30, 35, 37, and 40°C. The result was recorded after 1 week and 3 weeks. When testing on agar medium, a slant of Glucose-peptone-yeast extract agar was inoculated with cells of a young culture and incubated at the chosen temperature for 4 days and then inspected for growth.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Isolation and collection of yeasts

A total of 147 yeast strains isolated from various sources: fresh fruits, fermented fruits, fermented milk products, solid waste from food factory, etc (Appendix 1D). Most of yeast habitats were most often be rich in simple organic carbon, liquid or very high in moisture, acidic or occasionally alkaline, and nutritional complex (Kurtzman and Fell, 1998). Such conditions were met by plant tissue undergoing various forms of decay, as well as exudates of roots, leaves, or flowers and some yeasts might be regarded as extremophiles, particularly certain osmophillic yeasts, which were able to thrive in solute-rich environments. Several of these yeast species were encountered as food spoilage organisms (Walker, 1998).

The characteristics of the cultures were noted when the cells were examined after 3 days of cultivation at 30°C. The following features of the appearance of cultures were recorded in Appendix 1D. Distinctive colors of colonies, such as white, white-cream, yellow, orange, pink or red as well as were recorded, the surface of colony, whether glistening or dull, and the colony morphology, as described in Appendix 1D.

3.2 Screening and selecting of glycerol- and mannitol-producing yeast strains

One hundred and forty-seven yeast isolates from various sources and 15 type strains, were cultured in YEPD medium containing 5% glucose for 4 days, in order to study the sugar alcohol production capabilities by the medium with high concentration of sugar (Omori *et. al*., 1995) for the primary screening. Both yeast culture broth and cell lysate were determined for glycerol and mannitol by Thin-layer chromatography (TLC technique). One percent of sugar alcohol concentration: glycerol,

mannitol, xylitol, and sorbitol were used as standards for the comparison of R_f.. The standards gave R_f 0.741, 0.464, 0.433, 0.406, and 0.350 for glycerol, xylitol, glucose, mannitol, and sorbitol respectively, which corresponded to that of authentic sugar alcohols.

In culture broth, 117 isolates produced glycerol could be detected on TLC chromatogram, and 3 isolates could produced high concentration of mannitol on chromatograms. In cell lysate, the glycerol spot were found slightly from extracts of 2 isolates, and 13 isolates produced mannitol (Table 10). For 15 type strains, the culture broth of *Saccharomyces cerevisiae* var. *burgundi, S. cerevisiae* var. *champaign*, *H. anomala* TISTR 5082, *K. maxianus* TISTR 5270, and *S. baryanus* showed dense spots of glycerol on chromatograms. *Saccharomyces cerevisiae*, *C. utilis*, *C. famata*, *C. krusei*, and *C. tropicalis*, and *S. fibuligera* TISTR 5067 could also produce glycerol detected from their culture broth (Table 11, Figure 14, 15 and 16). In cell lysate, only *Rhodotorula rubra* TISTR 5067 produced mannitol detected on chromatogram (Figure 12 and 13). TLC chromatograms of yeast culture broth and cell lysates were shown in Figures 12-16. Mannitol was detected from 11 isolates (AFY4, LLY3, AFY5, SLY1, TAY7, WAY6, KAY1, TAY9, PIY2, PUY4, and Y60) and 1 type strain, *R. rubra* TISTR 5067 (Figure 12 and 13). Glycerol was detected 9 isolates (COY1, SWY1, BLY2, APY6, FAY1, FAY2, LIY2, TAY9 and CIY1) (Figures 14, 15, and 16).

The Glycerol combination test kit was also used for the determination of glycerol in the screening step. Thirty-three yeast isolates and 1 type strain, *K. maxianus* TISTR 5270, showed dark spots on TLC chromatograms were selected for quantitative analysis of glycerol. Nine yeast isolates with the four scores (Table 10) gave glycerol productivity approximate 1.86-4.64 g/l. The yeast isolate LIY2 gave the highest glycerol production, 4.64 g/l. The mannitol production was quantified by using High-Performance Liquid Chromatography (HPLC). Three yeast isolates (KAY1, PIY2, and PUY4) produced both of mannitol and glycerol in yeast cells and could also produced in cultured medium. At the three scores, the highest glycerol production were 2.66 g/l and 1.68 g/l respectively from yeast isolate APY6 and *K. maxianus* TISTR 5270 (Figure 17).

For the determination of glycerol and mannitol using High-Performance Liquid Chromatography (HPLC), the sugar alcohols production was compared with the spot on chromatogram. Yeast isolates KAY1, PIY2, and PUY4, and *R. rubra* TISTR 5067 could produce glycerol and mannitol in both cultured media and cell lysates (Table 12). In cultured medium, the

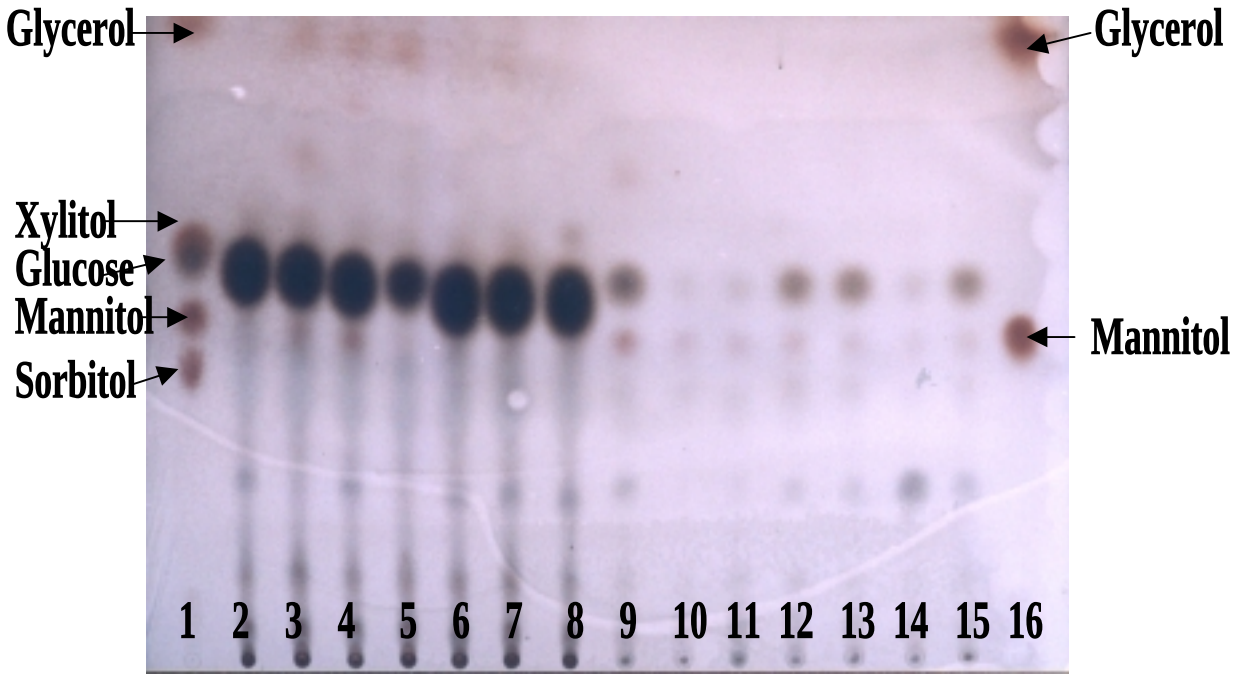


Figure 12. TLC chromatogram of glycerol and mannitol determination from yeasts when cultivating for 4 days in YEPD medium containing 5% glucose. Lanes 3-8 and 9-15 were samples from cultured broth and cell lysates respectively. Lanes: 1, standard mixture; 2, 5%YEPD broth; 3, KAY1; 4, PIY2; 5, PUY4; 6, WAY6; 7, AFY4; 8, *Rhodotorula rubra* TISTR 5067; 9, KAY; 10, PIY2; 11, PUY4; 12, WAY6; 13, AFY4; 14, *R. rubra* TISTR 5067; 15, LLY3; and 16, standard mixture.

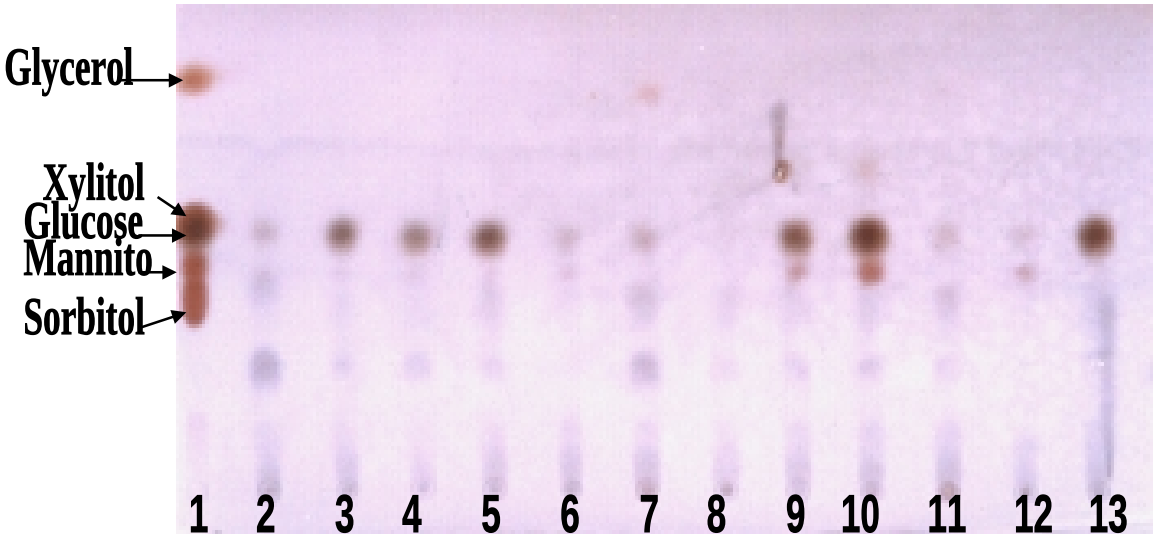


Figure 13. TLC chromatogram of yeast cell lysates when cultivating for 4 days in YEPD medium containing 5% glucose. Lanes: 1, standard mixture; 2, *Rhodotorula rubra* TISTR 5067; 3, AFY4; 4, LLY3; 5, AFY5; 6, PUY4; 7, SLY1; 8, TAY7; 9, WAY1; 10, KAY1; 11, TAY9; 12, PIY2, and 13, Y60.

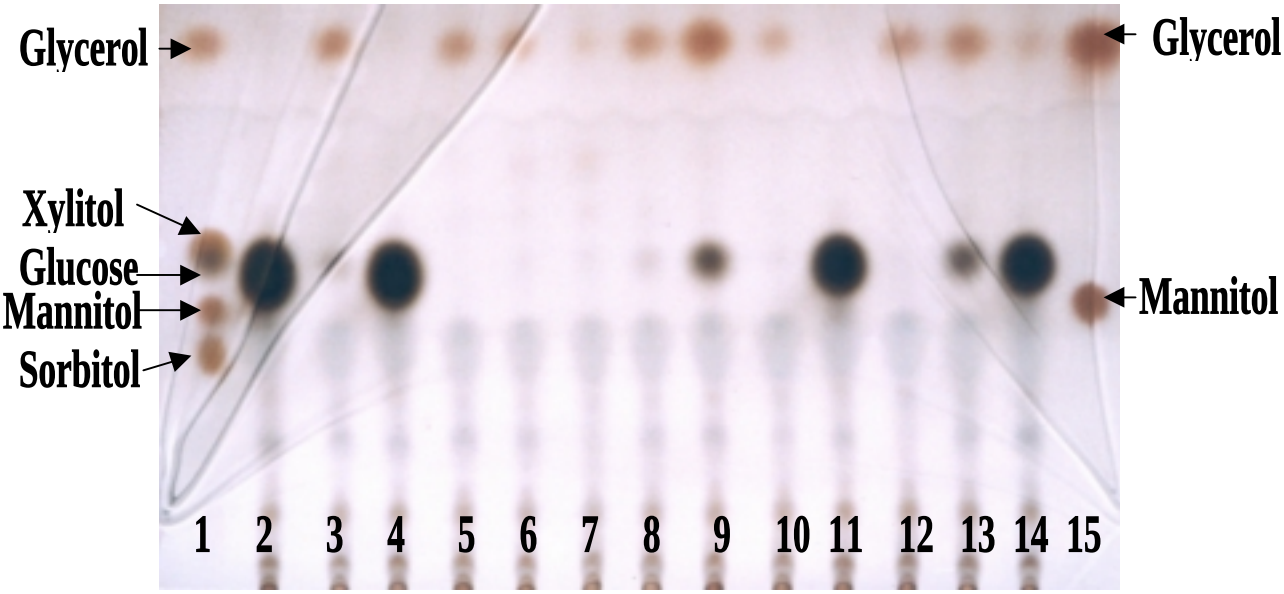


Figure 14. TLC chromatogram of glycerol determination from yeast cultured broth when cultivating for 4 days in YEPD medium containing 5% glucose. Lanes: 1, standard mixture; 2, YEPD broth containing 5% glucose. Yeast isolates: 3, CIY1; 4, WAY6; 5, APY5; 6, *Kluyveromyces marxianus* TISTR 5270; 7, SOY2; 8, PIY3; 9, LIY2; 10, YOY1; 11, MLY2; 12, SAY1; 13, APY2; 14, FAY4; and 15, standard mixture.

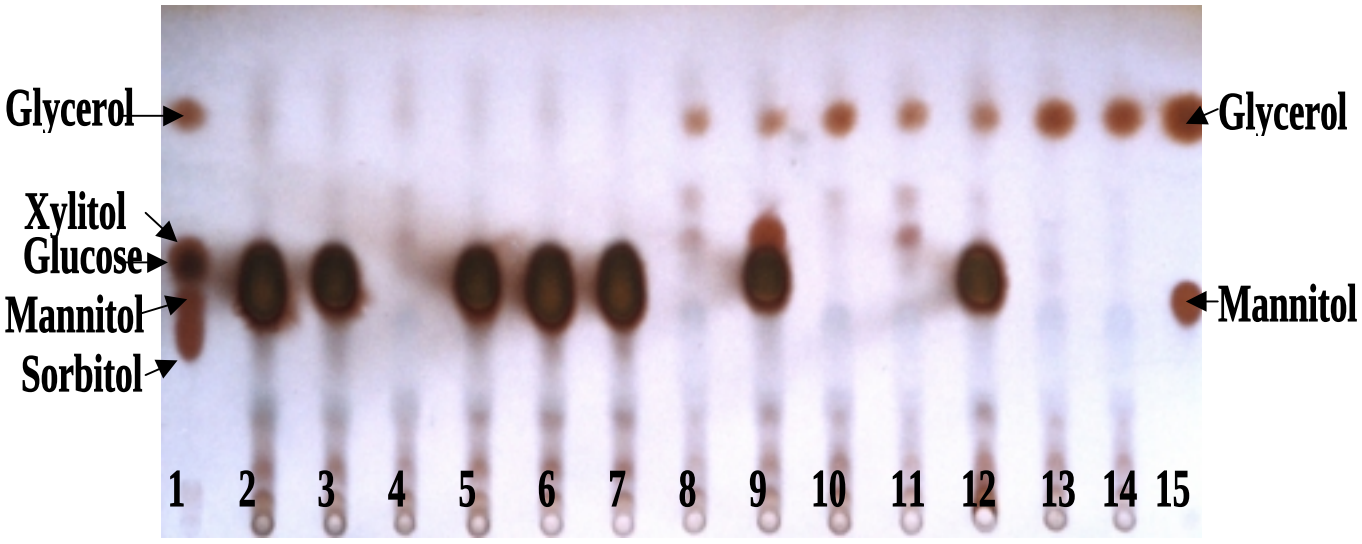


Figure 15. TLC chromatogram of glycerol determination from yeast cultured broth when cultivating for 4 days in YEPD medium containing 5% glucose. Lanes: 1, standard mixture; 2, YEPD broth containing 5% glucose. Yeast isolates: 3, MLY2; 4, AFY1; 5, OAY1; 6, SOY1; 7, SOY5; 8, SOY2; 9, LLY1; 10, LAY1; 11, TAY3; 12, TAY4; 13, *Saccharomyces cerevisiae*; 14, ***S****. bayanus*; and 15, standard mixture.

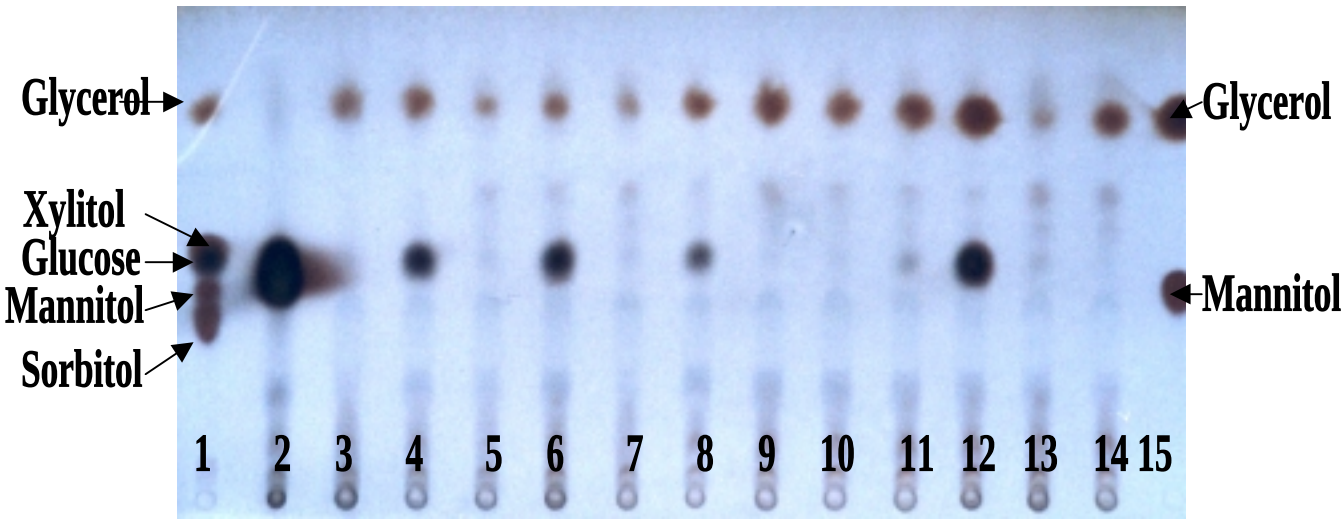


Figure 16. TLC chromatogram of glycerol determination from yeast cultured broth when cultivating for 4 days in YEPD medium containing 5% glucose. Lanes: 1; standard mixture; 2, YEPD broth containing 5% glucose. Yeast isolates: 3, RIY1; 4, ORY1; 5, ORY2; 6, AMY1; 7, AMY2; 8, CIY1; 9, TAY9; 10, APY6; 11, FAY1; 12, LIY2; 13, *Hansenula anomala* TISTR 5082; 14, *Kluyveromyces marxianus* TISTR 5270; and 15, standard mixture.

Table 10. Primary screening of glycerol and mannitol production by yeast strains when culturing in the medium containing 5% glucose for estimation concentration usingTLC analysis.

No.	Isolate number	Culture medium		Cell lysate		No.	Isolate number	Culture medium		Cell lysate	
		Gly[a]	Man[b]	Gly[a]	Man[b]			Gly[a]	Man[b]	Gly[a]	Man[b]
	Standard	++++	++++	++++	++++		Standard	++++	++++	++++	++++
1	AFY 1	+	-	-	-	26	CNY 2	+++	-	-	-
2	AFY 2	+++	-	-	-	27	CMY 1	+++	-	-	-
3	AFY 3	+++	-	-	-	28	CRY1	ND	ND	ND	ND
4	AFY 4	++	-	-	++	29	CRY2	ND	ND	ND	ND
5	AFY 5	++	-	-	++	30	DRY 1	+++	-	-	-
6	AMY 1	+++	-	-	-	31	FAY 1	++++	-	-	-
7	AMY 2	+++	-	-	-	32	FAY 2	++++	-	-	-
8	APY 1	+++	-	-	-	33	FAY 3	+++	-	-	-
9	APY 2	+++	-	-	-	34	FAY 4	++	-	-	-
10	APY 3	+++	-	-	-	35	GFY 1	+++	-	-	-
11	APY 4	NG	NG	NG	NG	36	GRY 1	ND	ND	ND	ND
12	APY 5	++	-	-	-	37	GRY 2	+++	-	-	-
13	APY 6	++++	-	-	-	38	GRY 3	+++	-	-	-
14	BAY 1	-	-	-	-	39	HUY 1	+	-	-	-
15	BAY 2	-	-	-	-	40	HUY 2	+++	-	-	-
16	BAY 3	+++	-	-	-	41	HUY 3	+++	-	-	-
17	BLY 1	NG	NG	NG	NG	42	HUY 4	+++	-	-	-
18	BLY 2	++++	-	-	-	43	KAY 1	+	+	-	+++
19	BLY 3	+++	-	-	-	44	LAY1	++	-	-	-
20	BLY 4	+++	-	-	-	45	LAY 2	NG	NG	NG	NG
21	CAY 1	++	-	-	-	46	LIY 1	+	-	-	-
22	COY 1	++++	-	-	-	47	LIY 2	++++	-	-	-
23	CHY 1	NG	NG	NG	NG	48	LIY 3	+++	-	-	-
24	CIY 1	++++	-	-	-	49	LIY 4	+++	-	-	-
25	CNY 1	++	-	-	-	50	LIY5	++	-	-	-

Table 10. (continued).

No.	Isolate number	Culture medium		Cell lysate		No.	Isolate number	Culture medium		Cell lysate	
		Gly[a]	Man[b]	Gly[a]	Man[b]			Gly[a]	Man[b]	Gly[a]	Man[b]
51	LLY 1	++	-	-	-	76	PAY4	ND	ND	ND	ND
52	LLY 2	++	-	-	-	77	PIY 1	+++	-	-	-
53	LLY 3	++	-	-	++	78	PIY 2	++	++	+++	++
54	LLY 4	+++	-	-	-	79	PIY 3	+++	-	-	-
55	MAY 1	+++	-	-	-	80	POY 1	+	-	-	-
56	MAY 2	+++	-	-	-	81	POY 2	++	-	-	-
57	MEY 1	+++	-	-	-	82	POY 3	+++	-	-	-
58	MEY 2	+++	-	-	-	83	PUY 1	++	-	-	-
59	MEY 4	+++	-	-	-	84	PUY 2	++	-	-	-
60	MEY 3	+++	-	-	-	85	PUY 3	+++	-	-	-
61	MIY1	ND	ND	ND	ND	86	PUY 4	+++	-	+	++
62	MLY 1	++	-	-	-	87	RIY 1	+++	-	-	-
63	MLY 2	+	-	-	-	88	RIY2	ND	ND	ND	ND
64	MLY 3	+++	-	-	-	89	RIY 3	+++	-	-	-
65	OAY 1	+	-	-	-	90	RIY4	ND	ND	ND	ND
66	OAY 2	+++	-	-	-	91	RIY5	ND	ND	ND	ND
67	OAY 3	+++	-	-	-	92	RIY 6	ND	ND	ND	ND
68	OAY 4	+	-	-	-	93	RIY7	ND	ND	ND	ND
69	ORY 1	+++	-	-	-	94	SAY 1	+++	-	-	-
70	ORY 2	+++	-	-	-	95	SCY 1	+++	-	-	-
71	ORY 3	+++	-	-	-	96	SCY2	ND	ND	ND	ND
72	ORY 4	+++	-	-	-	97	SCY3	ND	ND	ND	ND
73	PAY 1	++	-	-	-	98	SLY 1	+++	-	+	++
74	PAY 2	+++	-	-	-	99	SLY 2	+++	-	-	+
75	PAY 3	NG	NG	NG	NG	100	SOY 1	+	-	-	-

Table 10. (continued).

No.	Isolate number	Culture medium		Cell lysate		No.	Isolate number	Culture medium		Cell lysate	
		Gly[a]	Man[b]	Gly[a]	Man[b]			Gly[a]	Man[b]	Gly[a]	Man[b]
101	SOY 2	++	-	-	-	125	SYY 2	+++	-	-	-
102	SOY 3	+++	-	-	-	126	SYY 3	++	-	-	-
103	SOY4	++	-	-	-	127	TAY 1	+	-	-	++
104	SOY 5	+	-	-	-	128	TAY 2	+++	-	-	-
105	SOY 6	NG	NG	NG	NG	129	TAY 3	++	-	-	-
106	SOY 7	NG	NG	NG	NG	130	TAY 4	++	-	-	-
107	SPY 1	+++	-	-	-	131	TAY 5	+++	-	-	-
108	SPY 2	-	-	-	-	132	TAY 6	+	-	-	-
109	SMY 1	+++	-	-	-	133	TAY 7	+++	-	-	++
110	SMY 2	-	-	-	-	134	TAY 8	+++	-	-	-
111	SMY 3	+	-	-	+	135	TAY 9	++++	-	-	++
112	STY 1	+++	-	-	-	136	WAY 1	++	-	-	-
113	STY 2	-	-	-	-	137	WAY 2	++	-	-	-
114	STY 3	NG	NG	NG	NG	138	WAY 3	++	-	-	-
115	STY 4	+++	-	-	-	139	WAY 4	+	-	-	-
116	STY 5	-	-	-	-	140	WAY 5	NG	NG	NG	NG
117	STY 6	-	-	-	-	141	WAY 6	+	++	-	++
118	SUY 1	+++	-	-	-	142	VEY 1	+++	-	-	-
119	SUY 2	+	-	-	-	143	YOY 1	+		-	-
120	SWY 1	++++	-	-	-	144	Y 64	+++	-	-	-
121	SWY 2	+++	-	-	-	145	Y 24	NG	NG	NG	NG
122	SWY 3	+++	-	-	-	146	Y 60	+	-	-	+
123	SWY 4	++	-	-	-	147	Y 69	+++	-	-	-
124	SYY 1	+++	-	-	-						

ND= not detected, NG= not growth, Gly[a] = Glycerol, Man[b]= Mannitol.

++++ = Dark brown, +++ = Medium brown, ++ = pale brown, + = very slightly, and - = no spot.

Table 11. Primary screening of glycerol and mannitol production by type strains when culturing in the medium containing 5% glucose for estimation concentration usingTLC analysis**.**

No.	Type strains	Culture medium		Cell lysate	
		Glycerol	Mannitol	Glycerol	Mannitol
1	*Saccharomyces cerevisiae burgundi*	+++	-	-	-
2	*Saccharomyces cerevisiae champaign*	+++	-	-	-
3	*Saccharomyces cerevisiae*	++	-	-	-
4	*Saccharomycopsis fibuligera* TISTR 5033	+	-	-	-
5	*Rhodotorula rubra* TISTR 5067	-	-	-	+
6	*Hansenula anomala* TISTR 5082	+++	-	-	-
7	*Hansenula anomala* TISTR 5113	ND	ND	ND	ND
8	*Kluyveromyces maxianus* TISTR 5270	+++	-	-	-
9	*Geotricum candidum*	ND	ND	ND	ND
10	*Saccharomyces baryanus*	+++	-	-	-
11	*Candida utilis*	++	-	-	-
12	*Candida famata*	++	-	-	-
13	*Candida krusei*	++	-	-	-
14	*Candida tropicalis*	++	-	-	-
15	*Endomycopsis fibuligera*	ND	ND	ND	ND

ND= not detected, NG= not growth, Gly[a] = Glycerol, Man[b]= Mannitol.

++++ = Dark brown, +++ = Medium brown, ++ = pale brown, + = very slightly, and - = no spot.

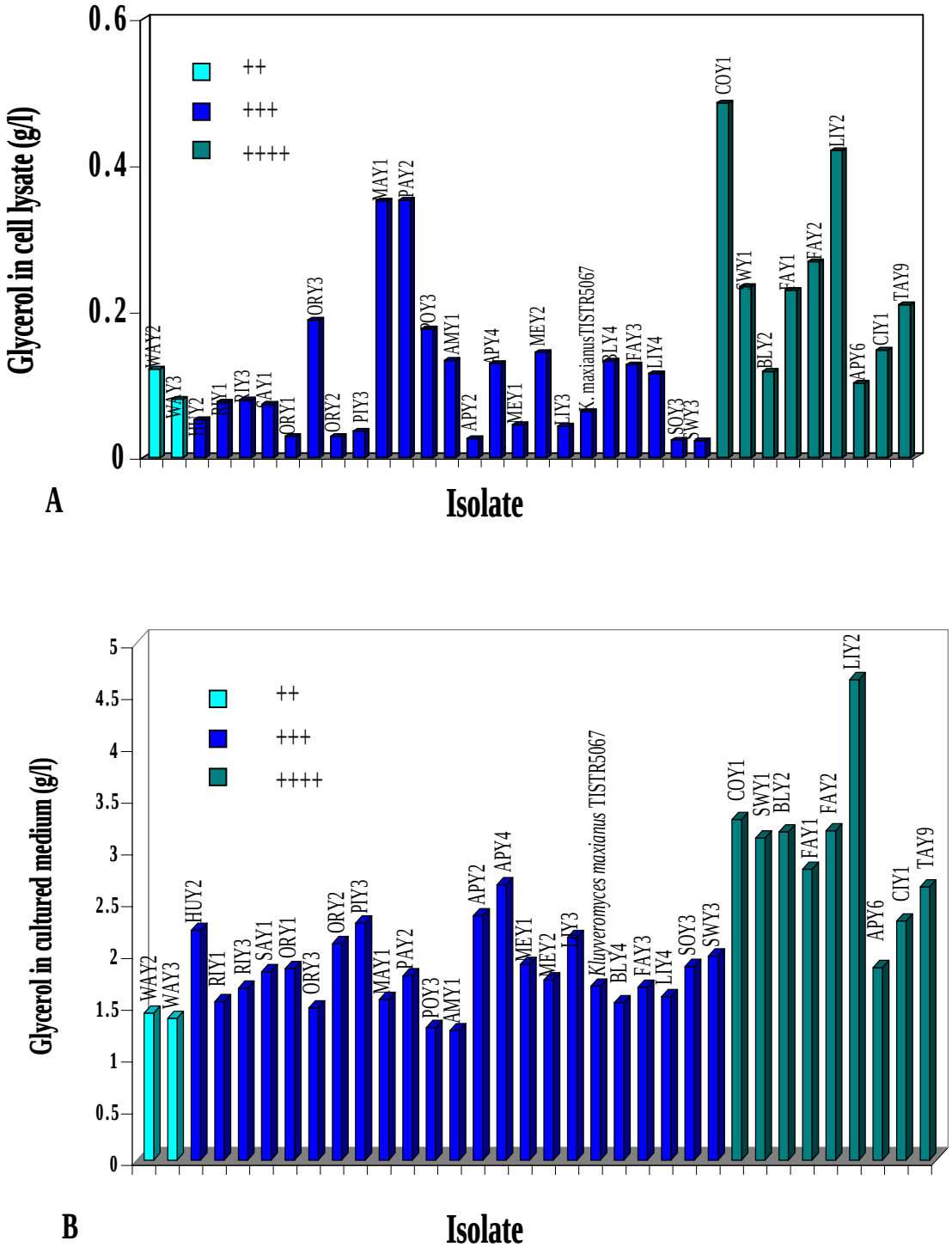


Figure 17. Glycerol concentrations in cell lysates (A) and in cultured media (B) of yeast when cultured in YEPD containing 5% glucose for 4 days, and detected by Glycerol combination test kit.

Table 12. Screening of glycerol and mannitol from 4 days YEPD medium containing 5% glucose assayed by High-Performance Liquid Chromatography (HPLC).

No.	Isolate	Glycerol (g/l)				Mannitol (g/l)				Wet weight (g)
		Score from TLC	Cul[a]	Score from TLC	Cell[b]	Score from TLC	Cul[a]	Score from TLC	Cell[b]	
	Standard	**++++**		**++++**		**++++**		**++++**		
1	**KAY1**	++	4.8	-	0.598	+	0.86	+++	0.825	ND
2	**PIY2**	+++	4.6	+++	0.265	++	1.13	++	0.336	0.0086
3	**PUY4**	+++	9.3	+	0.128	-	0.34	++	0.294	0.0116
4	**WAY6**	+	-	-		++	0.12	++	0.208	0.1650
5	**AFY4**	++	-	-	-	-	0.11	++	0.145	0.1915
6	**AFY5**	++	-	-	-	-	0.08	++	0.070	0.1602
7	**LLY3**	++	-	-	-	-	0.24	++	0.190	0.1711
8	**TAY1**	+	ND	-	ND	-	ND	+	ND	0.1062
9	**TAY7**	+++	ND	-	ND	-	ND	+	ND	0.1701
10	**SLY1**	+++	ND	-	ND	+	ND	+	ND	0.2120
11	**SLY2**	+++	ND	-	ND	-	ND	+	ND	0.2244
12	**SMY3**	+	ND	-	ND	-	ND	+	ND	0.0768
13	**Y60**	+	ND	-	ND	-	ND	+	ND	0.1417
14	***Rhodotorula rubra* TISTR 5067**	-	-	-	-	-	0.0814	+	0.159	0.2601

ND = not detected

Cul[a] = Cultured medium

Cell[b] = Cell lysate.

average were 4-10 g of glycerol per liter, and 0.3-0.9 g of mannitol per liter. In cell lysate showed the average were 0.1-0.6 g of glycerol per liter, and 0.2-0.9 g of mannitol per liter. In cell lysate, 6 isolates (TAY1, TAY7, SLY1, SLY2, SMY3 and Y60) gave negative results. Sugar alcohols played a role in osmoregulation (Brown, 1978). Type of sugar alcohols (glycerol, eryrhritol, arabitol, and mannitol) were found in yeast metabolism (Shallenberger, 1993; Christain, 1994; Voet, 1995). The yeast that could grow in high substrate concwentration, called osmophillic yeast. Osmophillic yeasts accumulate various intracellular sugar alcohols during growth in media containing high concentrations of substrate in order to counterbalance high extracellular osmotic pressure and sugar alcohol accumulation slightly reduces water activity without affecting enzyme activity (Grolean *et al*., 1995; Rapin *et al*., 1994). From a number of investigated experiments, *Sacchaomyces* was selected for their glycerol production (Onishi *et al*., 1968, Onishi *et al*., 1969; Omori *et al*., 1997; Lewis *et al*., 1995; Kajiwara *et al*., 2000), and other yeast strains were *H. anomala* (Parekh *et al*., 1984) and *K. maxianus* (Rapin *et al*., 1994). Mannitol producing yeast strains were *Torulopsis mannitofaciens* CBS 5981 (Onishi *et al*., 1970), *T. versatilis* and *T. anomala* (Onishi *et al*., 1968) and *Zygosacharomyces rouxii* (Groleau *et al*., 1995).

3.3 Screening and selecting of starch-utilizing yeast strains

Out of the 147 yeast strains isolated from various sources (Appendix D1), and 15 type strains, 22 yeast isolates: Y69, SOY6, SOY7, KAY1, PUY2, PUY4, DRY1, OAY1, OAY2, OAY3, APY3, AFY4, AFY5, SPY2, SYY1, SYY2, SYY3, HUY3, HUY4, LLY2, LLY3, and STY4 were able to grow on cassava starch agar medium, and gave clear zones of starch hydrolysis when the iodine test was performed (Table 13). The efficiency of yeasts for starch utilization was compared by diameter sizes of clear zones. Four isolates provided larger clear zone diameter which markedly different from others (Y69, SOY6, SOY7 and KAY1) and 2 type strains (*Saccharomycopsis fibuligera* TISTR 5033 and *Endomycopsis fibuligera*). *S. fibuligera* and *E. fibuligera* have been reported for their capabilities of starch utilization (Jarl, 1969). Isolate KAY1 could convert glucose to glycerol and mannitol (Section 3.2). Commonly, starch-utilizing yeasts were found in raw-starch materials and other starch manufactures. They have amylase enzymes to hydrolyse starch in to simple monosaccharide when are used as carbon source (Laluce, 1988; Jarl, 1969). From this study isolates SOY6, SOY7, and KAY1

Table 13. Clear zone diameter from iodine test on starch agar medium in preliminary screening test for starch hydrolyzation ability of yeasts.

No.	Source	Isolate number	Clear zone diameter of colony (cm)	Clear zone from edge of colony (cm)
1	**Type strain**	*Saccharomycopsis fibuligera* TISTR 5033	2.5-3	0.9-1
2	**Type strain**	*Endomycopsis fibuligera*	2.7	ND
3	**Cassava solid waste**	Y69	2.3-3	1-1.2
4	**Fruit1: Makampom (*Phyllanthus emblica* Linn.)**	SOY 6	2.4-2.5	0.5-0.7
5		SOY 7	2.4-2.5	0.5-0.7
6	**Fruit2: Rozelle**	KAY 1	2.3-2.5	0.5-0.7
7	**Fruit3: Phut-sa (*Zizyphus jujuba* Lamk.)**	PUY 2	0.7	0.1
8		PUY 4	1.2	0.2-0.3
9	**Fruit4: Dragon fruit**	DRY 1	1.1-1.2	0.1-0.2
10	**Oat meal**	OAY 1	1.4-1.5	0.1
11		OAY 2	0.5-0.6	<0.1
12		OAY 3	0.6	0.1-0.2
13	**Fruit4: Apple**	APY 3	1.2	0.2
14	**Fermented fruits**	AFY 4	1.2-1.5	0.1
15		AFY 5	1.4-1.5	0.1
16	**Tomatoes**	SPY 2	0.9	0.1-0.15
17	**Syrup**	SYY 2	0.8-0.9	<0.1
18		SYY 3	0.9-1.2	0.1-0.15
19		SYY 1	0.7-0.8	0.1
20	**Look-pang**	HUY 3	0.7-0.8	<0.1
21		HUY 4	0.5-0.6	<0.1
22	**Chilli**	LLY 2	1.3	<0.1
23		LLY 3	1.6	<0.1
24	**Fruit5: Strawberry**	STR 4	0.9	ND

ND= not detected.

were isolated from fresh fruits, Makhampom (*Phyllanthus emblica* Linn.) and Rozelle, and produce extracellular enzyme for starch breakdown. Some yeasts may be better adapted to condition met in association with tissue of certain plants, usually acting as hosts, and use a sugar or organic acid from plant exudated as carbon source (Kurtzman, 1998). A number of yeasts or yeast like organisms have been described that will degrade hemicellulosic materials to free sugars. A few yeasts also produce and/or metabolize cellooligosaccharides. In general, yeast capable of producing cellulase or xylanase are obligately aerobic (Verachtert *et al.*, 1990).

3.4 Starch hydrolysis by selected yeast strains

For obtaining the potential yeasts for glycerol and mannitol production from available starchy materials, selected yeast isolates were cultured in medium containing cassava starch, rice starch, and Khao-klong starch for 3 days. Eight isolates were Y69, SOY6, SOY7, KAY1, PUY4, AFY4, AFY5, and LLY3, and 2 type strains, *S. fibuligera* TISTR 5033 and *E. fibuligera* were selected from the previous study (Section 3.3). For 4 isolates (Y69, SOY6, KAY1, and SOY7) were able to utilize the three substrates, and gave the high concentration reducing sugars when compared with the 2 effective starch-utilizing type strains (Figure 18). But the other 4 isolates (PUY4, AFY4, AFY5, and LLY3) gave lower amount of reducing sugars than the first four yeast isolates. The yeast isolate Y69 produced the higher amount of reducing sugars than the 2 type strains when grown in the medium containing either cassava starch or rice starch. The yeast isolates KAY1 could produced glycerol and mannitol from glucose, and produced high amounts of reducing sugars in Khao-klong starch and cassava starch, and rice starch media. Yeast isolates PUY4, AFY4, AFY5, and LLY3 were unable to utilize starch.

When cultured selected the starch-utilizing yeast isolates in various concentrations of starch, the highest amount of reducing sugars from yeast isolate KAY1 was increased at the second day of cultivation (Figure 19, and 20). Reducing sugar concentration were related to starch concentrations. At the sixth day and the seventh day of cultivation of yeast isolate SOY6 in Khao-klong starch medium, the maximum concentration of reducing sugars was obtained at all concentrations of Khao-klong starch. When cassava starch was used as substrate, yeast isolate Y69 and the type strain, *E. fibuligera*, could produce the maximum concentration of reducing sugar at the second day and the

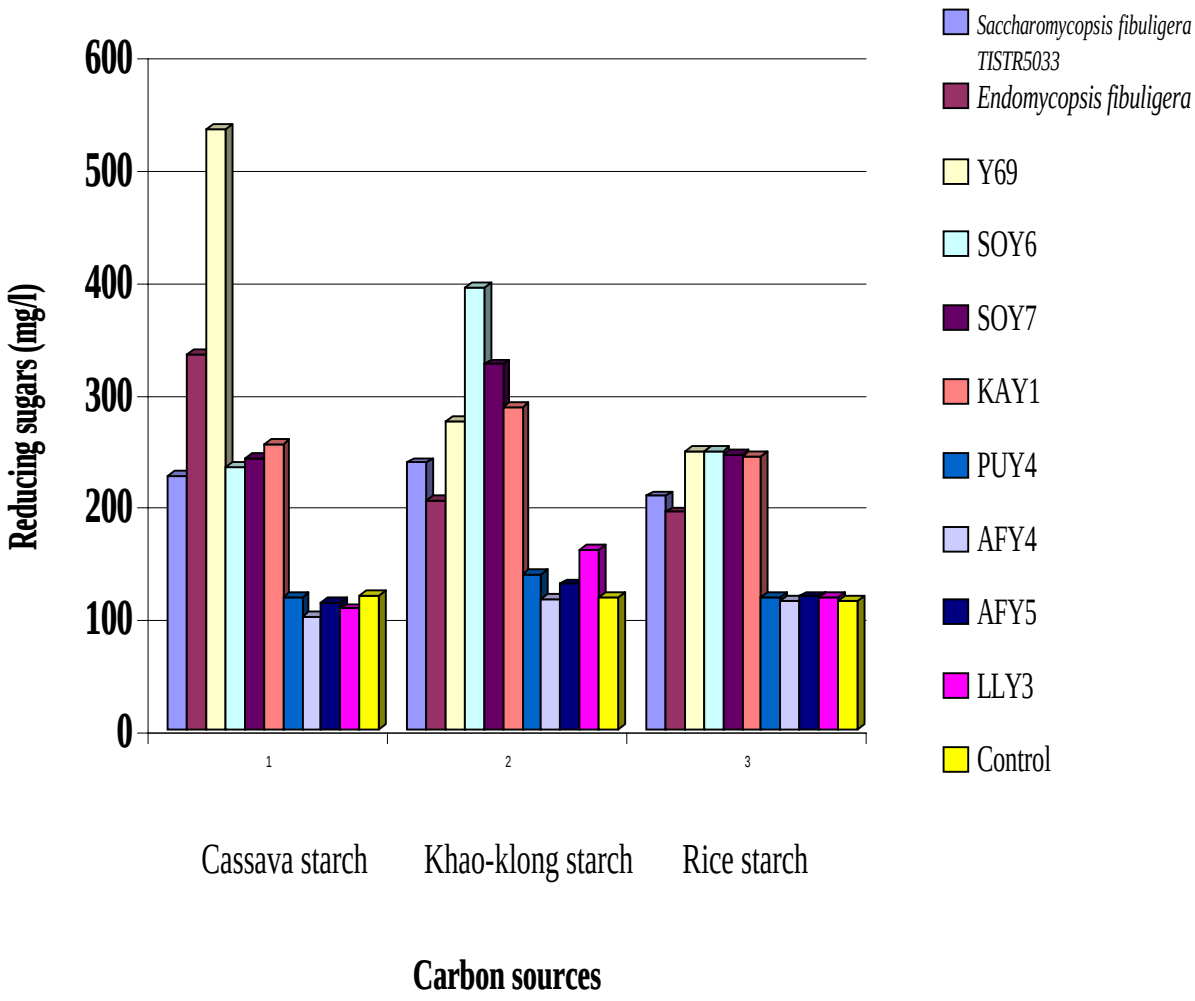


Figure 18. Hydrolysis of starch by 11 yeast strains when cultured in the medium containing various carbon sources (1% (w/v)) for 3 days at 30°C.

third day of incubation (Figure 21 and 22). The highest of reducing sugars was obtained at the 5% of cassava starch. For the rice starch, yeast isolate Y69 showed the maximum amount of reducing sugars at the second day and the third day of cultivation (Figure 23 and 24). The yeast isolate SOY6 gave the maximum amount of reducing sugars at the sixth day and the seventh day of growth in both cassava starch and Khao-klong starch. Yeast isolates Y69 and KAY1 were able to utilize the starch faster than isolate SOY6, but gave lower concentration of reducing sugars than isolate SOY6. Isolate Y69 and KAY1 were suitable when considered as the short time of fermentation, therefore, selected for starch hydrolysis.

The cultivation products were drawn aseptically from the cultured medium at the third day to determined the production of glycerol and mannitol produced by 10 yeasts isolates. Only yeast isolate KAY1 was found to produce mannitol accumulated in cells. No sugar alcohol was detected in cultured media.

3.5 Comparison of sugar alcohol production capability of selected starch-utilizing strains

Mixed cultures as well as the culture of selected yeasts (section 3.2 and 3.4) were tested for their ability to convert starch into glycerol and mannitol. Mixtures of the yeast culture: Y69 and LIY2, Y69 and COY1, Y69 and FAY2, Y69 and PIY2, Y69 and PUY4, Y69 and KAY1 were carried out using the similar process of Symba yeast process (Jarl, 1969). Yeast isolate Y69 was chosen for its ability to hydrolyse starch to produce reducing sugars. Then the second yeast isolate (either LIY2, COY1, FAY2, PIY2, PUY4, or KAY1) was applied to produce glycerol and mannitol. Cassava starch was chosen for the production of reducing sugars. Yeast isolates LIY2, COY1, and FAY2 were glycerol-producing and non-starch-utilizing yeasts (results from section 3.2). In the single culture, isolate KAY1 was selected for its starch-utilizing and mannitol-producing capability.

Sugar alcohol were analyzed at the second day of cultivation. Mixtures of yeast cultures Y69 and PIY2, Y69 and PUY4, Y69 and KAY1 could convert the cassava into mannitol accumulated only in yeast cells at the approximate concentration of 0.2-0.6 g/l. No sugar alcohol was detected in cultured media of mixed cultures of isolates Y69 and isolates LIY2, or COY1, or FAY2. Also, no sugar alcohol was found in both cultured media and cell lysates. For the single culture, yeast isolate KAY1 produced mannitol at the maximum concentration of 1.38 g/l.

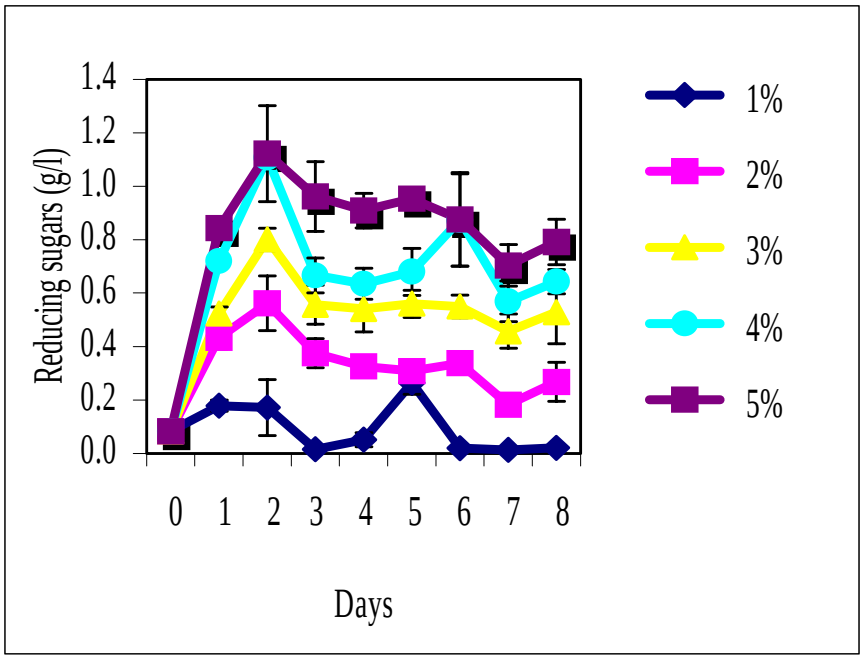


Figure 19. Reducing sugar production by yeast isolate KAY1 when cultured in the medium containing Khao-klong starch (1%, 2%, 3%, 4%, and 5% (w/v)) for 8 days at 30°C.

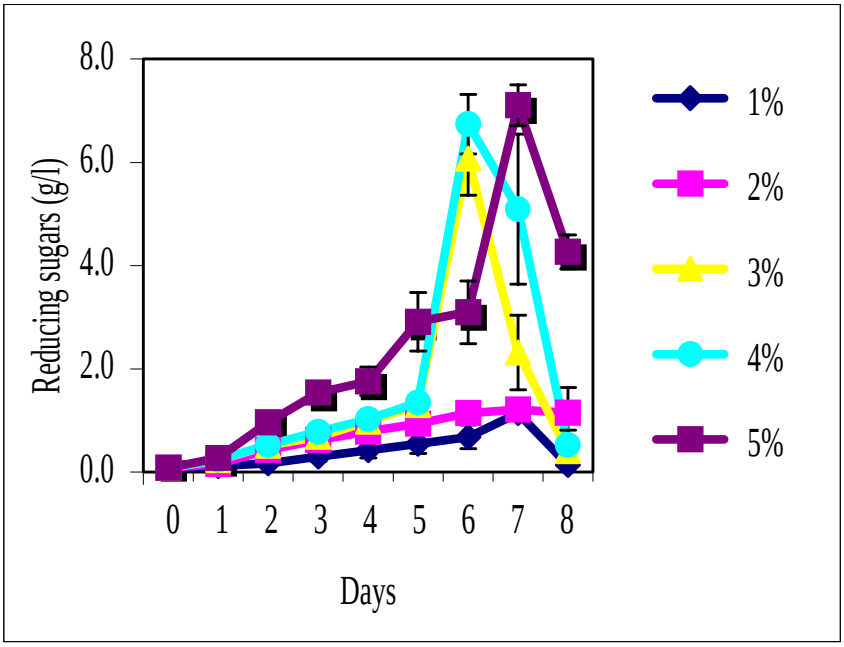


Figure 20. Reducing sugar production by yeast isolate SOY6 when cultured in the medium containing Khao-klong starch (1%, 2%, 3%, 4%, and 5% (w/v)) for 8 days at 30°C.

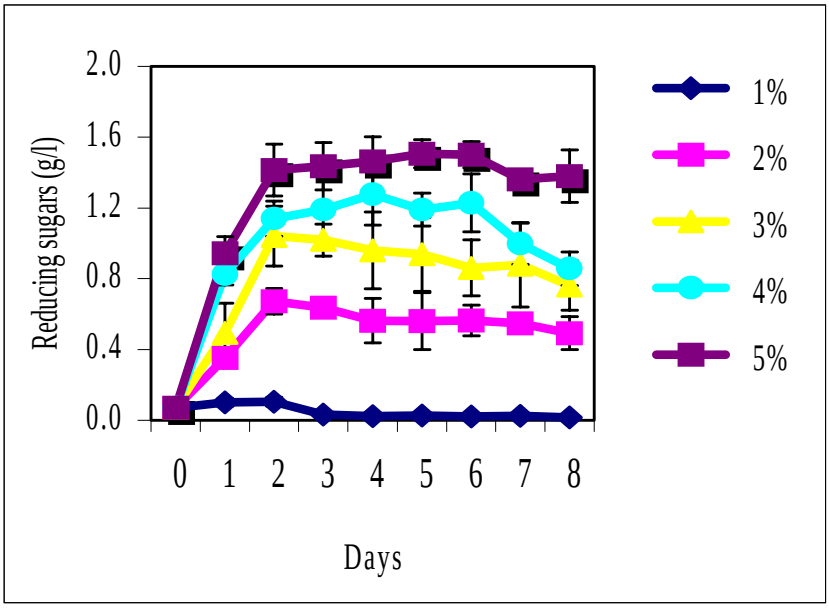


Figure 21. Reducing sugar production by yeast isolate Y69 when cultured in the medium containing cassava starch (1%, 2%, 3%, 4%, and 5% (w/v)) for 8 days at 30°C.

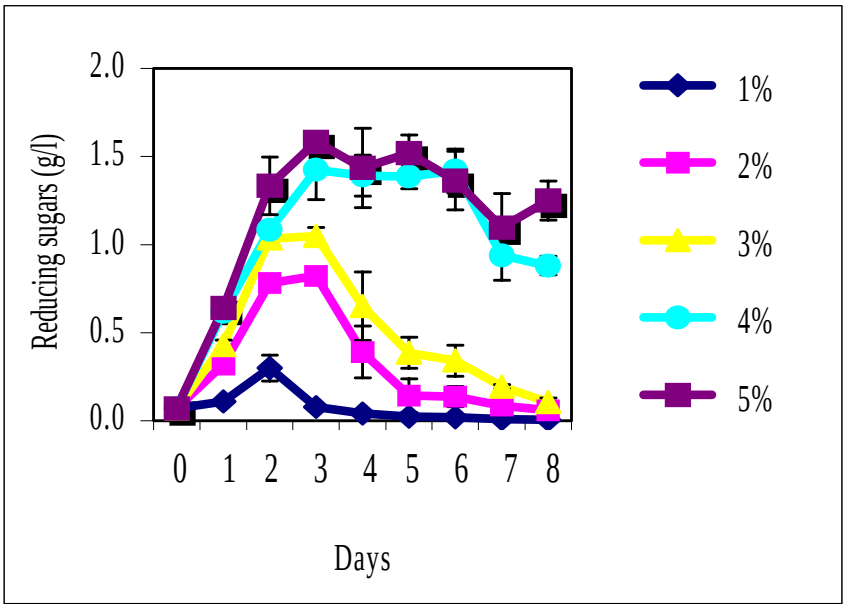


Figure 22. Reducing sugar production by yeast strain *Endomycopsis fibuligera* when cultured in the medium containing cassava starch (1%, 2%, 3%, 4%, and 5% (w/v)) for 8 days at 30°C.

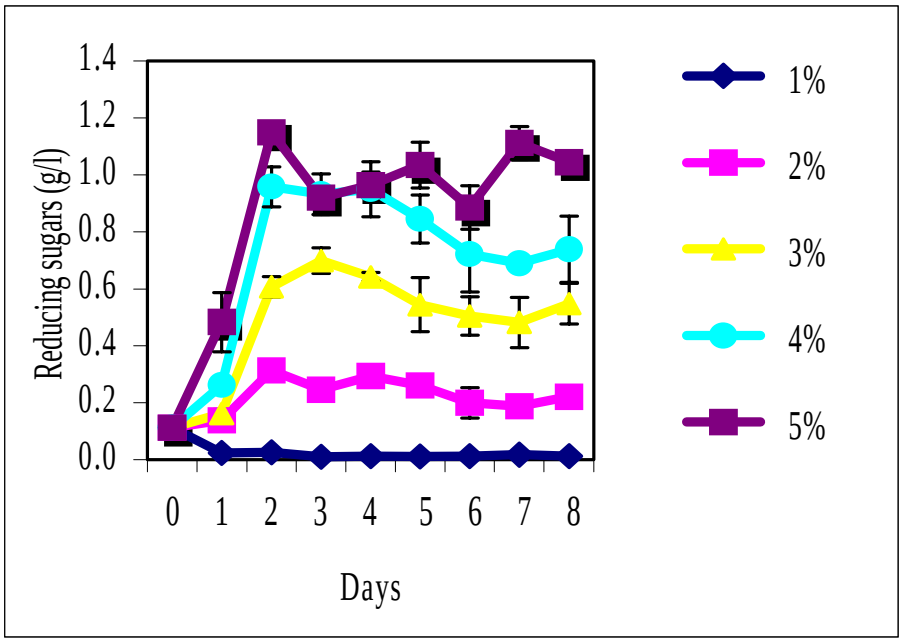


Figure 23. Reducing sugar production by yeast isolate Y69 when cultured in the medium containing rice starch (1%, 2%, 3%, 4%, and 5% (w/v)) for 8 days at 30°C.

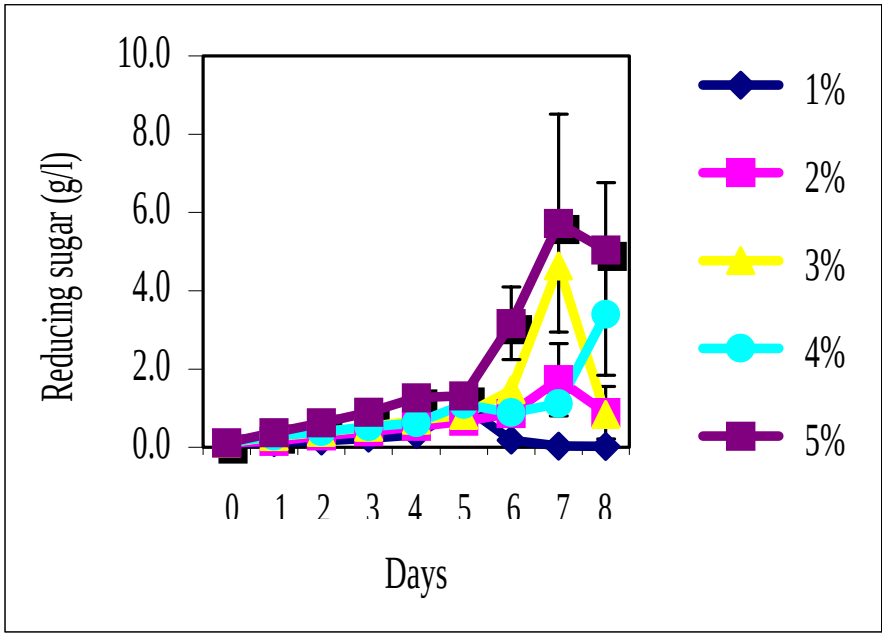


Figure 24. Reducing sugar production by yeast isolate SOY6 when cultured in the medium containing rice starch (1%, 2%, 3%, 4%, and 5% (w/v)) for 8 days at 30°C.

These results indicated that the cooperation of starch-utilizing strain and glycerol-producing isolate could not support the glycerol production. It might cause from the competition occuring when both population. The competition also determines the different species of yeast that are often found in mixed cultures such as in the lambic main fermentation (Granse, 1934).

Under competition conditions, organisms better adapted for new situations may be selected. From the result of section 3.2, 5% of glucose in YEPD medium were used to screen sugar alcohol production capability of yeasts. Yeast isolates LIY2, COY1, and FAY2 produced the high amount of glycerol in both cultured media and cell lysates. In term of"polyols or sugar alcohols", accumulated intracellular polyols have at least three major physiological functions: namely they act as: 1) osmoregulators, 2) food reserves, and 3) protectors of enzyme activity at low levels of a_w substances (Brown, 1978). Yeasts accumulated glycerol as the primary osmoregulatory solute (Adler *et al*., 1985). From results of this study the reducing sugar concentration in cultured medium was approximate 0.4-0.5% (w/v). It might not be sufficient for cells to accumulate sugar alcohols, but enough for growth. With regard to polyol catabolism, over 50% of yeasts surveyed could aerobically use glycerol, D-glucitol (sorbitol) and D-mannitol while fewer than 10% of species utilized galactitol (Walker, 1998). The glycerol produced and released into the cultured medium by glycerol-producing yeast isolates LIY2, COY1, and FAY1 could be used as the carbon source for both yeast isolate Y69 and the glycerol-producing strains. Thornton and Eustace (1986) suggested that glycerol formation is the result of competition between two enzymes, glycerol-3-phosphate dehydrogenase and alcohol dehydrogenase. An increase in the specific activity of the enzyme, glycerol-3-phosphate dehydrogenase, would increase the yield of glycerol. So the yeast strain might use the route of ethanol production via changes of reducing sugars to pyruvate, then pyruvate to acetaldehyde, and finally to ethanol.

3.6 Optimization of some glycerol and mannitol production conditions

To obtain the efficient production of glycerol and mannitol from starch, some intrinsic factor of fermentation, and inoculum sizes were investigated.

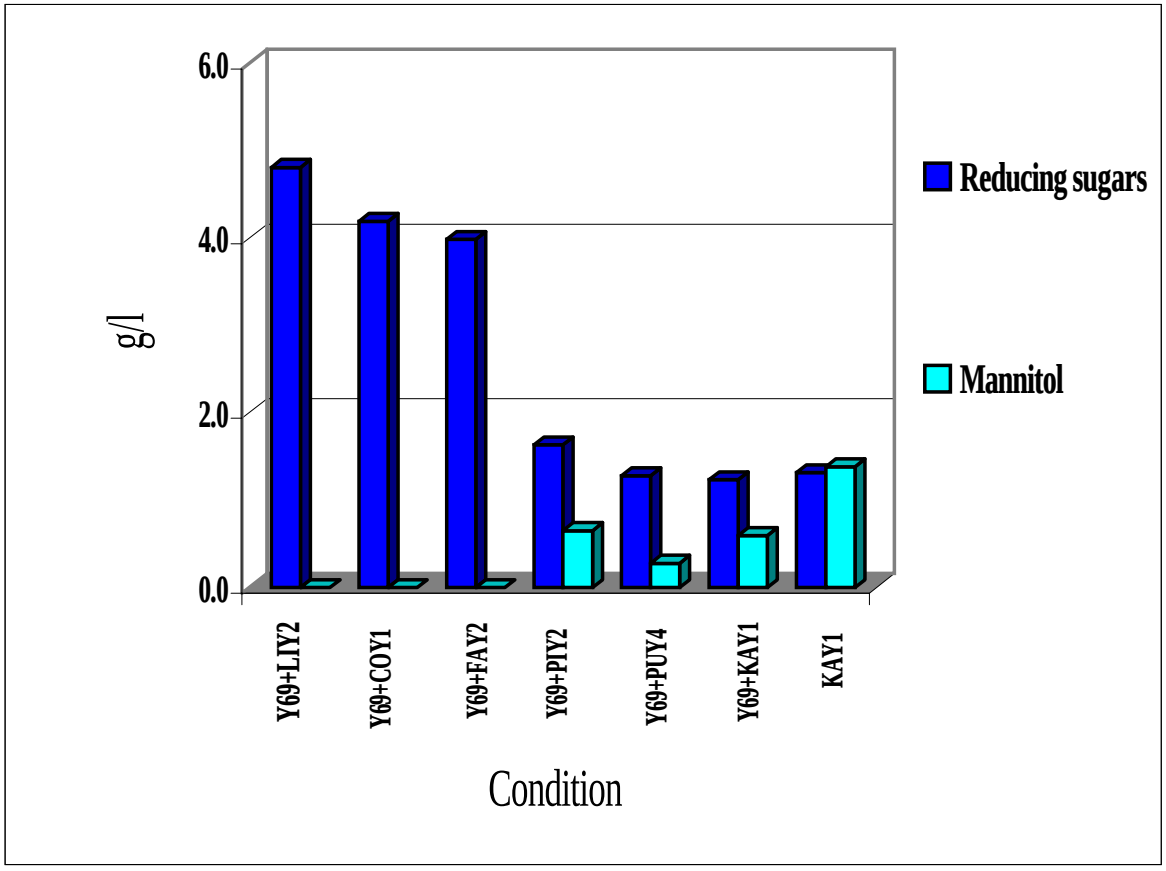


Figure 25. Comparison of sugar alcohol production capabilities of selected starch-utilizing strains when cultured in the medium containing 3% (w/v) cassava starch for 2 days at 30°C.

3.6.1 Determination of the suitable and optimum concentration of carbon sources

Yeast isolate KAY1 was selected to test its ability of mannitol production from starch. Three types of starch: Khao-klong starch, rice starch, and cassava starch were used as substrates for the selected starch-utilizing yeast isolate cultivation at various concentrations: 1, 2, 3, 4, and 5% (w/v). Two percent of inoculum size of 24-h were used. The cultivation products were measured at the maximum yield period during fermentation. The amounts of mannitol in cell lysate was increased correlation to the concentration of carbon source (Figure 26, 27, 28). At 2% of Khao-klong starch gave the similar amount of mannitol to 3% concentration (0.9 g/l-1.0 g/l of mannitol in cell lysate). For the rice starch medium, 2% starch concentrations gave 0.925 g/l of mannitol in cell lysate which was 2 times higher than the yield from 1% of starch.

For results of this study, the maximum amount of mannitol could be obtained when using 5% of either Khao-klong starch, or rice starch for production medium. However, for the cassava starch medium, the maximum yield of mannitol (around 1.17 g/l) was obtained at both 2% and 3% concentration of cassava starch. The suitable starch concentration used as sole source of carbon for the yeast isolate KAY1 was chosen at 2% (w/v) Khao-klong starch, rice starch, and cassava starch because the following factors have been considered. 1) The mannitol production cost would be high when using increased concentration of substrate without proportional yield 2) With regard to water requirements, yeast's (like all organisms) need water in high concentrations for growth and metabolism (Phaff *et al*., 1978). The term water potential (expressed in megpascals, Mpa) refers to the potential energy of water and was used to quantitate the availability of water in the presence of dissolved solutes. It, therefore, closely related to the osmotic pressure of yeast growth media (Walker, 1998). Pure water had a water potential of zero, while impure water would have a lower, negative water potential value. The 4% and 5% starch concentration had lower water potential than 2% and 3% starch concentration. Substrates and enzymes were all in aqueous solution or colloidal suspension, and no enzymic activity could occur in the absence of water (Jennings, 1995). Therefore 2% starch concentration would be suitable for the yeast isolate KAY1 growth.

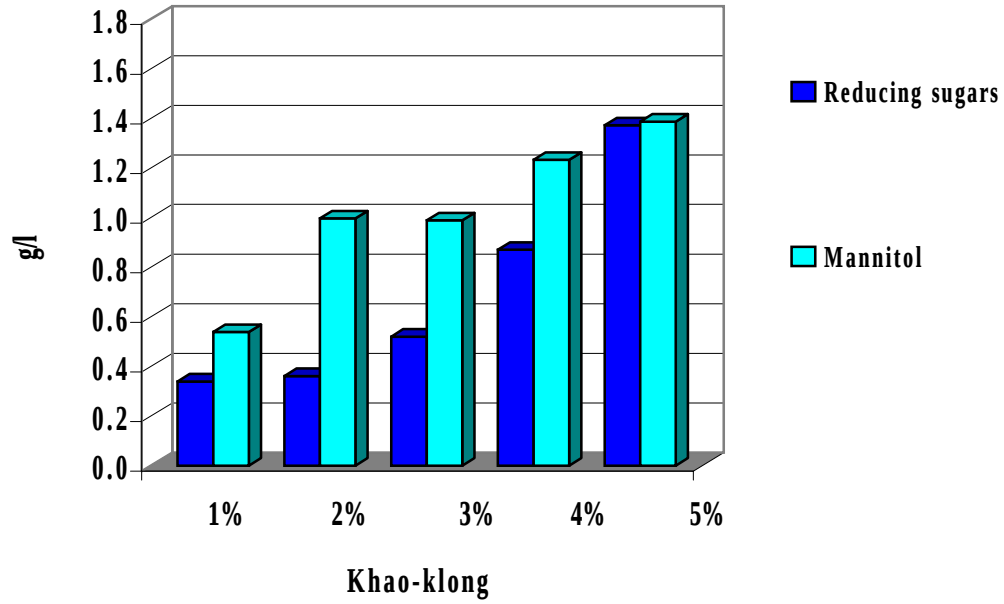


Figure 26. Effects of various concentrations: 1%, 2%, 3%, 4%, and 5% (w/v) of Khao-klong starch on mannitol and reducing sugar production by yeast isolate KAY1 for 2 days at 30°C.

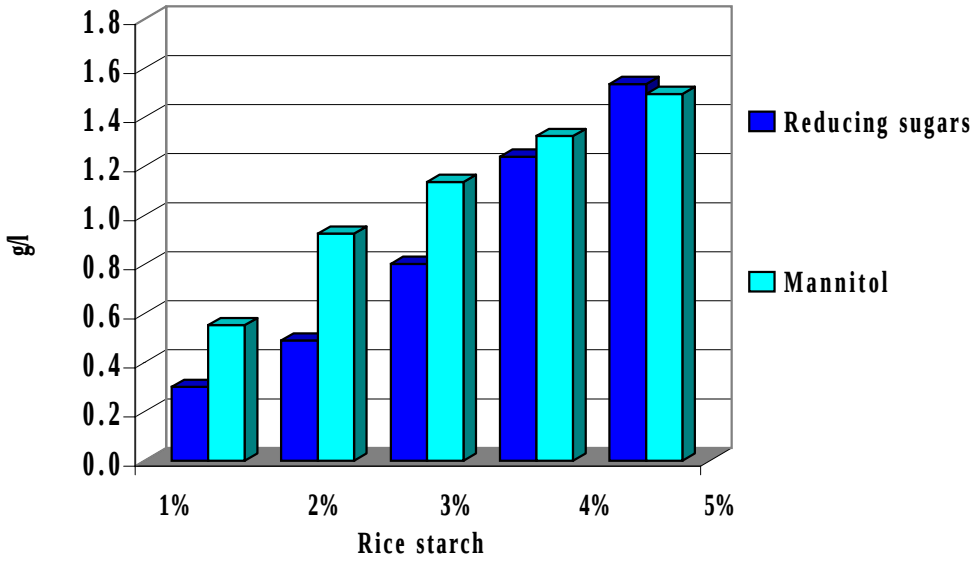


Figure 27. Effects of various concentrations: 1%, 2%, 3%, 4%, and 5% (w/v) of rice starch on mannitol and reducing sugar production by yeast isolate KAY1 for 2 days at 30°C.

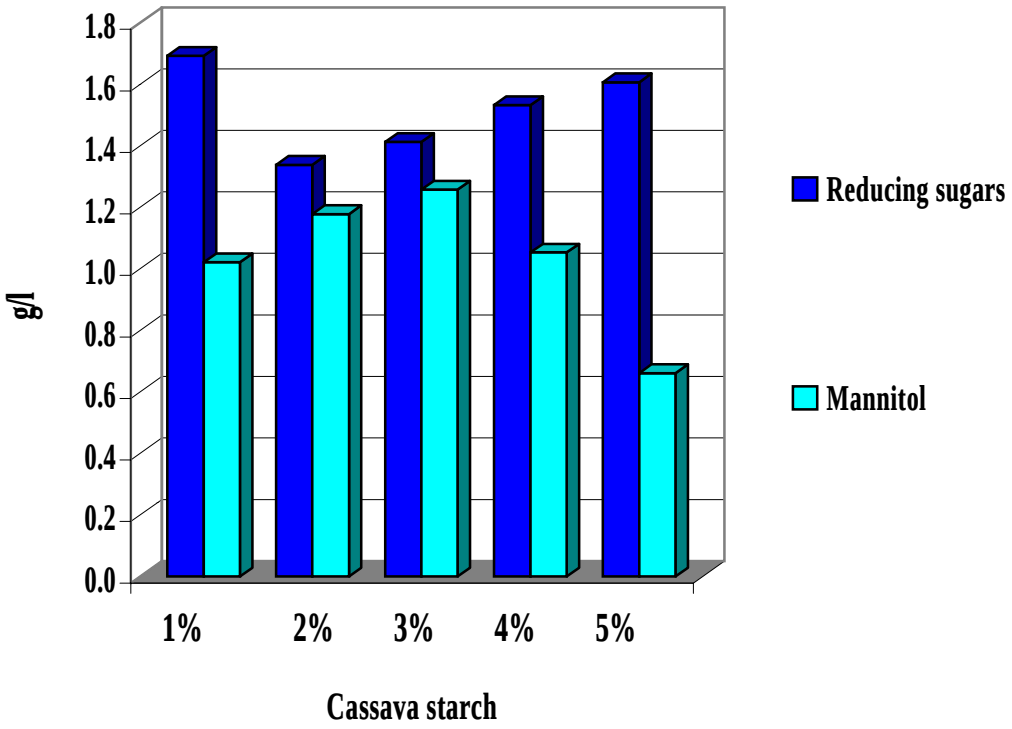


Figure 28. Effects of various concentrations: 1%, 2%, 3%, 4%, and 5% (w/v) of cassava starch on mannitol and reducing sugar production by yeast isolate KAY1 for 2 days at 30°C.

3.6.2 Determination of the suitable and optimum concentration of nitrogen source

The 2% starch concentration of either Khao-klong starch, rice starch, or cassava starch were selected for the direct mannitol production by yeast isolate KAY1. The 2% inoculum size of 24 h culture was used for the production. Two nitrogen sources: ammonium sulfate and urea were tested for their suitable for sugar alcohol production. Changes in the cultivation product were measured each day for 4 days.

The 5 concentrations of nitrogen sources: 0, 0.1, 0.3, 0.5, and 0.7% were added into the 2% starch medium and adjust aseptically in the proper volume. Figure 29 showed the effects of ammonium sulfate on reducing sugars and mannitol in cell lysate. The reducing sugars in Khao-klong starch medium were highest at the first day at 2 g/l of mannitol in cell lysate. After the first day, its gradually decreased at the end during fermentation. For the effects on mannitol in cell lysate (Figure 30), it gave the maximum amounts of mannitol at the second day of cultivation, which containing 0% (no added) ammonium sulfate, but the mannitol yield were decreased when the higher concentrations of ammonium sulfate were added.

As the same results, in rice starch medium was not added any inorganic nitrogen source gave the highest amounts of mannitol in cell lysate at the second day of incubation at 2.1 g/l of mannitol in cell lysate (Figure 31 and 32). The amounts of mannitol were decreased after 2 day of incubation. The reducing sugars gave the maximum at the first day and gradually decreased after the first day of incubation.

The effects of inorganic nitrogen sources on cassava starch medium was the slowly of fermentation and mannitol production was inhibited. (Figure 33 and 34). The reducing sugars was highest at the third day with no added any nitrogen source. Relative to the amounts of mannitol in cell lysate. It has been shown that 0% ammonium sulfate gave the maximum yield at 1.343 g/l of mannitol in cell lysate at the third day of cultivation. It could not detect the mannitol in cell lysate at the first day in the level of 0.1, 0.3, 0.5, and 0.7% (w/v) concentration of ammonium sulfate. The organic nitrogen source urea various concentrations in 5 levels were 0, 0.1, 0.3, 0.5, and 0.7% (w/v). From the results only the 0% urea concentration gave the highest amounts of mannitol in cell lysate.

Onishi and Suzuki (1968) reported that when an inorganic ammonium salt was used as a nitrogen source, the rapid decrease in pH of the medium during fermentation might reduced sugar

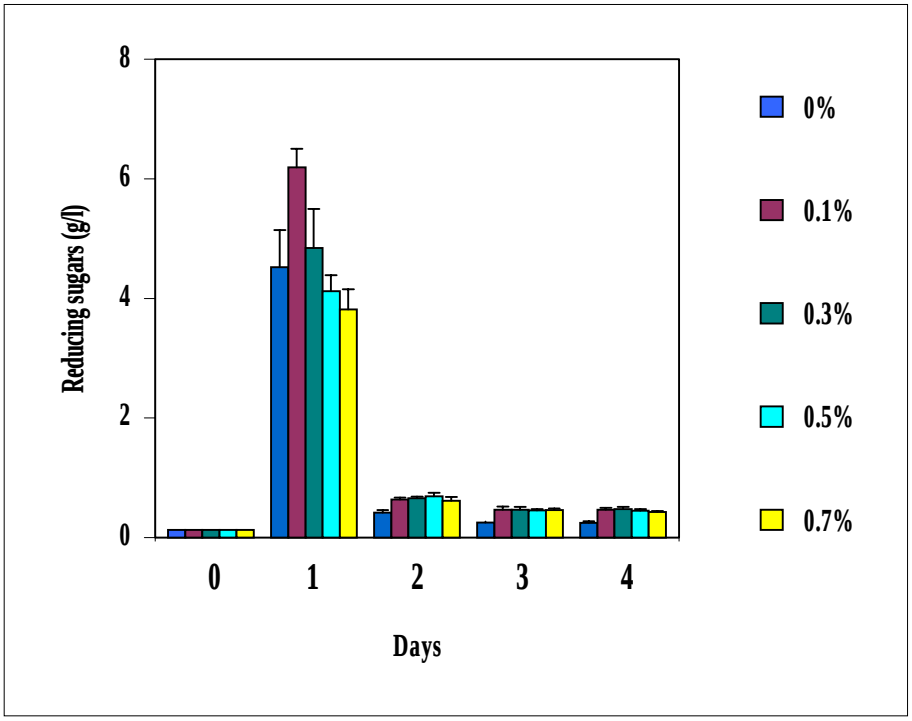


Figure 29. Effects of various ammonium sulfate concentrations on reducing sugar production by yeast isolate KAY1 when cultured in 2% Khao-klong starch for 4 days at 30°C.

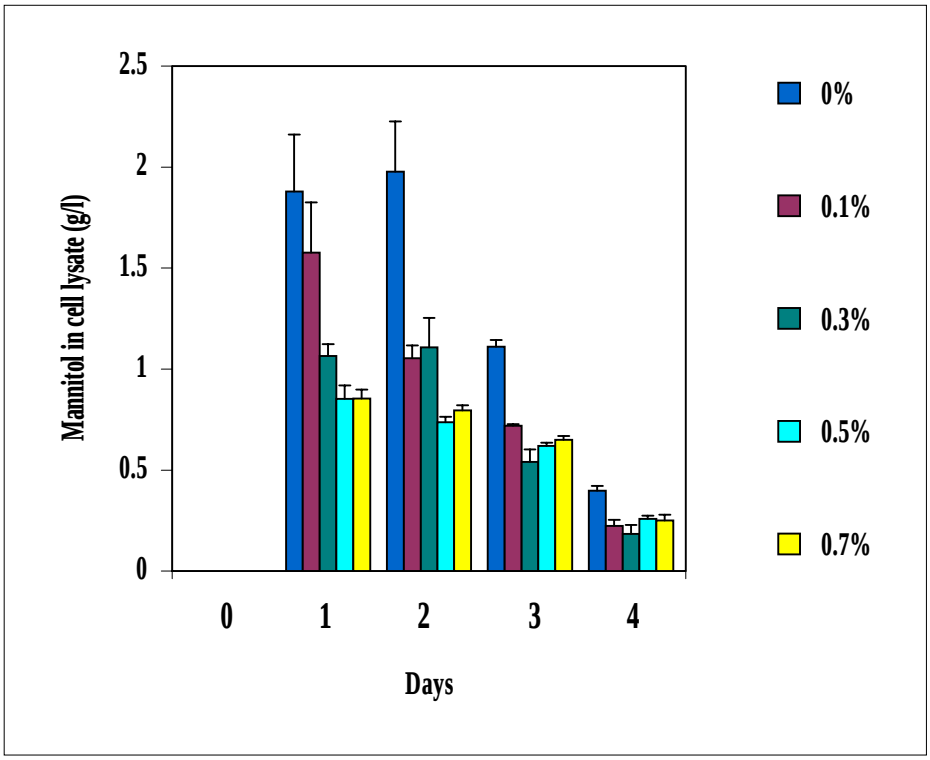


Figure 30. Effects of various ammonium sulfate concentrations on mannitol production in cell lysate of yeast isolate KAY1 when cultured in in 2% Khao-klong starch for 4 days at 30°C.

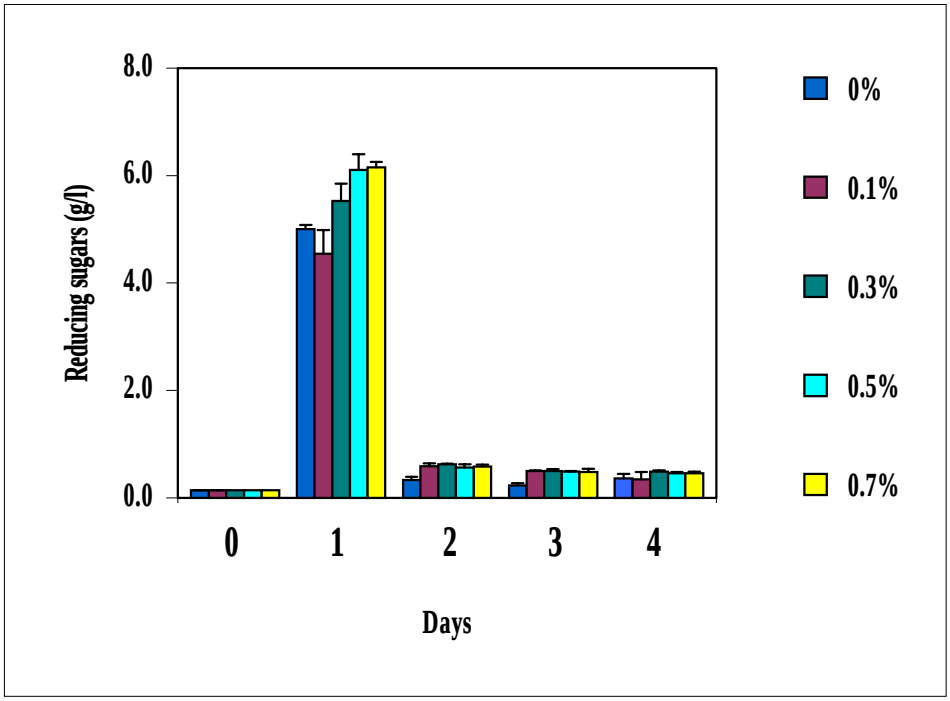


Figure 31. Effects of various ammonium sulfate concentrations on reducing sugar production by yeast isolate KAY1 when cultured in in 2% rice starch for 4 days at 30°C.

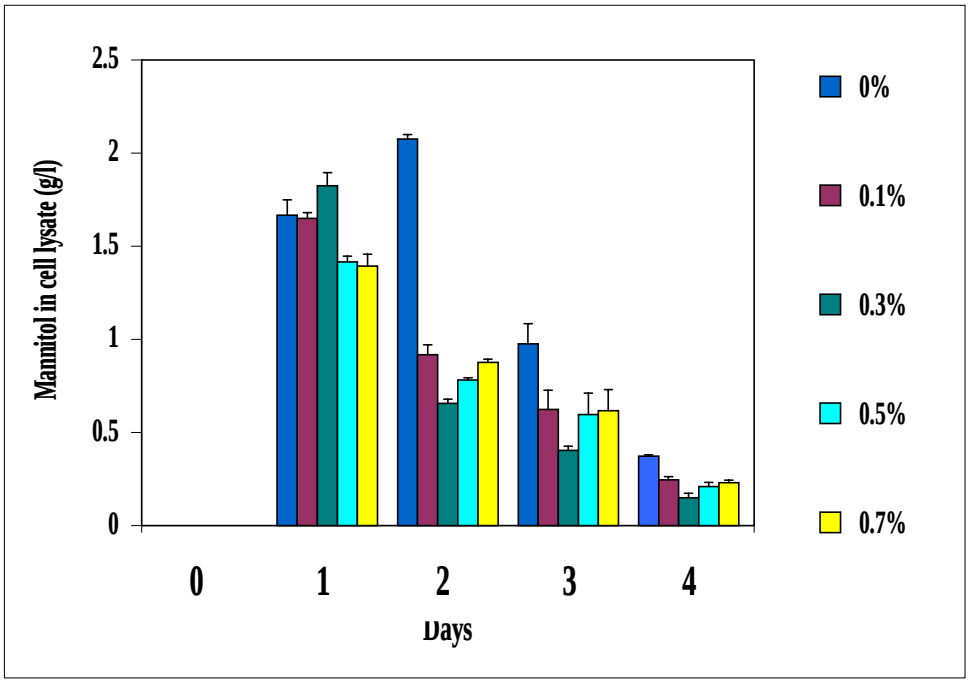


Figure 32. Effects of various ammonium sulfate concentrations on mannitol production in cell lysate of yeast isolate KAY1 when cultured in in 2% rice starch for 4 days at 30°C.

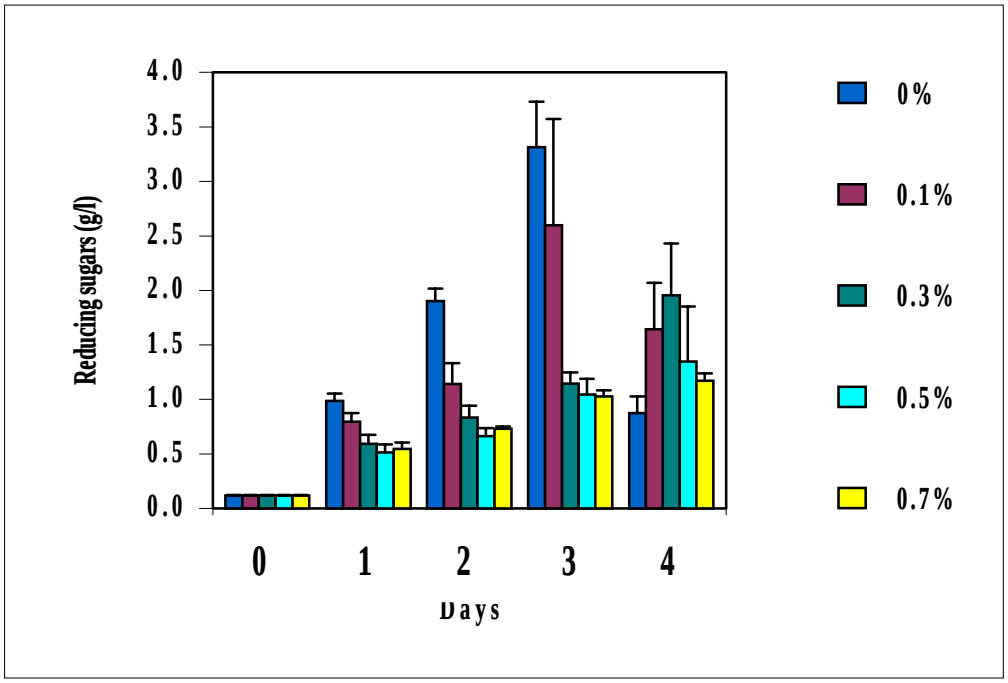


Figure 33. Effects of various ammonium sulfate concentrations on reducing sugar production by yeast isolate KAY1 when cultured in in 2% cassava starch for 4 days at 30°C.

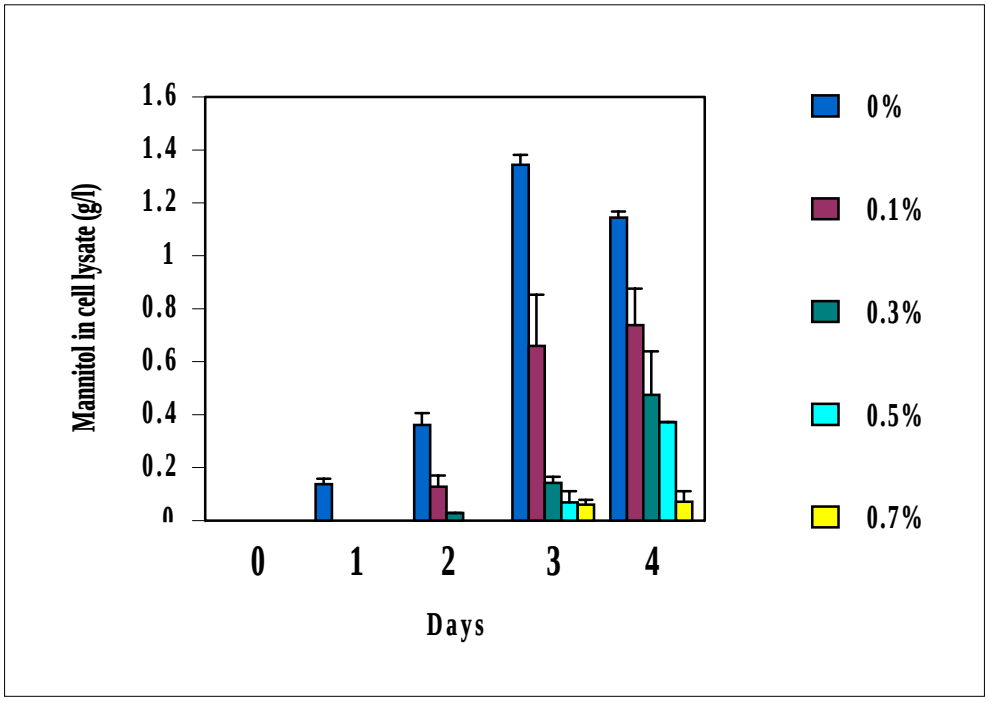


Figure 34. Effects of various ammonium sulfate concentrations on mannitol production in cell lysate of yeast isolate KAY1 when cultured in in 2% cassava starch for 4 days at 30°C.

alcohol yield. Similarly, From this experiment, the results showed that the ammonium sulfate could not supported the growth of yeast isolate KAY1 and reduced the mannitol production in cell lysate. However, in 0% ammonium sulfate concentration in starch medium, the amounts of mannitol in cell lysate increased higher than when ammonium sulfate added and not dropped in the latter day. In starch medium formula containing 0.3% yeast extract. It might enhanced the yeast growing well and supported the sugar alcohol production in yeast. So yeast extract should be selected to optimize in further experiment.

3.6.3 Determination of the suitable and optimum concentration of yeast extract

The yeast isolate KAY1 could utilized the starch as sole carbon source. The starch concentration was 2% which suitability for their growth and sugar alcohol production. When added inorganic substance such as ammonium sulfate or organic nitrogen source: urea, it stopped their growth and the ability to produce mannitol in yeast cell. In the contrast, the starch medium that no added the ammonium sulfate showed the higher yield than the condition added with nitrogen source. So that the yeast extracts were selected for nitrogen source. In this experiment, two conditions were tested for sugar alcohol production. The first, various yeast extract concentrations were 0, 0.1, 0.3, and 0.5% (w/v). The second condition were added 0.1% ammonium sulfate in the starch medium and various concentrations of yeast extract in 4 levels were 0, 0.1, 0.3, and 0.5% (w/v). Changes in the amounts of mannitol in cell lysate and reducing sugars were measured at the maximum period during fermentation.

From the results in Figure 35, when yeast isolate KAY1 cultured in Khao-klong starch medium. It has been shown that 0.3% of yeast extract gave the maximum yield of mannitol in cell lysate at 1.25 g/l. As the same results in Khao-klong starch medium, cassava starch medium containing 0.3% gave the maximum amounts of mannitol higher than other concentrations (Figure 36). In the concentration of 0% and 0.5% yeast extract could not detect any sugar alcohol in cell lysate and cultured medium. From Figure 37, for the first tested in the rice starch medium, 0.5% yeast extract gave the highest amounts of mannitol in cell lysate at 2.0 g/l. In the separate study, when varied the concentrations of yeast extract in the higher levels were 0.4, 0.5, 0.6, and 0.7% (w/v).

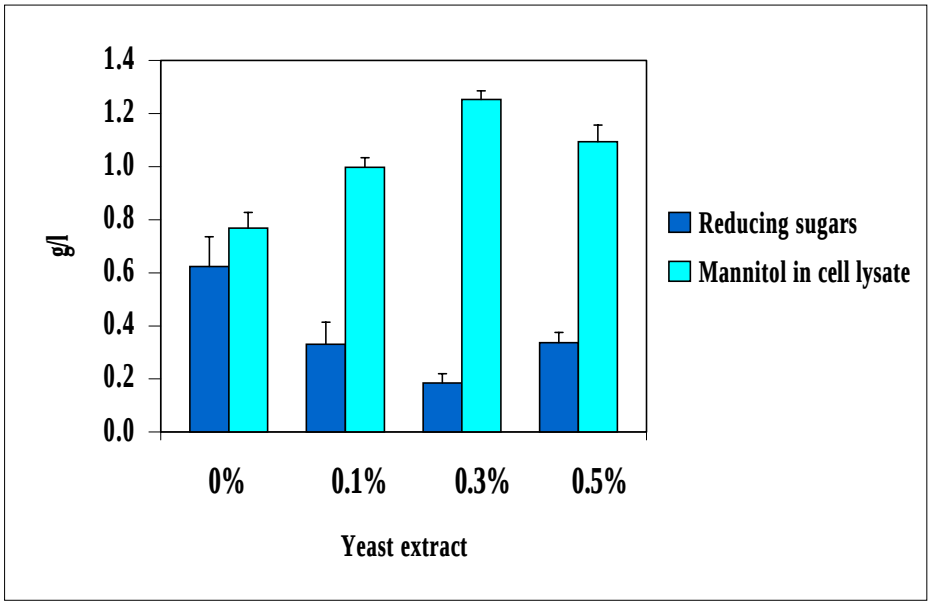


Figure 35. Effects of various yeast extract concentrations: 0%, 0.1%, 0.3%, and 0.5% on mannitol and reducing sugar production by yeast isolate KAY1 when cultured in the medium containing 2% Khao-klong starch for 2 days at 30°C.

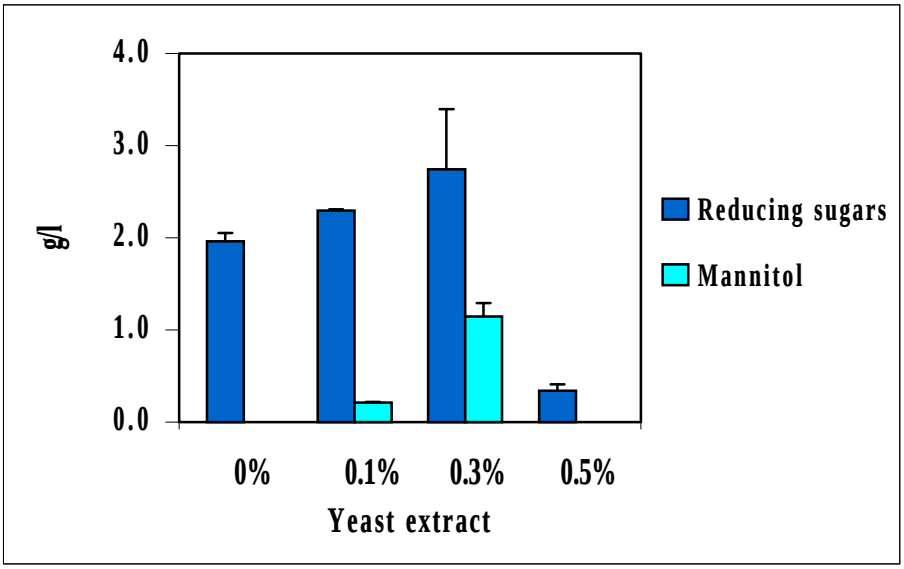


Figure 36. Effects of various yeast extract concentrations: 0%, 0.1%, 0.3%, and 0.5% on mannitol and reducing sugar production by yeast isolate KAY1 when cultured in the medium containing 2% cassava starch for 3 days at 30°C.

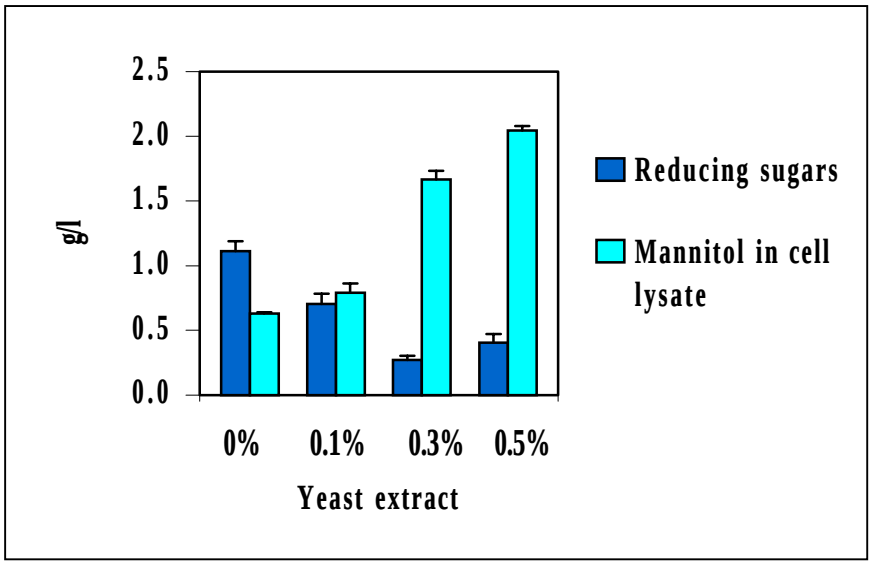


Figure 37. Effects of various yeast extract concentrations: 0%, 0.1%, 0.3%, and 0.5% on mannitol and reducing sugar production by yeast isolate KAY1 when cultured in the medium containing 2% rice starch for 2 days at 30°C.

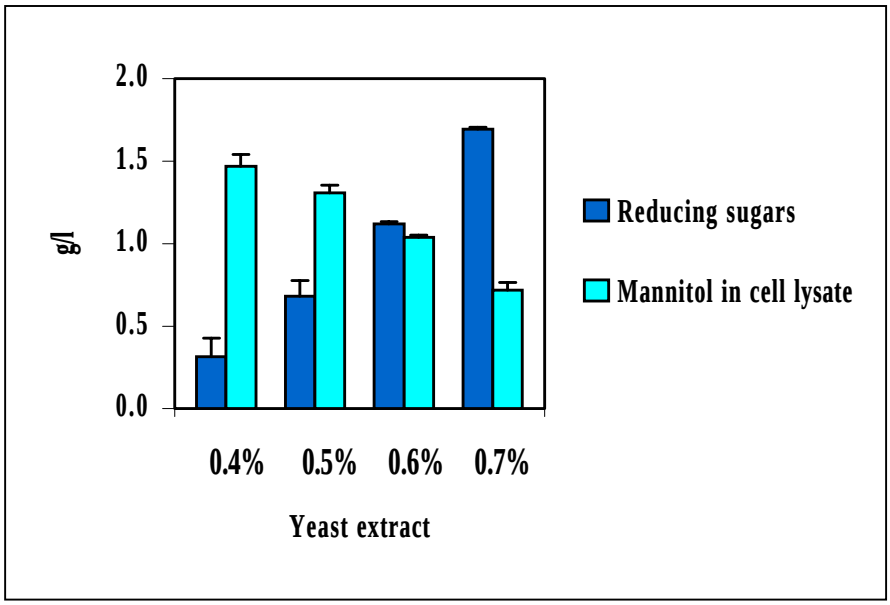


Figure 38. Effects of various yeast extract concentrations: 0.4%, 0.5%, 0.6%, and 0.7% on mannitol and reducing sugar production by yeast isolate KAY1 when cultured in the medium containing 2% rice starch for 2 days at 30°C.

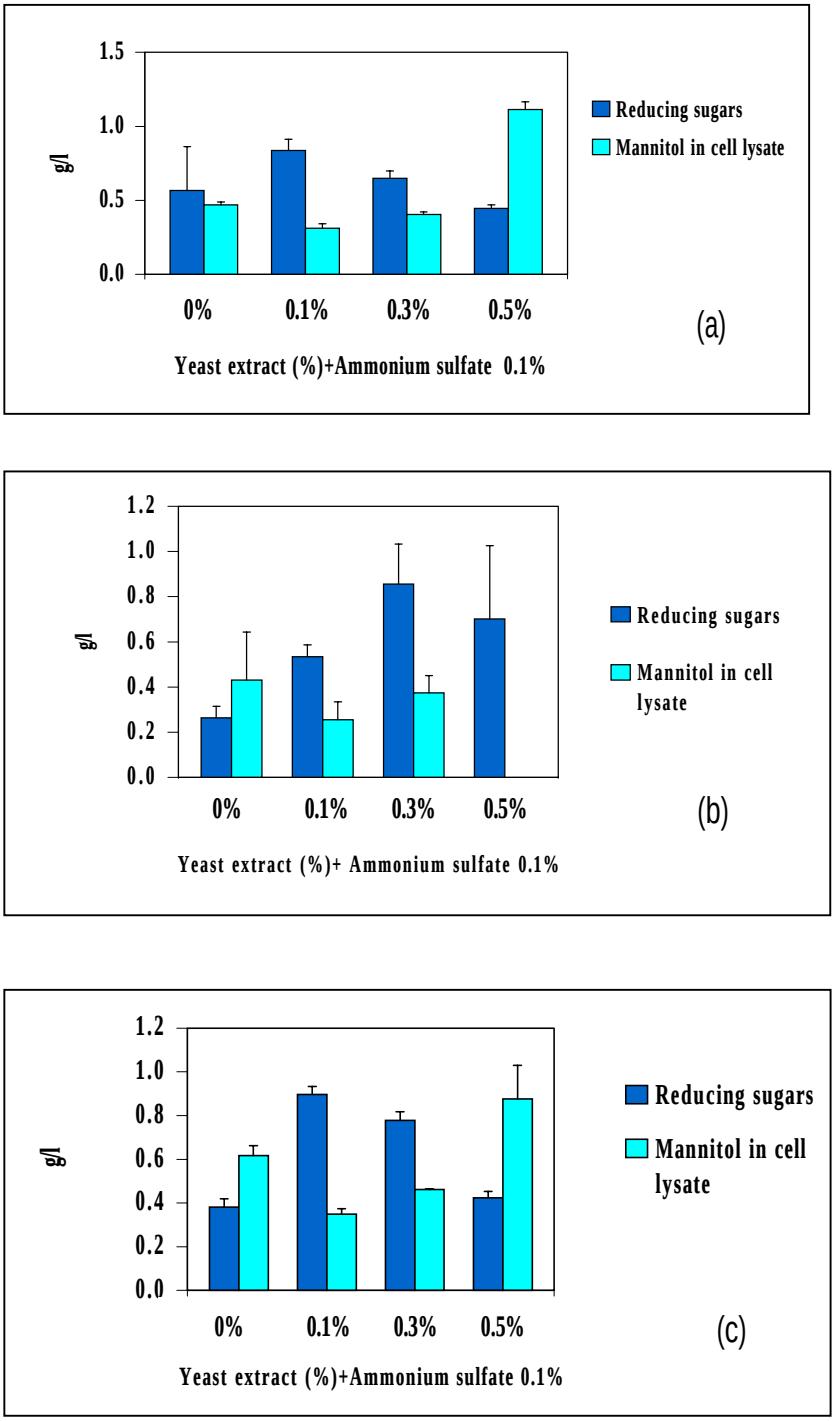


Figure 39. Effects of various yeast extract concentrations: 0%, 0.1%, 0.3%, and 0.5% on mannitol and reducing sugar production by yeast isolate KAY1 when cultured in the medium containing 0.1% ammonium sulfate for 2 days at 30°C. (a) 2% Khao-klong starch, (b) 2% rice starch, and (c) 2% cassava starch

These results could investigated that 0.4% yeast extract gave the amounts of mannitol in the cell lysate higher than 0.5%, 0.6%, and 0.7% yeast extract in rice starch medium (Figure 38).

The second conditions were shown in Figure 39 (a), (b) and (c). The 0.1% ammonium sulfate was added and varied the concentration of yeast extract. The results showed that at the 0.5% yeast extract plus 0.1% ammonium sulfate gave the maximum yield of mannitol higher than 0.1%, and 0.3% yeast extract (plus 0.1% ammonium sulfate) when cultured in the medium containing 2% Khao-klong starch and rice starch medium. Except in cassava starch medium, in the 0.5% yeast extract could not found the mannitol in cell lysate. When compared between two condition. The condition that varied only yeast extract gave the mannitol in cell lysate greater than the condition which added with 0.1% ammonium sulfate.

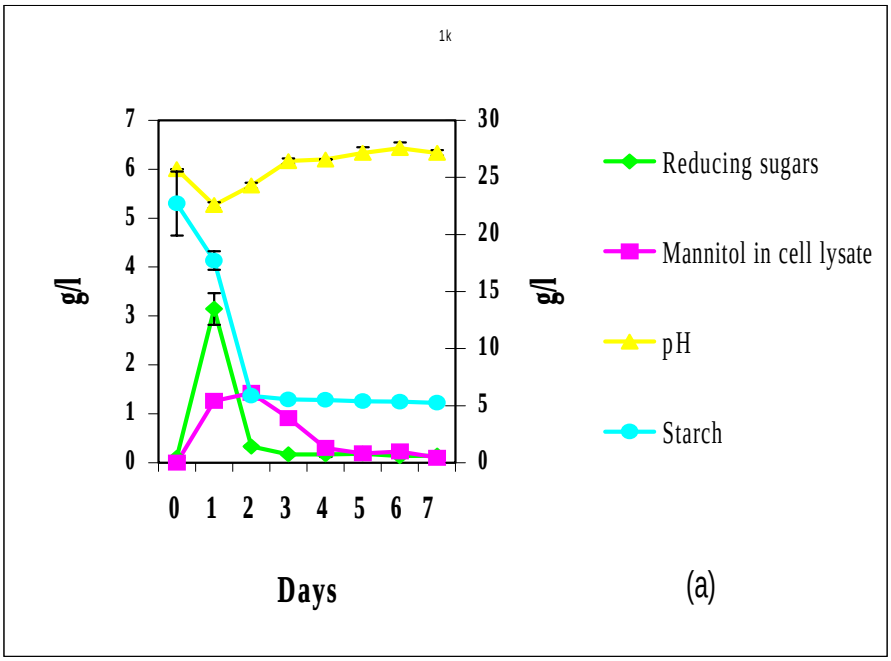
Extracted yeast cell products are rich sources of proteins, nucleic acids, vitamins and minerals but with negligible levels of triglycerides (Walker, 1998). For the previous study from other authors were reported as the same resulted for using yeast extract as sole source of nitrogen. The addition of natural nutrients (yeast extract) led to vigorous growth and it resulted in an increase in mannitol production (Hattori and Suzuki, 1973). At the level of 0.4% yeast extract appeared to be best for mannitol production (Onishi and suzuki, 1968). Groleau *et al*., (1995) noticed that a relatively high concentration of yeast extracts, around 1% is critical for maximal glucose consumption, and for ethanol and polyols accumulation. Similarly, from this experiment, the best level of 0.3% and 0.4% yeast extract in the starch medium enhanced the mannitol production in the cell lysate. The reducing sugars at 0.3% and 0.4% yeast extract was reduced lower than other levels. It was therefore reasoned here the suitable level of yeast extract stimulated growth and favoring first glucose utilization.

3.6.4 Determination of the suitable inoculum size

Results obtained from optimization condition could be concluded that 2% starch concentration was the most suitable as carbon source and yeast extract was considered to be the best nitrogen source at concentration of 0.3% by using 2% inoculum size. However, the good yield of mannitol gave from the suitable inoculum size. Therefore, the inoculum size of yeast isolate KAY1 was necessary for further analysis.

Starch fermentation was conducted in lab scale by using simple process, non-controlled pH, the flask was shaking with 200 rpm, at 28°C. The forth levels: 1, 2, 3 and 4% of inoculum sizes were used in this study. Figure 40 and 41 showed the time courses of mannitol production in 2% Khao-klong starch medium. The effects of inoculum sizes were demonstrated in the same pattern of fermentation. The pH level was rapidly decreased from initial pH at 6.0 to 5.3 in the first day of cultivation and again increased to 6.4 throughout fermentation period. It was suggested that when the reducing sugars was consumed, the main product was organic acids that decreased pH value. Relatively, the reducing sugars was reached maximum at the first day of cultivation. The high amounts of starch were rapidly hydrolysed from the initial culture to the second day of incubation. Mannitol in the yeast cell were observed for the high amounts at the first day and gave the maximum yield at the second day of fermentation. After that it gradually decreased at the end of fermentation.

The results clearly suggested that at the first day of cultivation, starch was vigorously hydrolysed by the amylase-producing yeast KAY1. At the same time, the high amounts of reducing sugars flow into the medium, and was absorbed by yeast cell for its energy sources. The concentrations of reducing sugars in the medium which defined as osmotic pressure (Reed *et al*., 1987) were supported the construction of polyols in the yeast cell. For this experiment, the yeast isolate KAY1 could produced the mannitol inside the cells. However, after the reducing sugars gradually declined, consequently the mannitol in the cell was also decreased. It was noticed that mannitol is a common reserve product of fungi (Lee, 1907). When the osmotic pressure in the cultured medium reduced. Mannitol intracellular was converted to the energy sources. Low molecular mass compounds were accumulated intracellularly in most organisms when exposed to osmotic stress to equilibrate the cytoplasmic water activity (a_w) with the a_w of the surrounding environment (Yancey *et al*., 1982; Csonka, 1989). The main solutes accumulated in yeasts exposed to osmotic stress are polyhydroxy alcohols (polyols) such as glycerol, D-arabitol, D-mannitol, and meso-erythritol (Spencer, 1978), and are compatible with metabolic activity (Brown, 1978). The polyol accumulated is related to the yeast species, the growth phase of the yeast (Nobre and da Costa, 1985) and the carbon source used for growth (Van Eck *et al*., 1989). The terms "osmophilic" and "osmotolerant" have been widely used to described the water relations of yeasts.



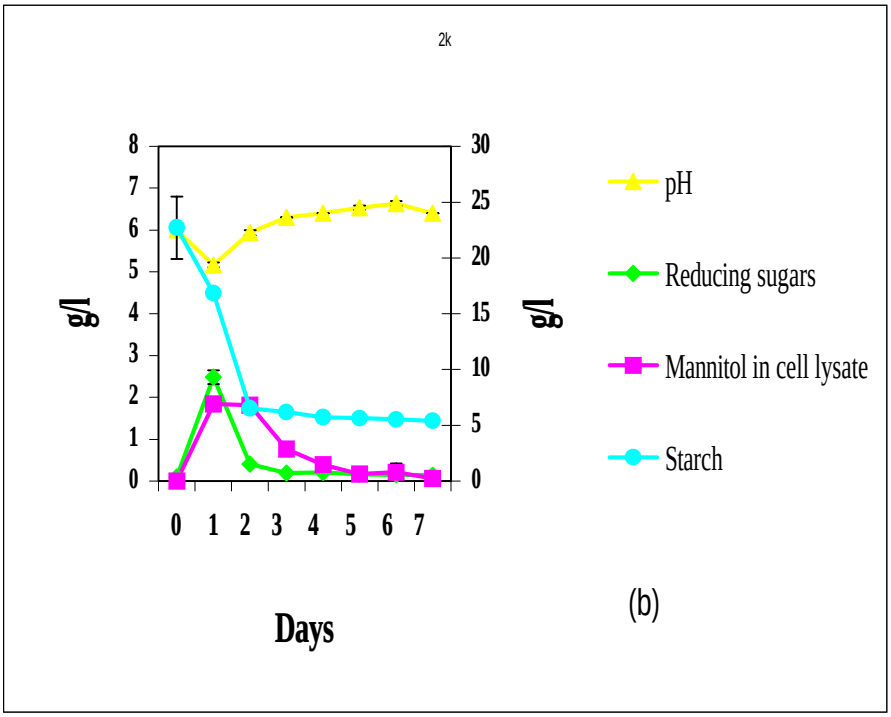
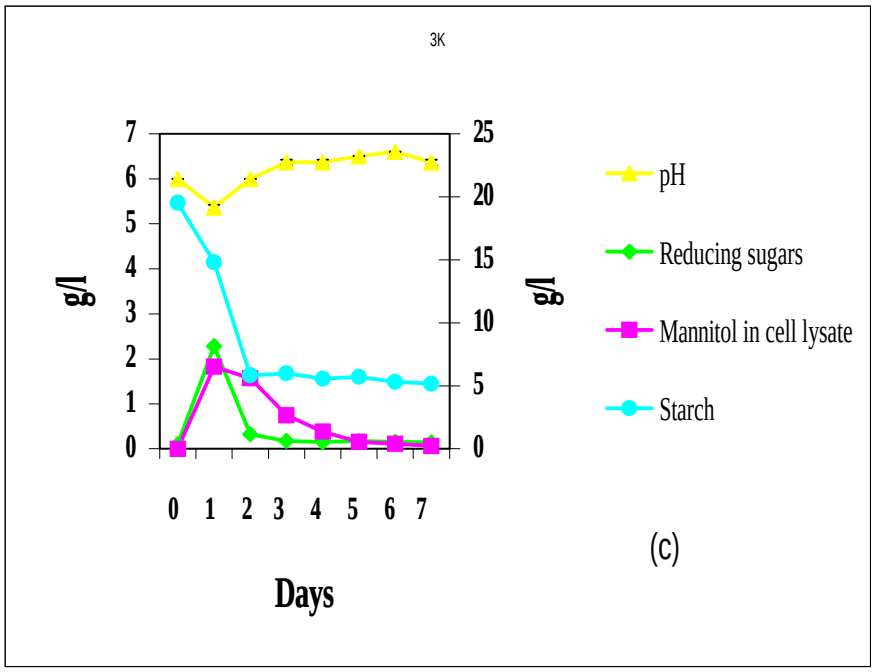


Figure 40. Time courses of mannitol production by yeast isolate KAY1 when using the medium containing 2% Khao-klong starch and different inoculum sizes; (a) 1%, and (b) 2%.



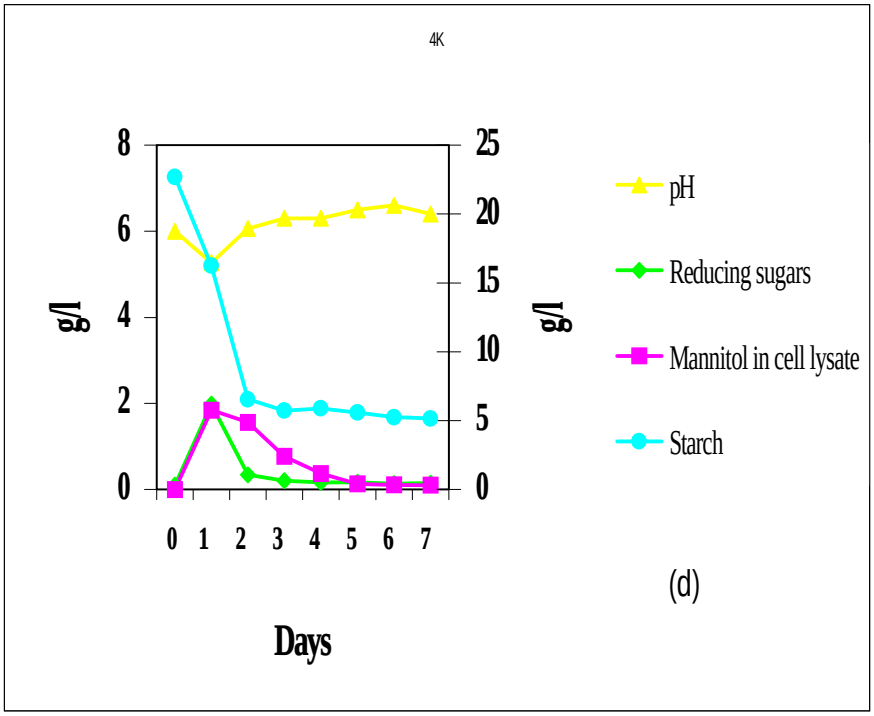


Figure 41. Time courses of mannitol production in cell lysate by yeast isolate KAY1when using the medium containing 2% Khao-klong starch and different inoculum sizes; (c) 3%, and (d) 4%.

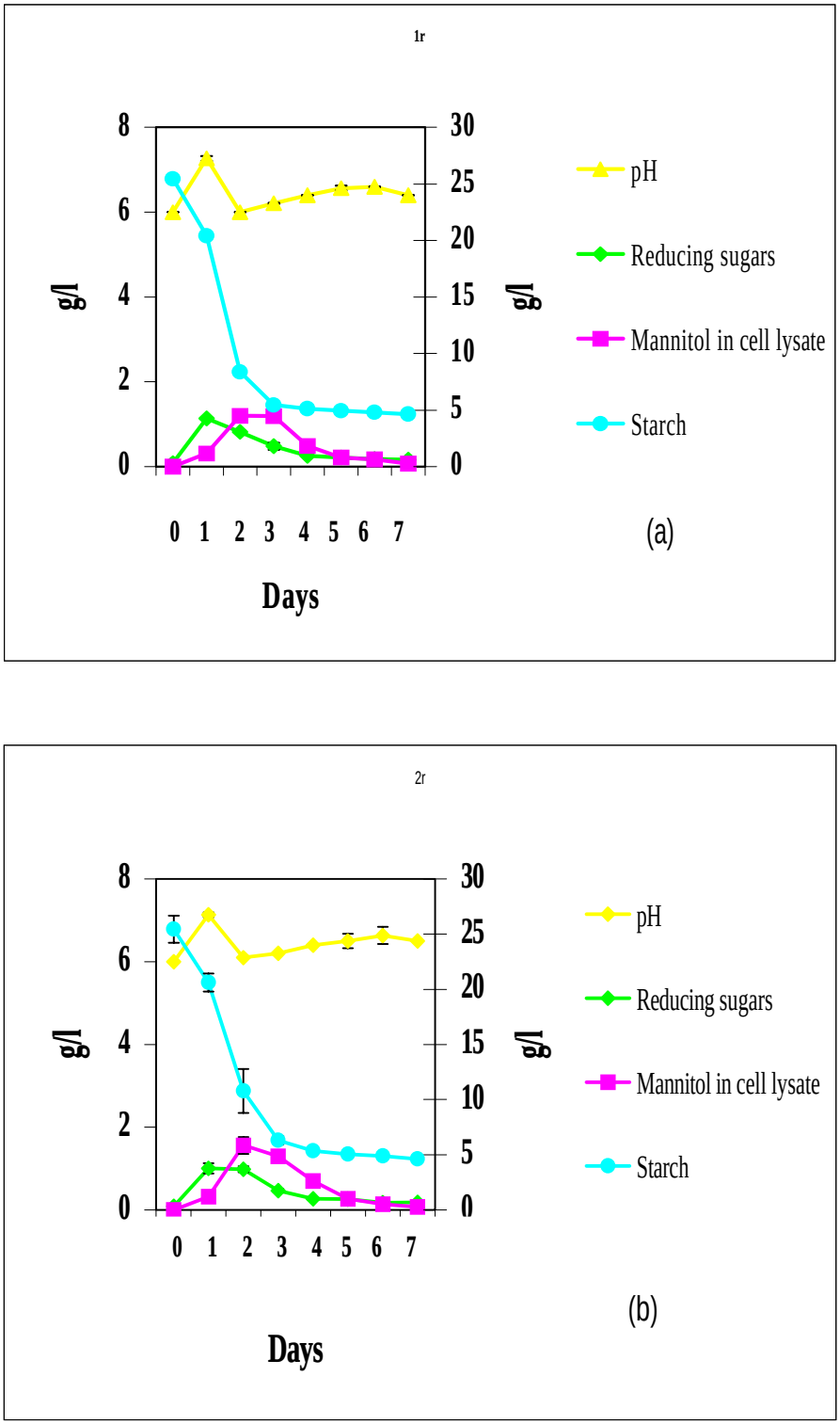
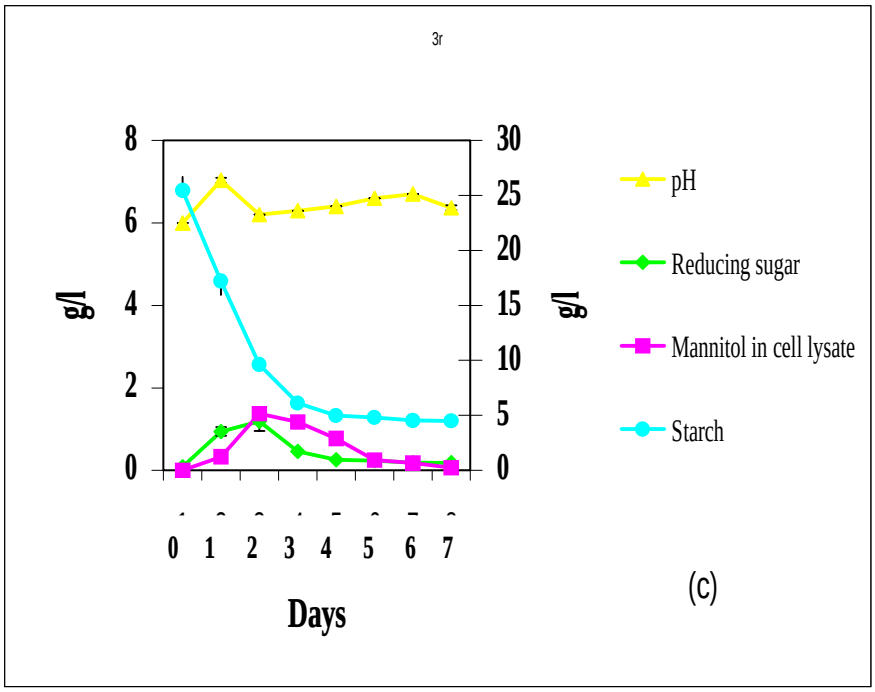


Figure 42. Time courses of mannitol production in cell lysate by yeast isolate when using the medium containing 2% rice starch and different inoculum sizes; (a) 1%, and (b) 2%.



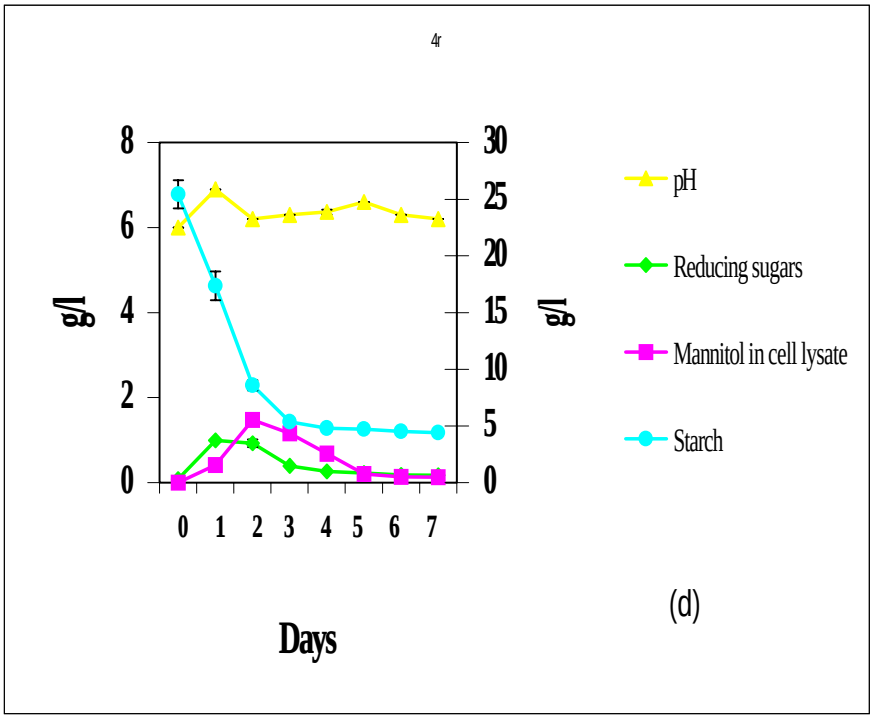


Figure 43. Time courses of mannitol production in cell lysate by yeast isolate when using the medium containing 2% rice starch and different inoculum sizes; (c) 3%, and (d) 4%.

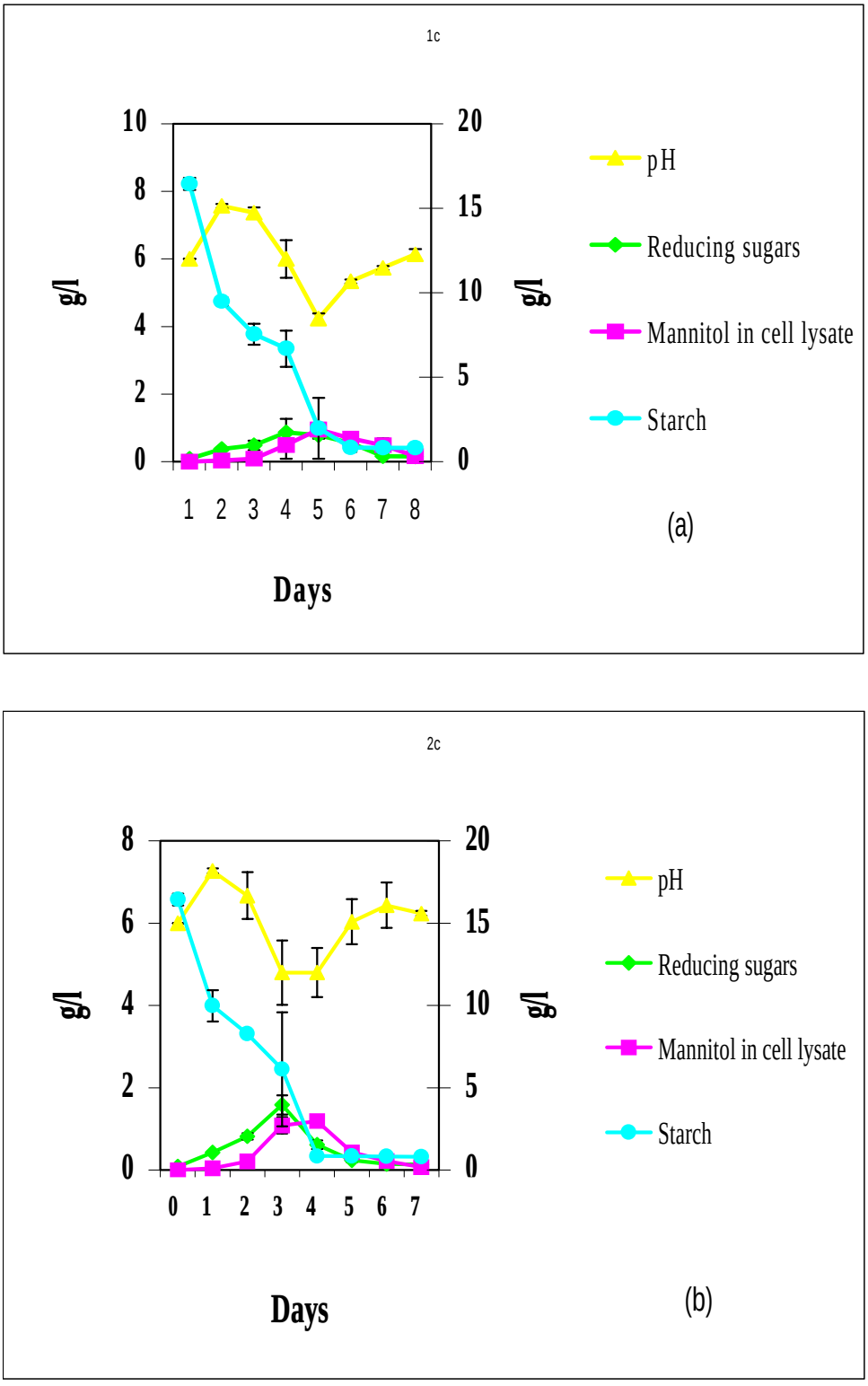
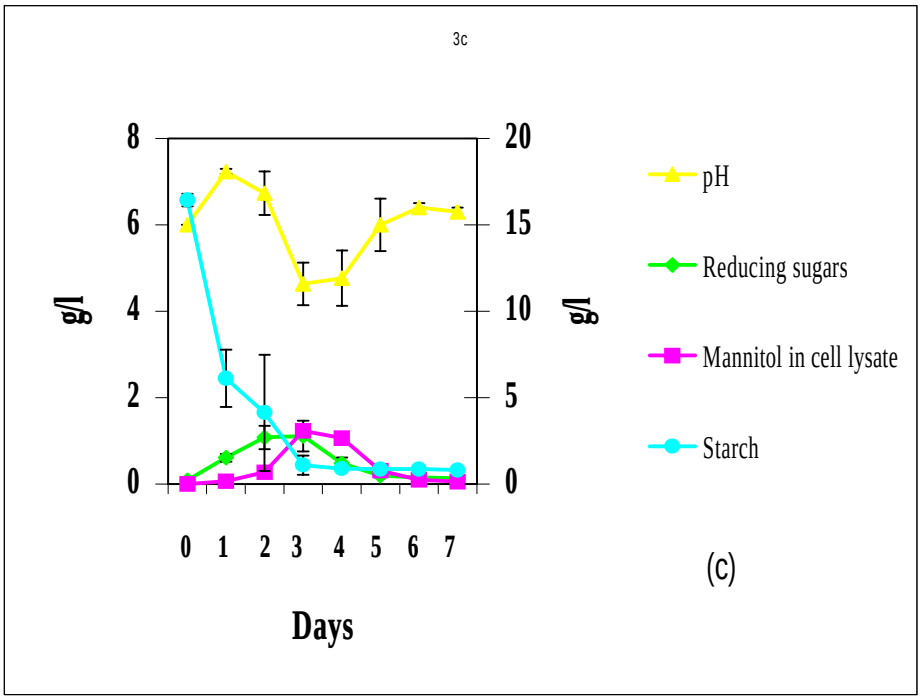


Figure 44. Time courses of mannitol production in cell lysate by yeast isolate when using the medium containing 2% cassava starch and different inoculum sizes; (a) 1%, and (b) 2%.



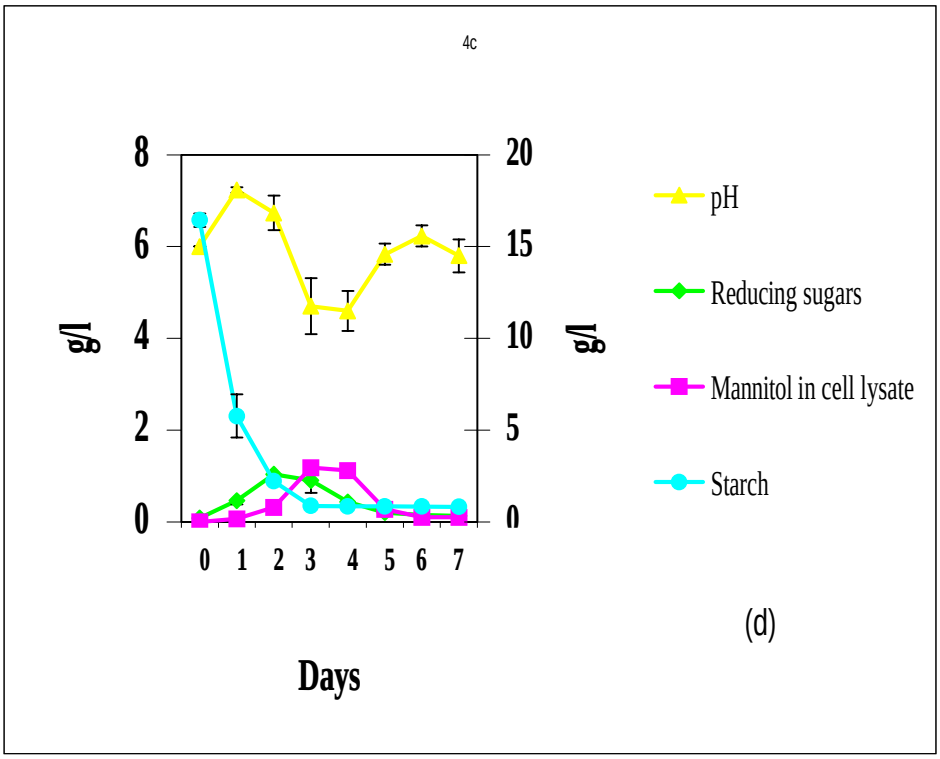


Figure 45. Time courses of mannitol production in cell lysate by yeast isolate when using the medium containing 2% cassava starch and different inoculum sizes; (c) 3%, and (d) 4%.

In the separate experiments, in 2% rice starch (Figure 42 and 43) and 2% cassava starch media were also tested (Figure 44 and 45). The inoculum sizes were used in this experiment. During the incubation of 2% rice starch medium, the time courses of 1, 2, 3, and 4% inoculum sizes were demonstrated at the similar graph pattern. Without pH control, pH level was rapidly increased at the first day from initial pH at 6.0 to 7.2 and pH decreased at the minimum at pH 6.0 at the second day of cultivation. The reducing sugar was reached the maximum at the first day of incubation. The residual starch was rapidly dropped at the third day of fermentation The production of mannitol in the cell was maximum at the second day of cultivation and gradually decreased at the end of fermentation. In the 2% cassava starch medium, the pH level was slowly decreased from initial pH at 6.0 to 4.3 at the forth day of cultivation. During the cultivation, the residual starch was slowly decreased and reducing sugar was observed at very high level at the third day of cultivation. The mannitol was maximum accumulated at the third day and the fourth day of cultivation.

The results showed that the mannitol production in cell lysate in 2% Khao-klong starch medium was maximum amount at the first day and second day of cultivation, in 2% rice starch medium was highest amounts at the second day and the third day of cultivation, and 2% cassava starch gave the maximum yield at the third day and the fourth day of incubation. However, the mannitol production in this experiment showed it was not different in the yield of mannitol in cell lysate because the range of various of inoculum sizes was narrow. Thus, the wide range of inoculum sizes might be tested for the further experiment.

The 4 levels of inoculum sizes: 2, 6, 8, and 10% were also tested. From Figure 46 (a) and (b) showed the effects of various inoculum sizes on mannitol and reducing sugars produced when they were cultured in starch medium containing 2% Khao-klong starch. At 6% inoculum sizes showed the highest amounts of mannitol at 2.367 g/l at the first day and 1.658 g/l at the second days of incubation, respectively. The extracellular and intracellular glycerol were found at the first day by using 6% and 8% of inoculum sizes (Figure 47). During the fermentation in 2% rice starch medium (Figure 48 (a) and (b)), 2% inoculum sizes gave the maximum amounts of mannitol in cell lysate at the third day. And when yeast isolate KAY1 cultured in 2% cassava starch medium (Figure 49 (a) and (b)), it was found that 2% inoculum sizes gave the maximum yields of mannitol in cell lysate at the forth days of incubation.

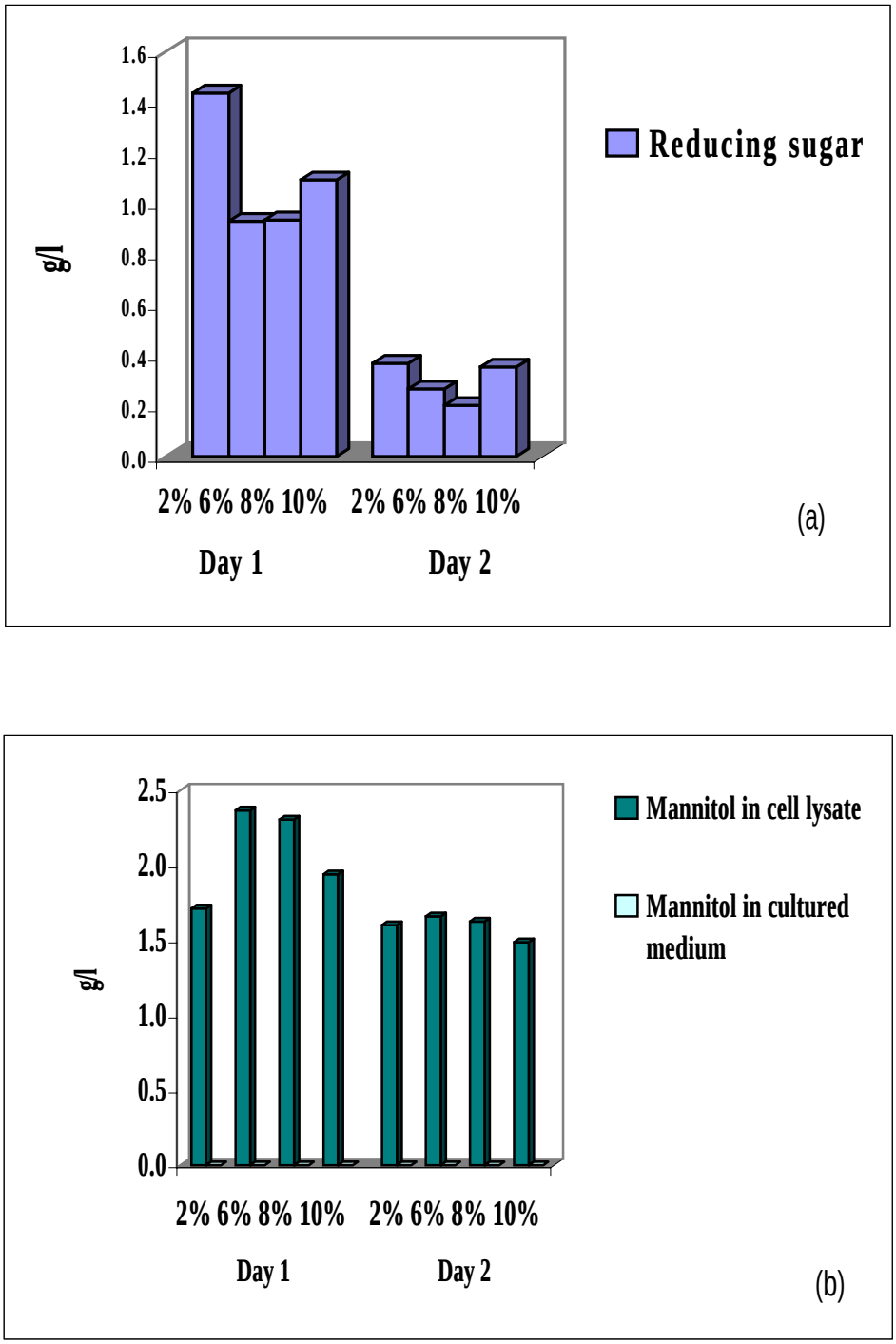


Figure 46. Effects of various inoculum sizes (2%, 6%, 8%, and 10% (v/v)) on the production of (a) reducing sugars and (b) mannitol by yeast isolate KAY1 when cultured in the medium containing 2% Khao-klong starch for 2 days at 30°C.

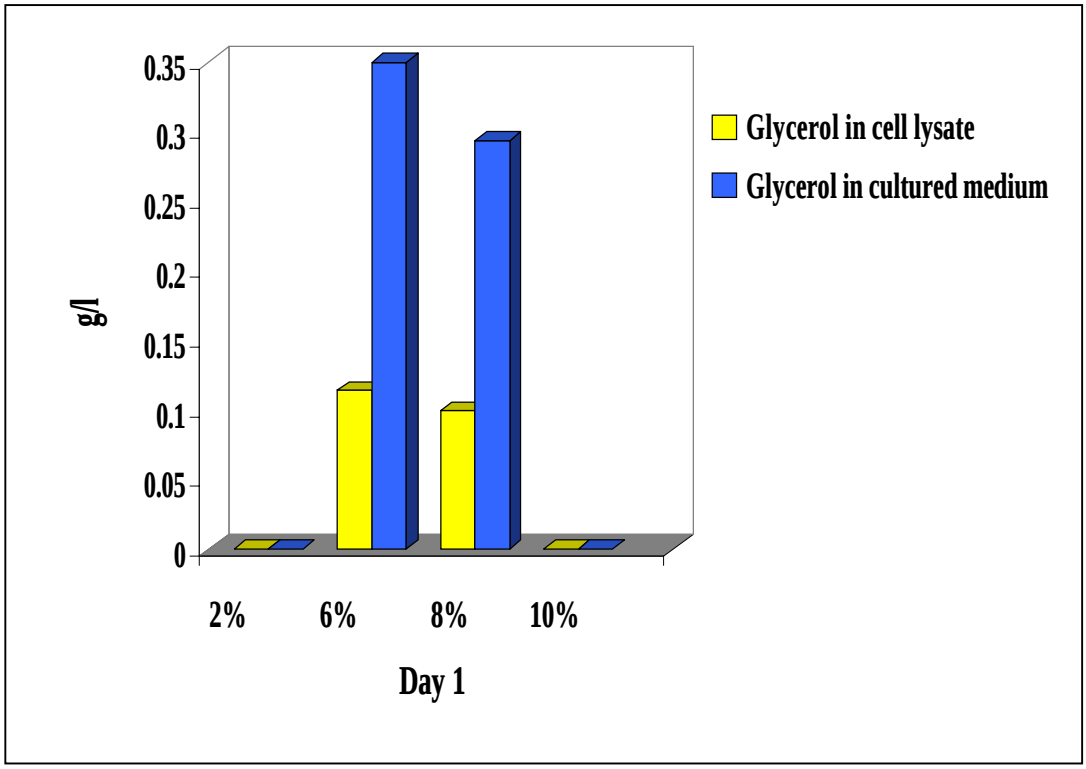
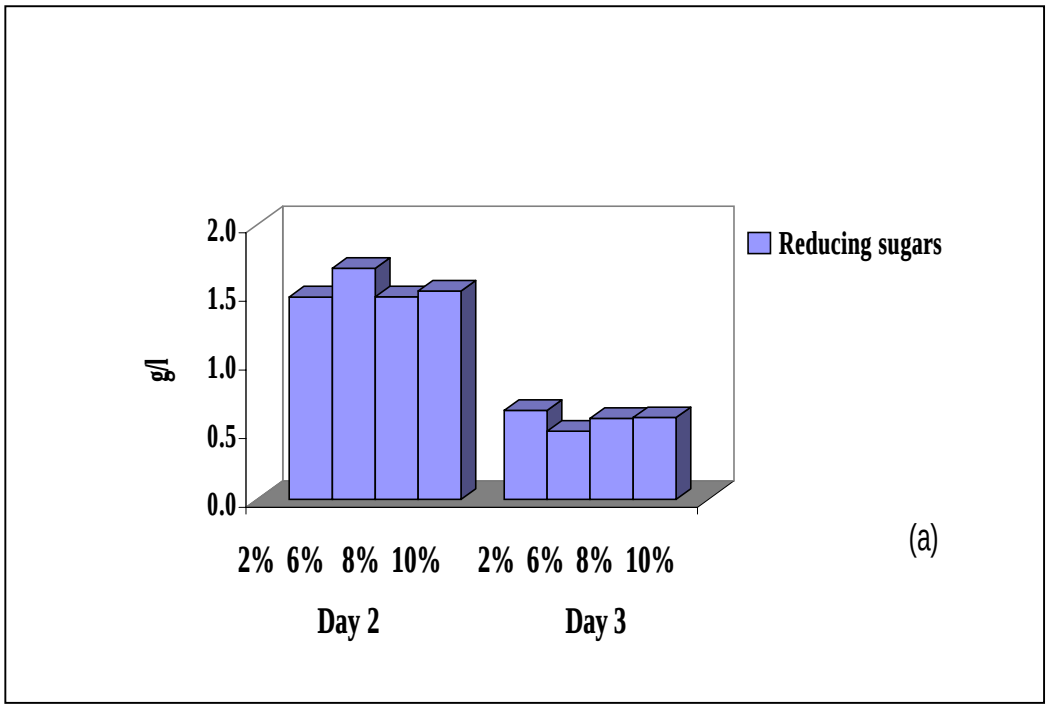


Figure 47. Effects of various inoculum sizes (2%, 6%, 8%, and 10% (v/v)) on the production of glycerol by yeast isolate KAY1 when cultured in the medium containing 2% Khao-klong starch for 2 days at 30°C.



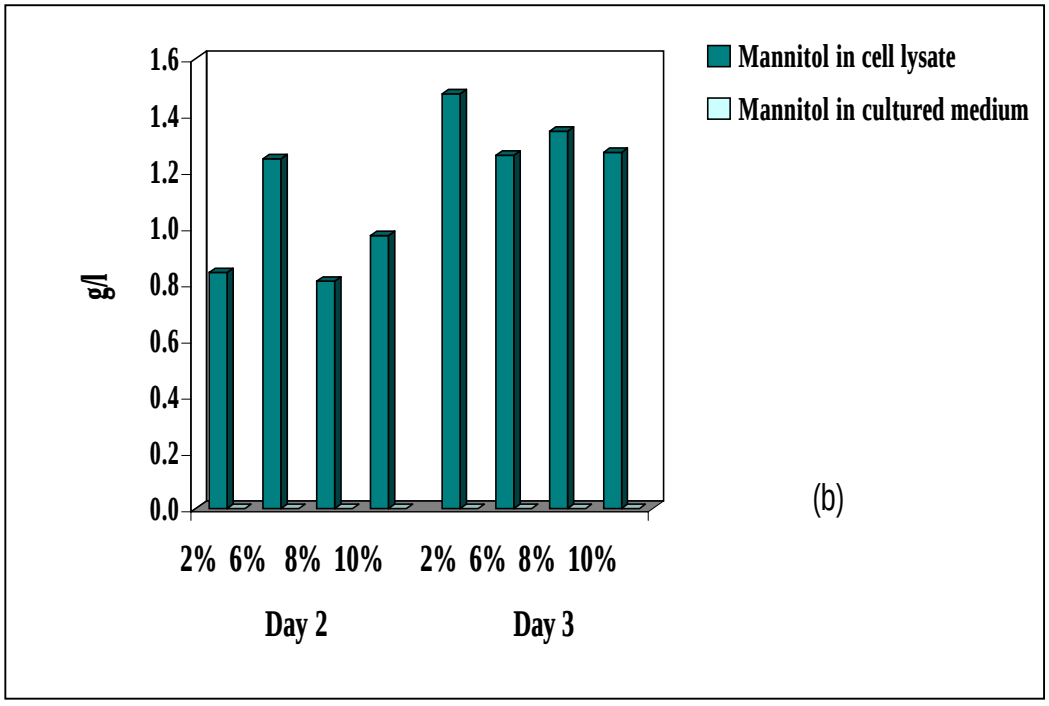
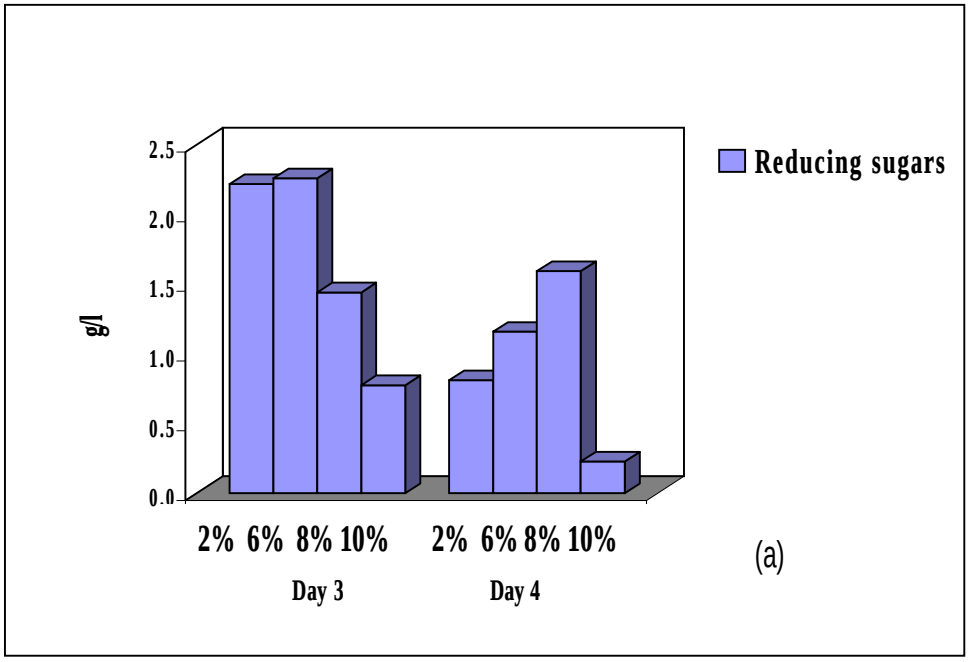


Figure 48. Effects of various inoculum sizes (2%, 6%, 8%, and 10% (v/v)) on the production of (a) reducing sugar and (b) mannitol by yeast isolate KAY1 when cultured in the medium containing 2% rice starch for 2 days at 30°C.



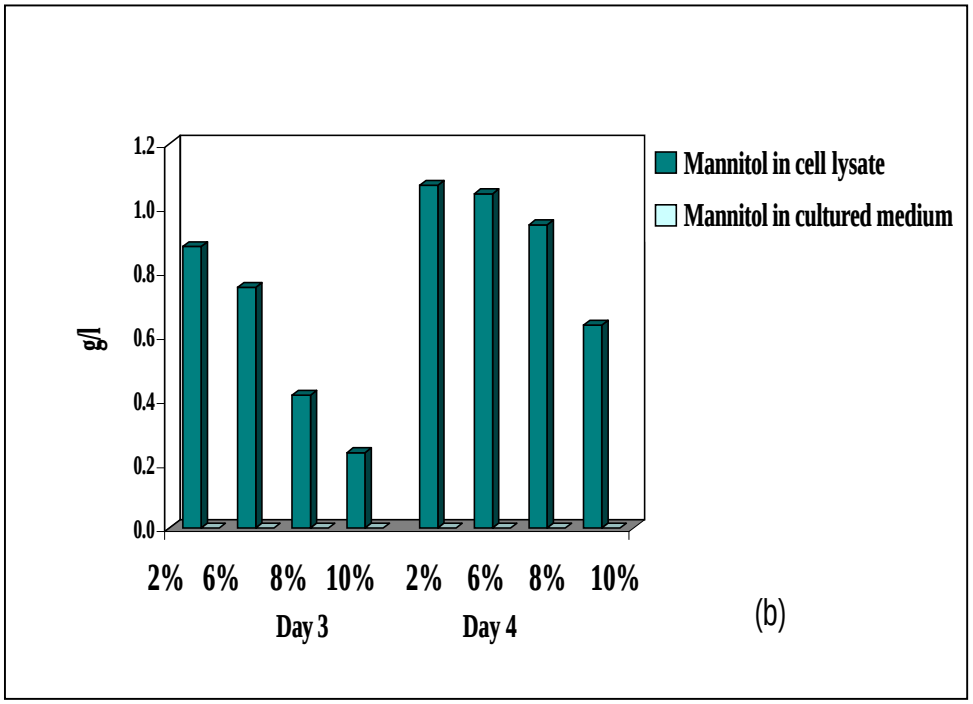


Figure 49. Effects of various inoculum sizes (2%, 6%, 8%, and 10% (v/v)) on the production of (a) reducing sugars and (b) mannitol by yeast isolate KAY1 when cultured in the medium containing 2% cassava starch for 4 days at 30°C.

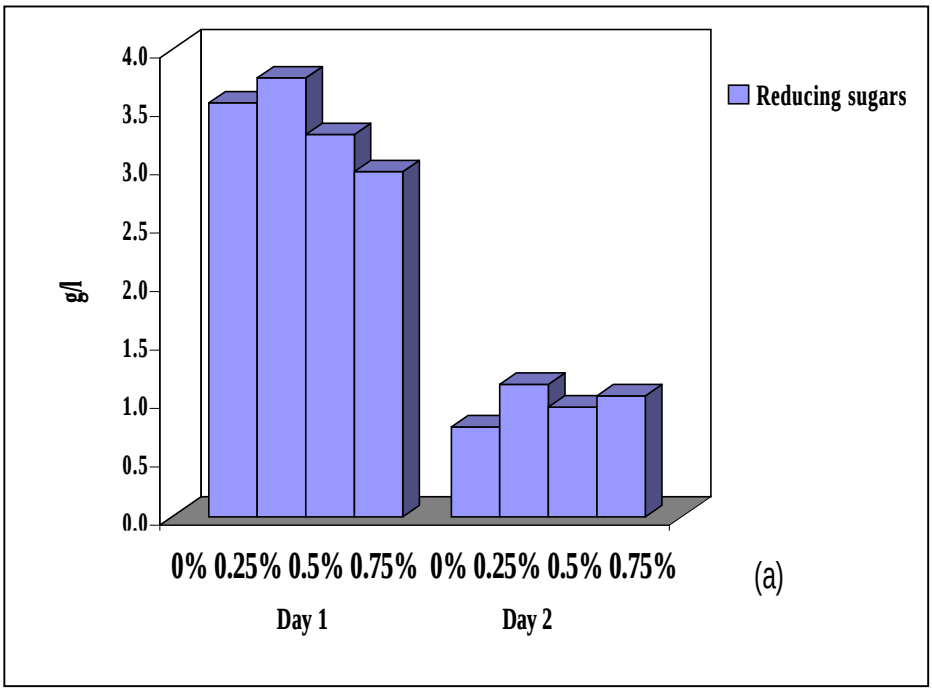
The results showed that amounts various inoculum sizes, the suitable inoculum sizes for 2% Khao-klong starch medium was 6%. For 2% rice starch medium, the maximum amounts of mannitol in cell lysate was gave from 2% inoculum size and 4% inoculum size enhanced the highest amounts of mannitol in 2% cassava medium starch. From this study, each substrate have the different in the quantitation of inoculum size and incubation time which gave the maximum yield of the cultivation products, it could be noticed that amylase enzyme from yeast isolate KAY1 have the specific to the starch composition of each substrate. The ability of an enzyme to digest raw starch appears to be associated with its ability to strongly absorb onto the granules (Ueda *et al*., 1984). The composition of the nutrient sources in the substrate might be important for the growth of yeasts. From the Table 6 showed the cassava starch had the highest starch content in major components of the structure, but it had the organic substances such as protein or fat lower than the other raw materials. From the results, the maximum yield of mannitol in cell lysate gave at the first and the second day of cultivation. The Khao-klong starch obtained from polished rice which had so much vitamins and coenzyme, it might be enhanced the utilization of starch and glucose consumption similar to the high minerals, and vitamins from yeast extract.

3.7 Sugar alcohol overproduction by physical and chemical stress

To study the effects of sugar alcohol overproduction rate, various conditions heat shock treatment, salt-stress, and pH regulation by addition of $CaCO_3$ were investigated.

3.7.1 Sugar alcohol overproduction by the addition of calcium carbonate

The 2% starch medium of either Khao-klong starch, rice starch, or cassava starch was used as the substrate, and the suitable inoculum sizes: 2%, 4% and 6% used for rice starch, cassava starch, and Khao-klong starch medium, respectively. Effects of various $CaCO_3$ concentrations (0%, 0.25%, 0.5%, and 0.75%(w/v)) on the production of reducing sugars and mannitol by yeast isolate KAY1 when cultured in the medium containing 2% Khao-klong starch were illustrated in Figure 50. The results showed that when yeast isolate KAY1cultured in 2% Khao-klong starch medium, the cultivation products was compared. Control which no calcium carbonate added gave the maximum amounts of mannitol in cell lysate at the first day and the second day of cultivation. The reducing sugars was maximum amounts at the first day and rapidly decreased in the second day of



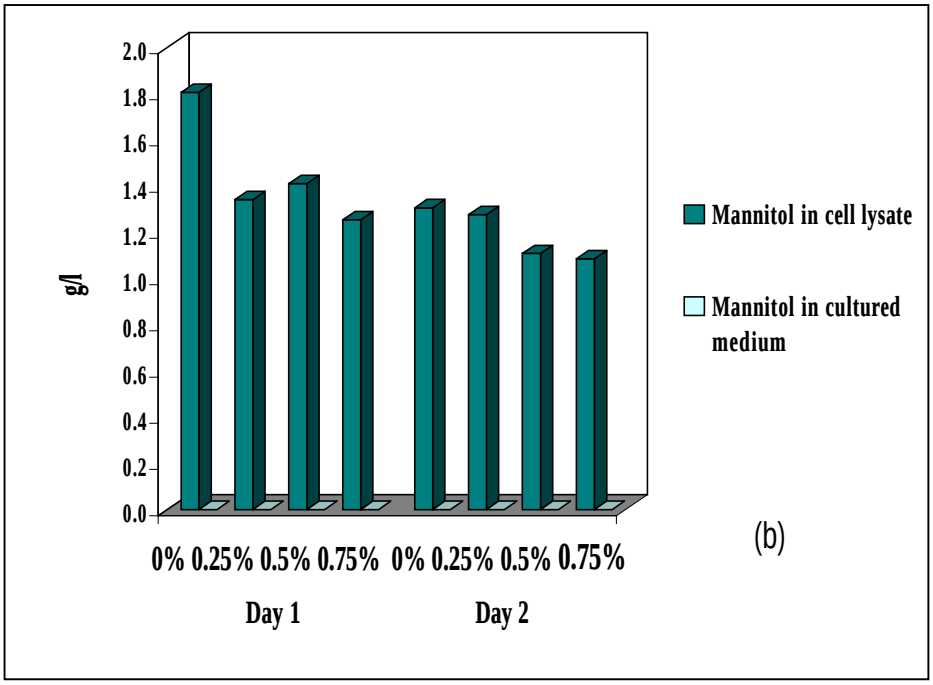
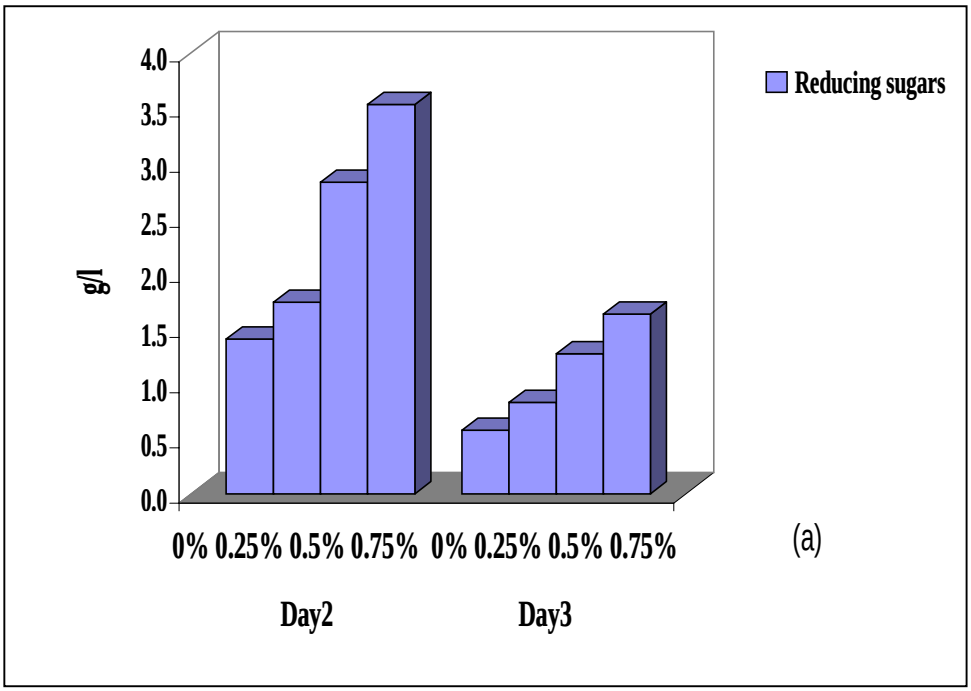


Figure 50. Effects of various $CaCO_3$ concentrations (0%, 0.25%, 0.5%, and 0.75%(w/v)) on the production of (a) reducing sugars and (b) mannitol by yeast isolate KAY1 when cultured in the medium containing 2% Khao-klong starch for 2 days at 30°C.



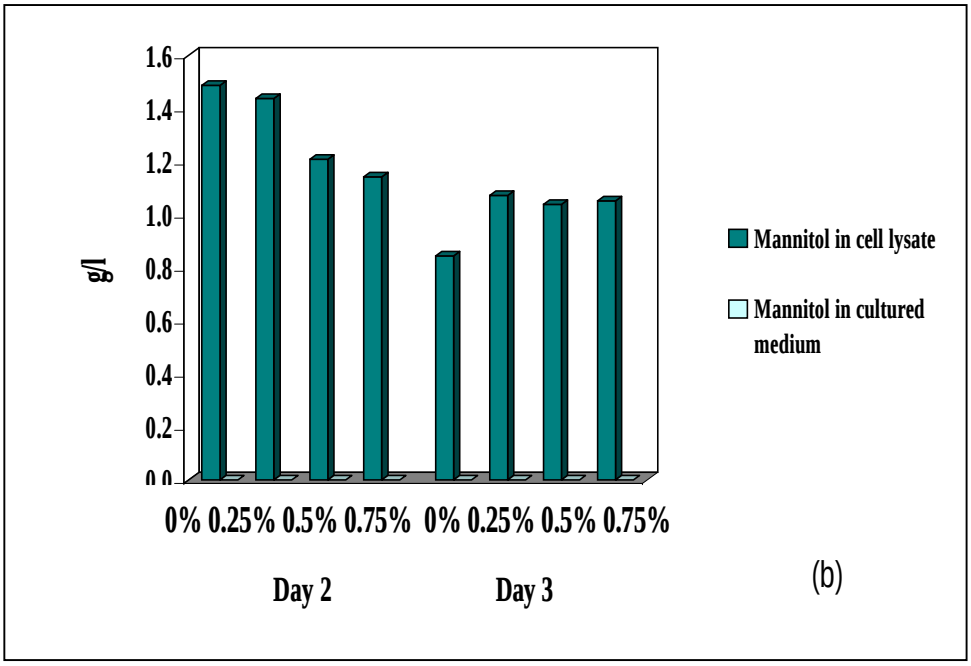
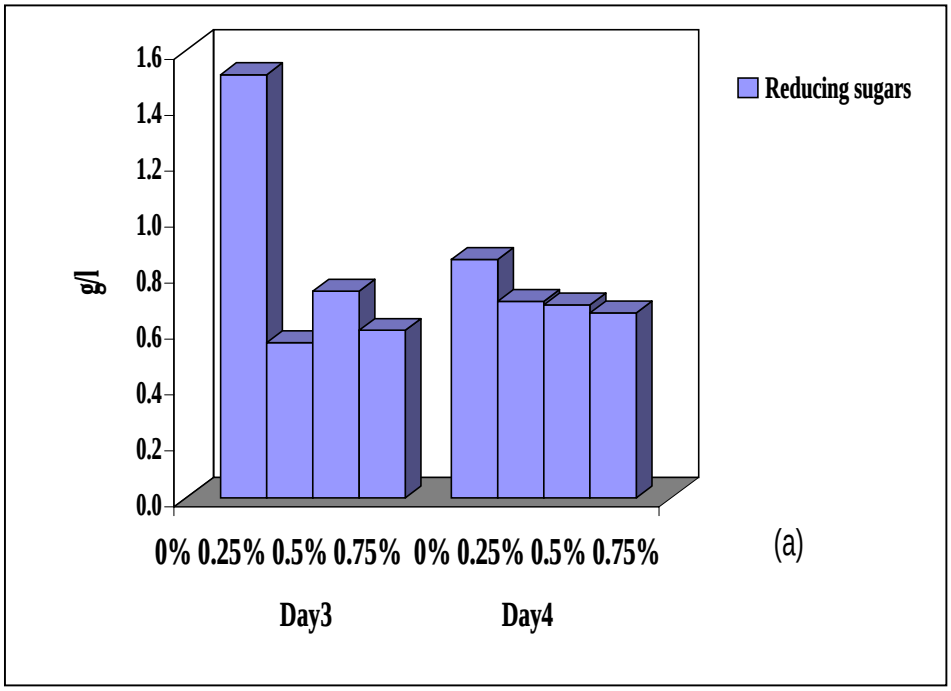


Figure 51. Effects of various $CaCO_3$ concentrations (0%, 0.25%, 0.5%, and 0.75%(w/v)) on the production of (a) reducing sugars and (b) mannitol by yeast isolate KAY1 when cultured in the medium containing 2% rice starch for 3 days at 30°C.



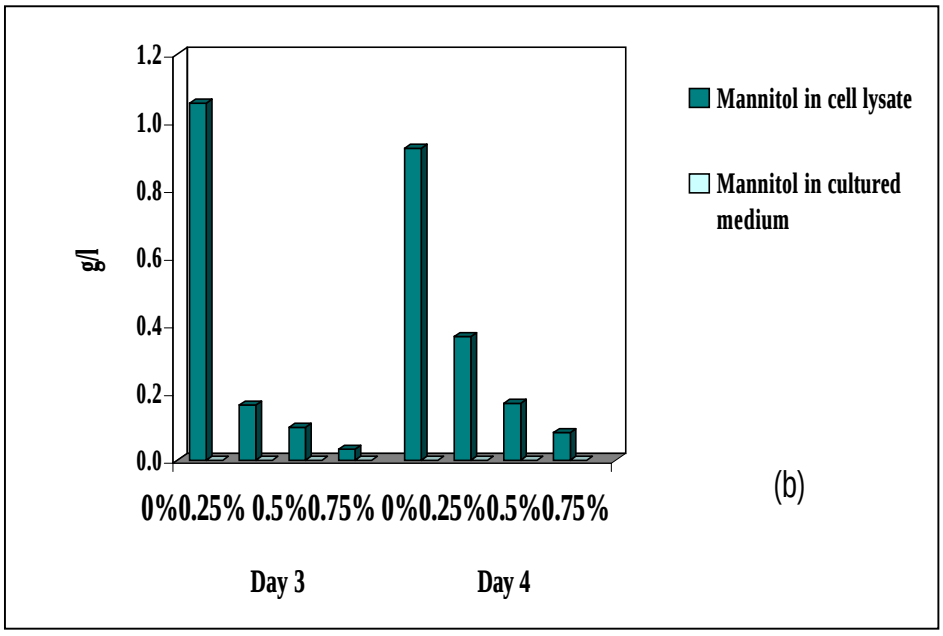


Figure 52. Effects of various $CaCO_3$ concentrations (0%, 0.25%, 0.5%, and 0.75%(w/v)) on the production of (a) reducing sugars and (b) mannitol by yeast isolate KAY1 when cultured in the medium containing 2% cassava starch for 4 days at 30°C.

fermentation, but the incubation products from the medium which containing the calcium carbonate gave the amounts of mannitol lower than the control. Similarly, when cultured in 2% cassava starch medium, at 0% of calcium carbonate gave the higher amounts of mannitol than the medium containing calcium carbonate at the third and the forth days of cultivation. In the same resulted, the control gave the highest amounts of mannitol at the second days of fermentation when cultured in 2% rice starch medium. It was clearly shown that calcium carbonate was not supported the production of mannitol in yeast cell.

Calcium carbonate is a classical buffering agent. The addition of calcium carbonate was order to maintain adequate pH values for the enzymatic activity. Additionally calcium had a specific effect on enzyme stability to extreme pH and temperatures (Pintado *et al*., 1999). Calcium carbonate was used since it was found that under alkaline conditions increased concentrations of glycerol could be obtained (Lie *et al*., 1991). From the results, 2% Khao-klong starch medium showed the initial pH at 6.0 and pH level was decreased at 5.8 at the first day, but 2% rice starch medium containing calcium carbonate was maintained at pH 6.2-6.4 along the incubation. In 2% cassava starch medium, pH level was 4.4 at the third and the forth day. When added with calcium carbonate, the pH was maintained at pH 7.7-8.2. Similarly, 2% rice starch (Figure 51) gave the same result as 2% cassava starch (Figure 52) and 2% Khao-klong starch medium.

The best yield of mannitol gave at the pH 4-5, but when added with calcium carbonate, the pH level was maintained at pH 6.2 or higher than control. It was suggested that in generally, yeast grow well at pH in the range of 3.5 and 5.0 (Kurtzman and Fell, 1998). The optimum pH values for most yeast amylases were found in the 4-6.5 ranges (Verachtert *et al*., 1990). For the yeast isolate KAY1, its enzymes might performed good activity was at pH 4-6.5. Thus, when calcium carbonate was added, the pH in the medium culture was reached and remained at between 6.0-8.0. In this condition, it could not grow well and amylase activity was reduced. Consequently, the cultivation products such as mannitol was decreased.

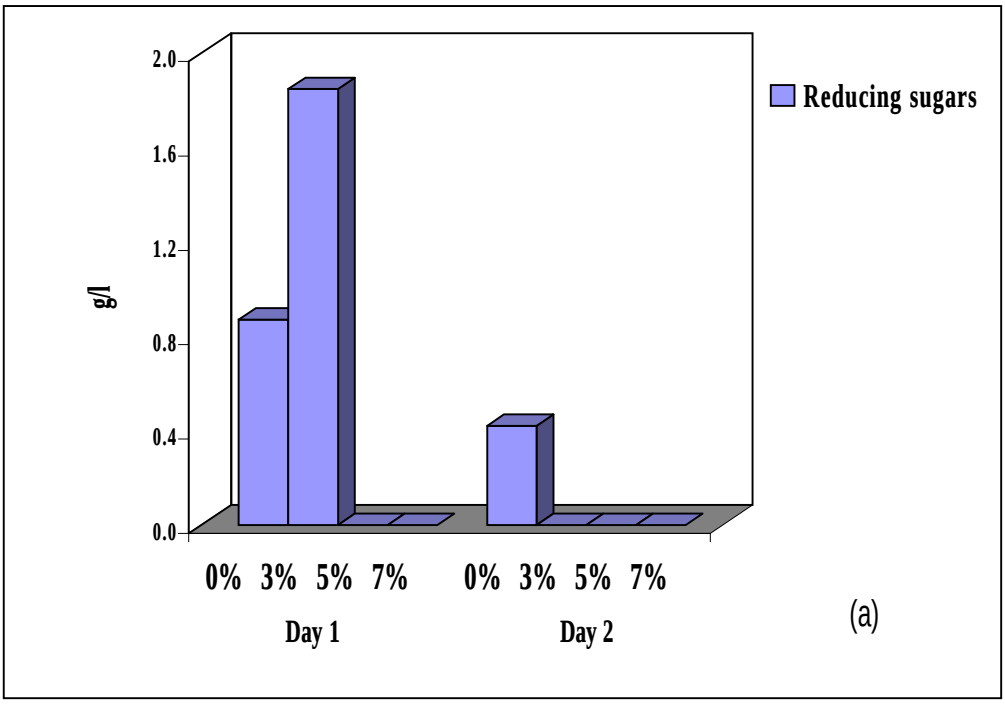
3.7.2 Salt-stress condition for sugar alcohol overproduction

The fifty millilitres of starch medium containing 2% (w/v) of either cassava starch, rice starch, or Khao-klong starch medium were added with sodium chloride at 0, 3, 5, and 7% (w/v). The

appropriate concentration of inocula which was 24-48 hours olds were inoculated into the starch medium.

From the results as shown in Figure 53 and 54, when yeast isolate KAY1 was cultured in 2% Khao-klong starch medium. The maximum yield of mannitol in cell lysate was detected in 5% sodium chloride at 2.837 g/l of mannitol when incubated for 2 days. The reducing sugars disappeared in the 5% and 7% sodium chloride concentrations. Extracellular and intracellular glycerol was found the maximum amounts at the second day of cultivation. In the contrast, when cultured in 2% rice starch medium (Figure 55 (a) and (b)) and 2% cassava starch medium (Figure 56). The yeast cell could not grow well in the 2% starch medium containing sodium chloride, but the reducing sugars and mannitol in cell lysate were observed the highest in the control.

The results clearly suggested that the yeast isolate KAY1 could grow well on 2% Khao-klong starch medium containing sodium chloride, but not grow well on 2% rice starch and 2% cassava starch medium containing sodium chloride. From previous study, in Khao-klong starch medium showed the highest yield of mannitol production at the first day of cultivation correlate to the amylase enzyme specific to the substrate. Relatively, at the first day of cultivation the starch was rapidly hydrolyzed, and the reducing sugars was highly absorbed in to the yeast cell as sole carbon source. The high amounts of reducing sugars at the first day of fermentation enough to maintained the energy and converted into the mannitol which protected the cell from salt-stress. Extracellular and intercellular glycerol were detected at the high amounts of 5% and 7% sodium chloride correlate to the previous study from other authors. Van Eck *et al*., (1993) reported that glycerol was the principle osmolyte in some filamentous fungi, algae, insects, crustaceans and vertebrates, which indicates that there was a selective advantage to organisms using such low molecular mass solutes (Yancey *et al*., 1982). As on osmolyte, glycerol offers a number of advantages: high glycerol concentrations confer a remarkable degree of protection to enzymes and macromolecular structure (Brown, 1978). From the results in Figure 54, the amounts of glycerol were increased correlated to the increasing concentration of sodium chloride. The ability to retained a greater proportion of a constant total (intra-and extracellular-) amounts of glycerol intracellularly was proposed by Brown (1978) as an essential characteristic of an osmotolerant yeast such as *Zygosaccharomyces rouxii*, whereas a non-osmotolerant yeast such as *S. cerevisiae* produced increasing amounts of glycerol and retained



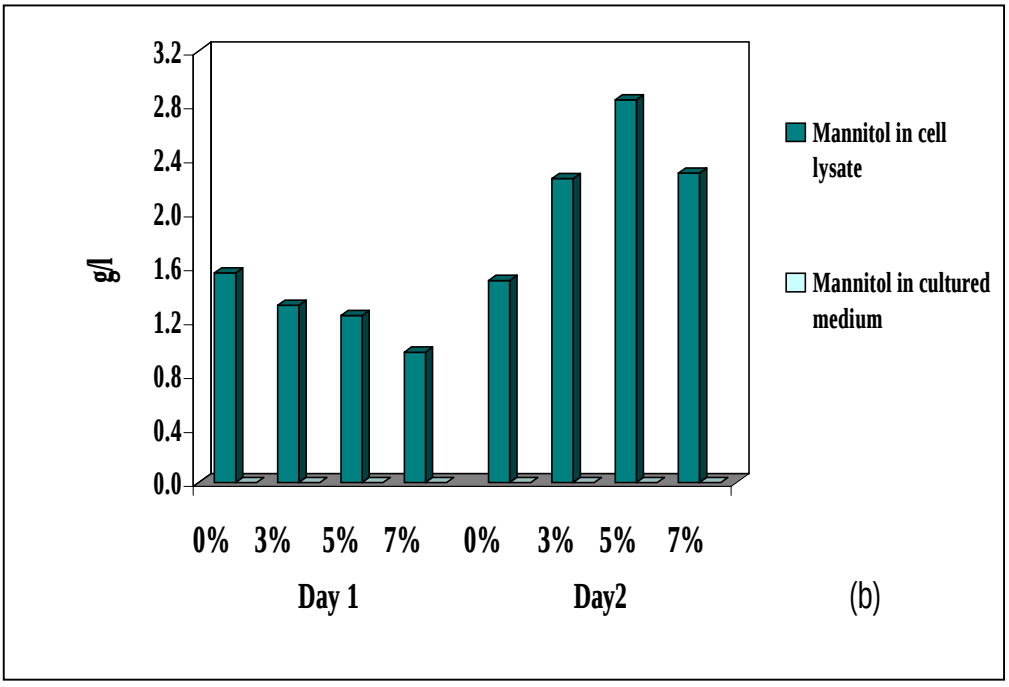


Figure 53. Effects of various NaCl concentrations (0%, 3%, 5%, and 7% (w/v)) on the production of (a) reducing sugars and (b) mannitol by yeast isolate KAY1 when cultured in the medium containing 2% Khao-klong starch for 2 days at 30°C.

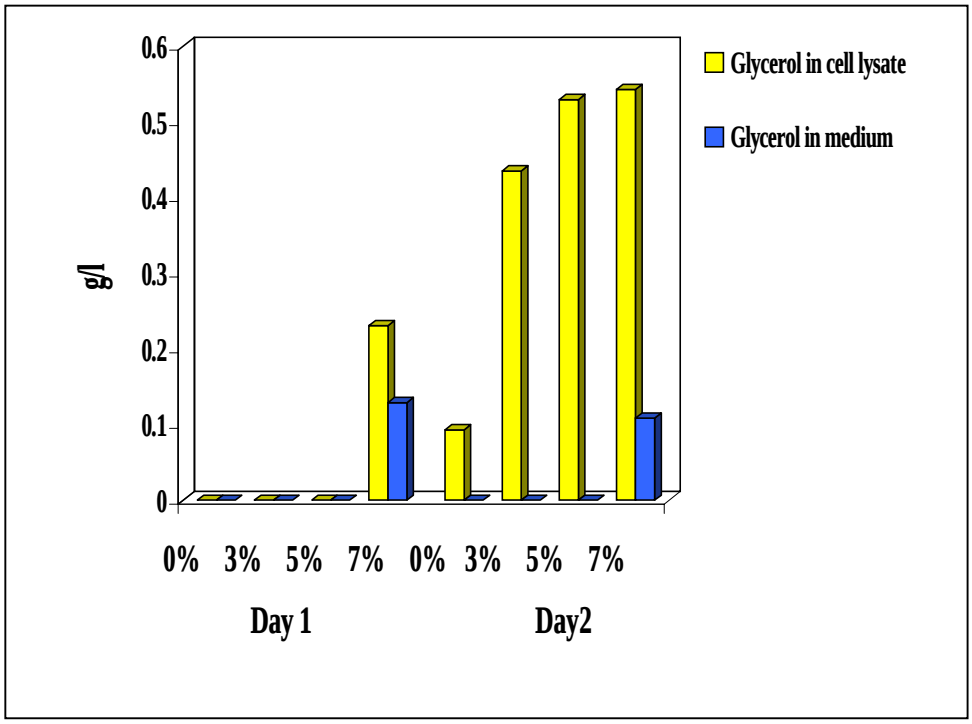
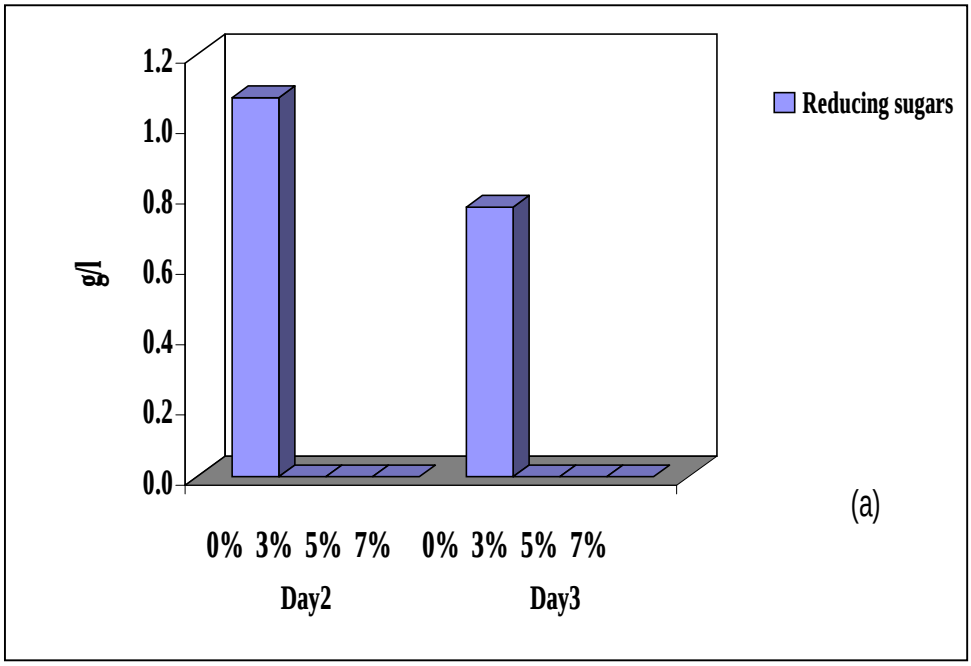


Figure 54. Effects of various NaCl concentrations (0%, 3%, 5%, and 7% (w/v)) on the production of glycerol by yeast isolate KAY1 when cultured in the medium containing 2% Khao-klong starch for 2 days at 30°C.



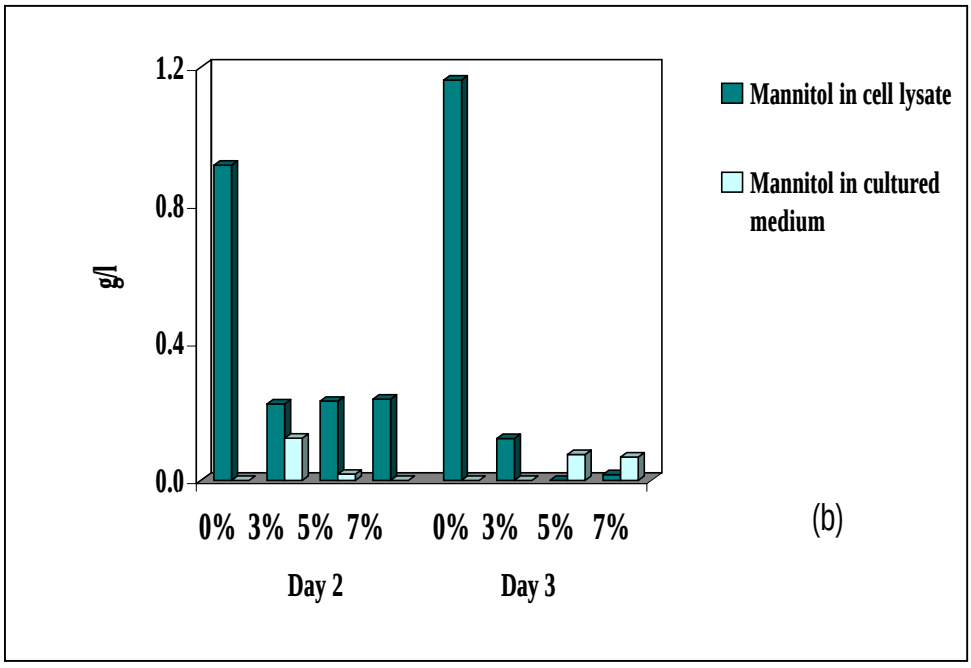


Figure 55. Effects of various NaCl concentrations (0%, 3%, 5%, and 7% (w/v)) on the production of (a) reducing sugars and (b) mannitol by yeast isolate KAY1 when cultured in the medium containing 2% rice starch for 3 days at 30°C.

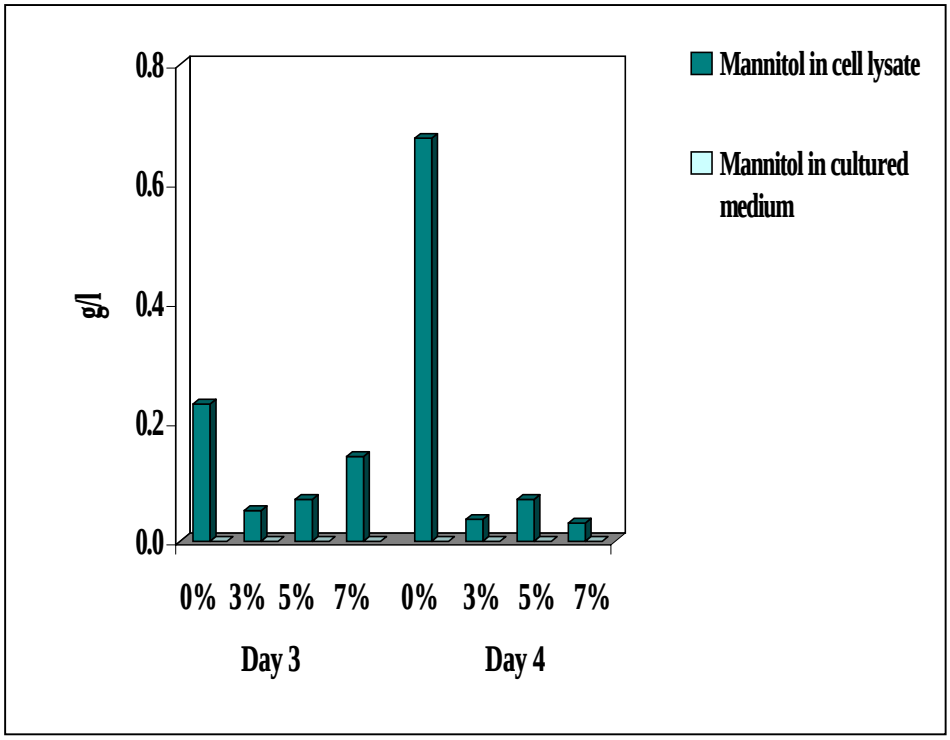


Figure 56. Effects of various NaCl concentrations (0%, 3%, 5%, and 7% (w/v)) on the production of (a) reducing sugars and (b) mannitol by yeast isolate KAY1 when cultured in the medium containing 2% cassava starch for 4 days at 30°C.

intracellularly a decreasing proportion of the total. The current division of yeasts into osmotolerant and non-osmotolerant had no clear physiological basis. (Van Eck *et al*., 1993). The increasing ratio (intra/extracellular concentration) of glycerol with a decrease in a_w indicated that the permeability of the plasma membrane was involved in the water relations of yeast (Brown, 1978). During osmotic stress, the permeability of the yeast membrane to glycerol and other solutes might be reduced by changes in the phospholipid composition (Watanabe and Takakuwa, 1984; Tunblad-Johansson and Adler, 1987) thus allowing solute accumulation. Alternatively, yeast might maintained high intracellular concentrations by means of active transport of glycerol. Active transport mechanisms regulated by osmotic stress have been described in *Debaryomyces hansenii* (Adler *et al*.,1985; Lucas *et al*., 1990) and *Z. rouxii* (Van Zyl *et al*., 1990) which enable glycerol to be accumulated intracellularly up to 5,000-fold.

3.7.3 Heat shock treatment

Heat shock treatment was performed at 45°C for 0, 20, 40, and 60 min. Yeast cells were grown in 50 ml of pre-culture medium (starch medium containing the optimal concentration of either cassava starch, or rice starch, or Khao-klong starch).

From the results in Figure 57, when cultured in 2% Khao-klong starch medium, the maximum reducing sugars was gave from 20 min heat shock treatment and gave the high amounts of reducing sugars at the second day of cultivation. The mannitol in cell lysate was rapidly increased at the second day by using heat shock treatment at 45°C for 20 min, but control gave the high amounts of mannitol at the first day and gradually decreased during the cultivation.

The Figure 58 showed the reducing sugars was increased correlate to heat shock treatment at 0, 20, 40, and 60 min at the first day on 2% rice starch medium. The reducing sugar was decreased at the third day of incubation. At the second day when cultured in 2% rice starch medium, the amounts of mannitol in yeast cell was maximum at 45°C for 20 min heat shock treatment. Similarly, the reducing sugar was increased maximum at the second day of cultivation at 45°C for 20 min heat shock treatment and gradually decreased at the end of fermentation when cultured in 2% cassava starch medium (Figure 59). The mannitol in cell lysate was highest at the third day of

incubation. The results clearly suggested that the heat shock treatment performed at 45°C for 20 min was effectively improved mannitol production by using yeast isolate KAY1.

Several authors reported that heat shock at 45 and 50°C was effective for glycerol production by Brewing yeast *S. cerevisiae* (Omori *et al*., 1996) and the effectively performed under moderate heat shock treatment was 45°C for 20 min. (Omori *et al*., 1997). The heat shock treatment (45°C, 1 h) caused a transient delay in cell growth, but the amounts of glycerol produced by heat shock-treated cells was 20% higher than that by control cells. (Kajiwara *et al*., 2000).

Yeast cells exhibited a rapid molecular response when exposed to elevated temperature. This was called the heat-shock response and was a ubiquitous regulatory phenomenon in all living cells (Walker, 1998). Sublethal heat-shock treatment of yeast leads to the induction of synthesis of a specific set of proteins, the highly conserved “heat-shock proteins” (Hsps). In addition to the induction of Hsps following heat shock, yeast cells also respond by accumulating other putative to the induction of Hsps following heat shock, yeast cells also respond by accumulating other putative protective compounds such as trehalose (Van Laere, 1989; Wiemken, 1990; Neves and Francois, 1992), glycerol (Omori *et al*., 1996) and enzymes such as catalase and mitochondrial superoxide dismutase (Costa *et al*., 1993). From the method of sugar alcohol overproduction by physical and chemical stress, various conditions which were heat shock treatment, salt-stress, and pH regulation by the addition of calcium carbonate were tested.

These results revealed that the addition of calcium carbonate could not supported the sugar alcohol production when compared with the control. The good yields of mannitol gave at 5% NaCl concentration when cultured in 2% Khao-klong starch medium and also detected either extracellular cellular and intracellular glycerol in the medium. However, the high salt concentration might caused rapid loss of viability of rhizobia (Vincent, 1982). A novel method, heat shock treatment at 45°C for 20 min was effectively improved mannitol production with yeast isolate KAY1. When the heat shock treatment was achieved in this experiment. The high amounts of sugar alcohols in both yeast cells and cultured medium was aimed. Therefore, further work was needed to elucidate this area.

The results obtained from optimization condition could be concluded that 2% starch concentration was the most suitable as sole carbon source and yeast extract was considered to be the

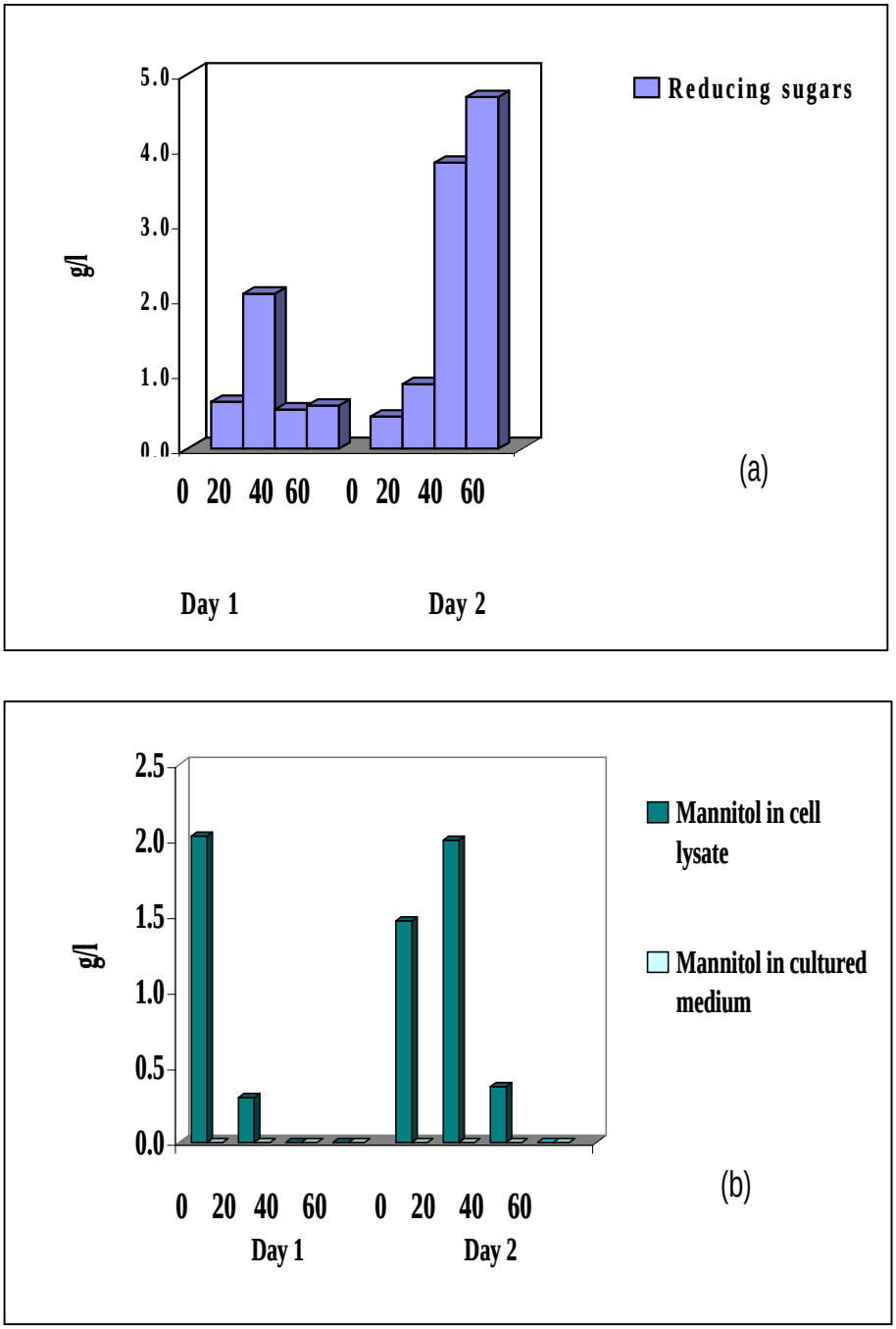
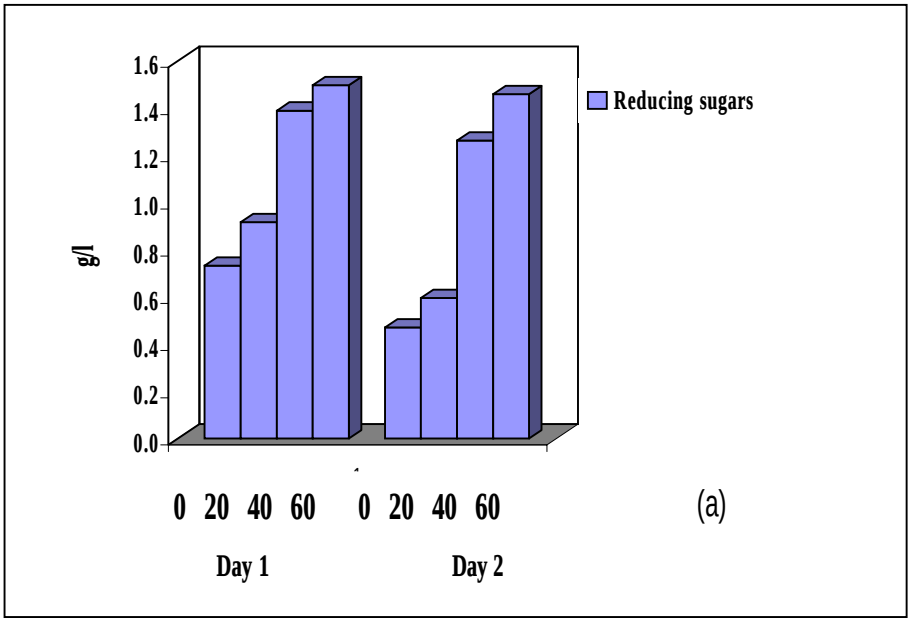


Figure 57. Effects of various heat shock treatment conditions (0 min, 20 min, 40 min, and 60 min) on the production of (a) reducing sugars and (b) mannitol by yeast isolate KAY1 when cultured in the medium containing 2% Khao-klong starch for 2 days at 30°C.



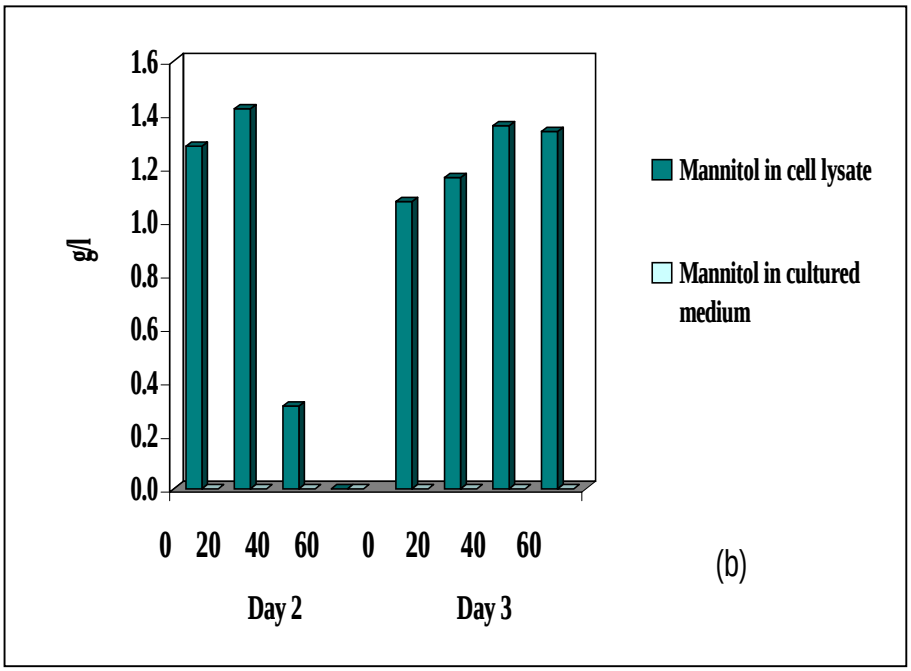


Figure 58. Effects of various heat shock treatment conditions (0 min, 20 min, 40 min, and 60 min) on the production of (a) reducing sugars and (b) mannitol by yeast isolate KAY1 when cultured in the medium containing 2% rice starch for 2 days at 30°C.

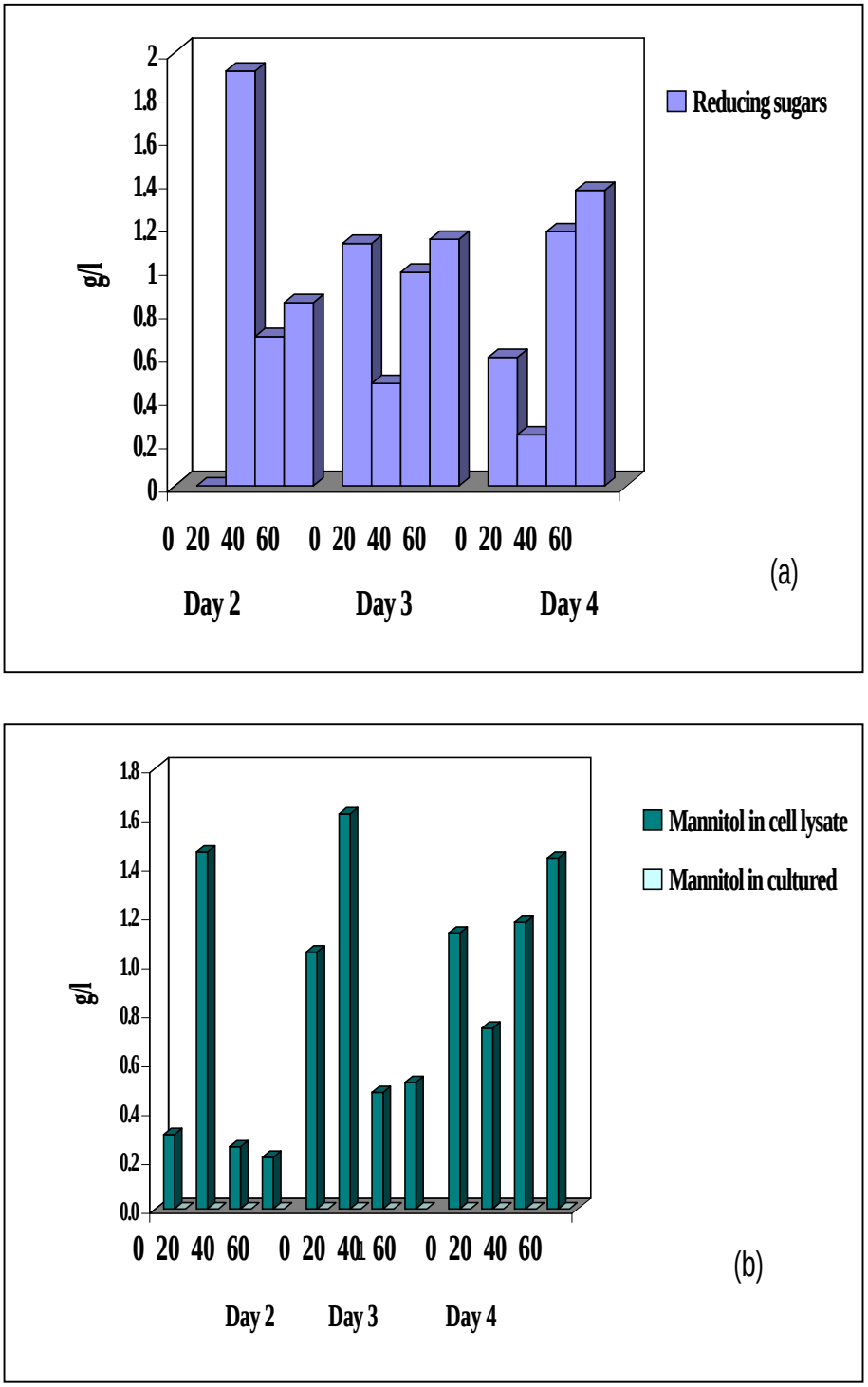


Figure 59. Effects of various heat shock treatment conditions (0 min, 20 min, 40 min, and 60 min) on the production of (a) reducing sugars and (b) mannitol by yeast isolate KAY1 when cultured in the medium containing 2% cassava starch for 2 days at 30°C.

best nitrogen source at concentration of 0.3% by using 2%, 4%, and 6% inoculum sizes for either rice starch, cassava, or Khao-klong starch medium, respectively. Heat shock treatment at 45^{o}C for 20 min was effective for sugar alcohol production. Starch fermentation was conducted in lab scale by using the process that non-controlled pH at 30^{o}C incubation. Figure 60, 61, and 62, showed the time courses of mannitol in cell lysate by heat shock treatment. Yeast isolate KAY1 was cultured in the medium containing 2% of either Khao-klong starch, rice starch, or cassava starch. Changes in reducing sugars, mannitol, pH, and residual starch were observed in the same pattern of the experiments obtained from the condition without heat shock treatment. The bioconversion was proceeded and the highest reducing sugars was obtained at approximately 1.7-2.2 g/l in the first day of incubation and completely utilized after 4 days of incubation time for either Khao-klong starch, rich starch, and cassava starch medium. Similarly, the mannitol production in yeast cell was directly increased correlate to reducing sugars. The maximum amounts of mannitol was obtained approximately 1.3-1.8 g/l in cell lysate at the first day of fermentation for either Khao-klong starch, rich starch, or cassava starch medium. The residual starch was observed correlate to reducing sugars and mannitol production, the residual starch was rapidly decreased from the initial values at 20 g/l to approximately 5-6 g/l at the second days of incubation. Especially, in 2% rice starch and 2% cassava starch medium, the residual starch was dropped into 6-8 g/l at the first day of incubation. pH levels was minimum at the first day, the second day, and the third day of cultivation of the either Khao-klong starch, rice starch, or cassava starch medium, respectively. Glycerol was detected in the cultured medium at the first day and rapidly decreased during the day of incubation.

This observation might explained that changes in reducing sugars, mannitol, residual starch, and pH of the either Khao-klong starch, rice starch, or cassava starch medium were observed in the same pattern. Surprisingly, in this study the rapid starch hydrolyzation and the high amounts of reducing sugars were shown at the first day of incubation. It was very interesting which might caused of the heat shock treatment power at 45^{o}C for 20 min. The previous study from other authors, The moderate heat shock treatment increases the NAD^{+}-dependent glycerol-3-phosphate dehydrogenase (GPDH, EC 1.1.1.8) activity in brewing yeast (*S. cerevisiae*) cells, consequently, brewing yeast cells produced a large amounts of glycerol (Omori *et al.*, 1996). Although heat shock treatment was tested in this experiment, glycerol could detected a little amounts and appeared at the first day. It could be

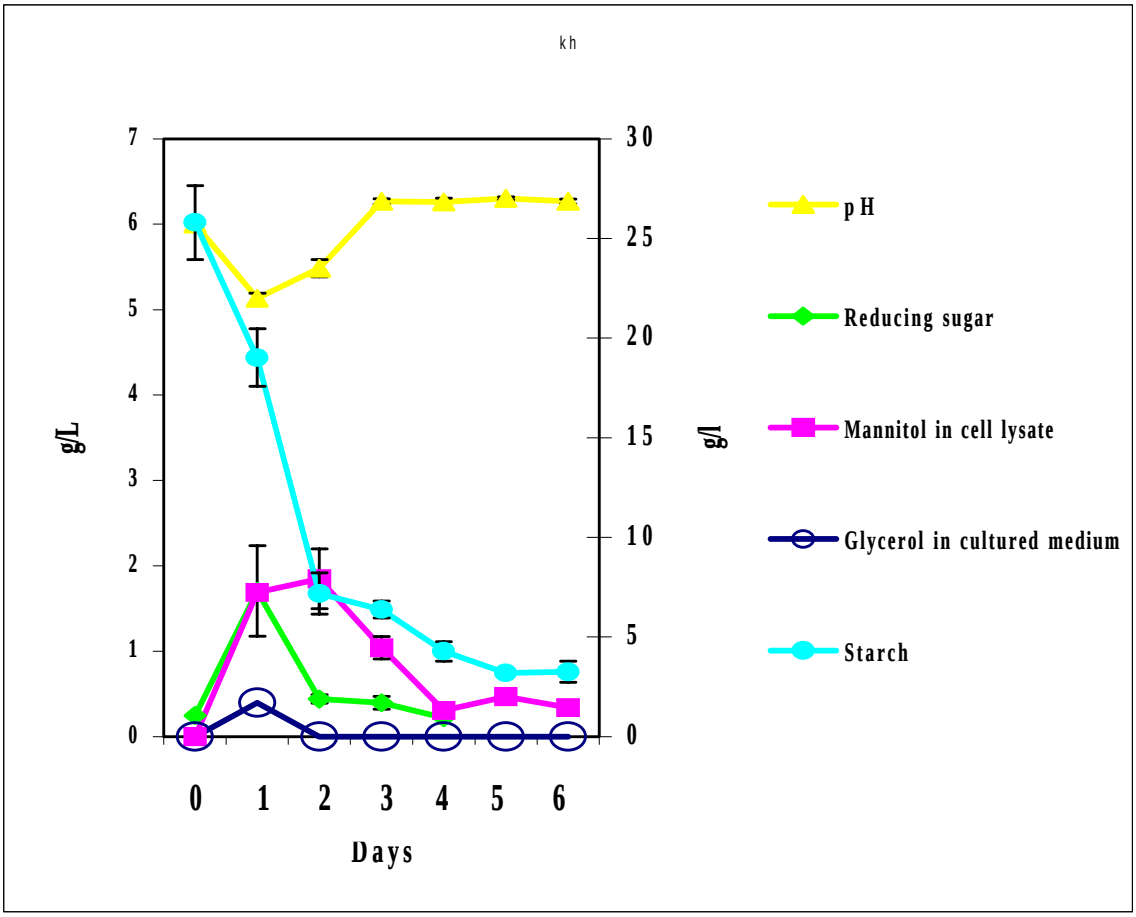


Figure 60. Time courses of mannitol production in cell lysate by heat shock treatment of yeast isolate KAY1 when cultured in the medium containing 2% Khao-klong starch.

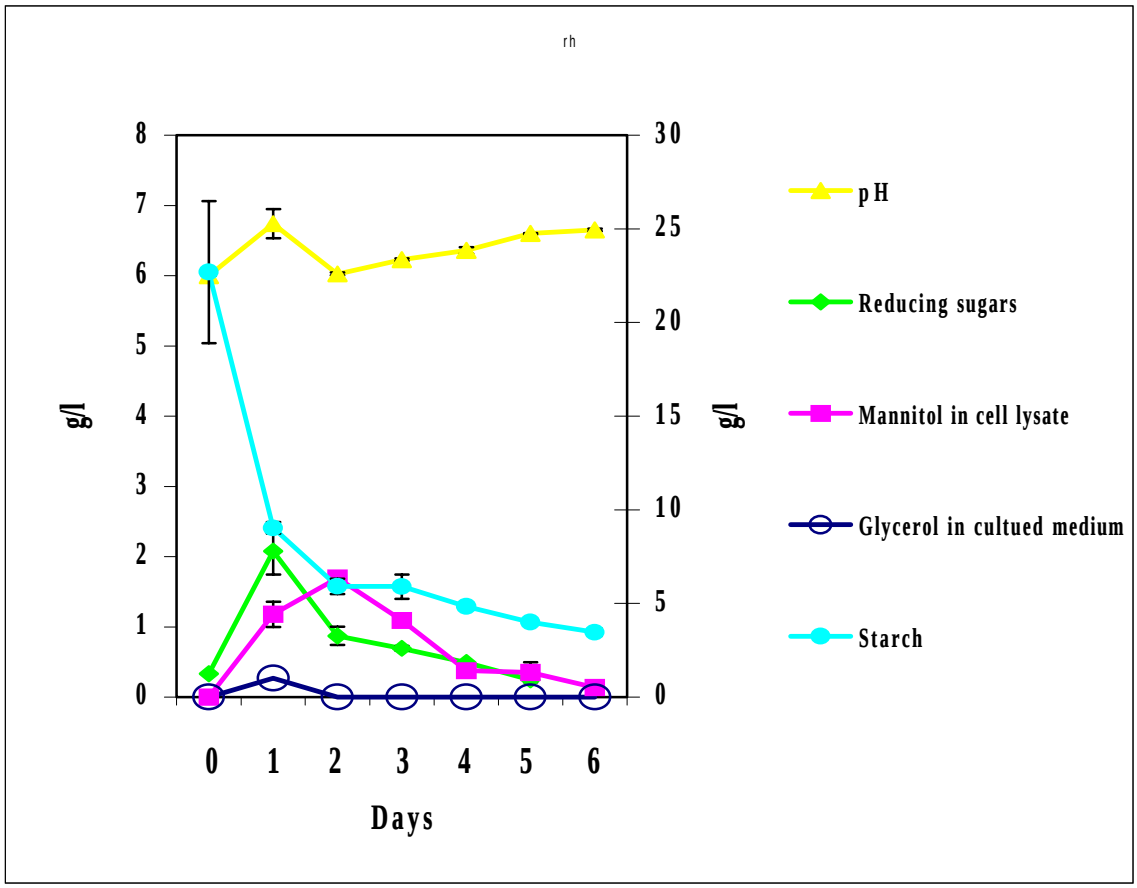


Figure 61. Time courses of mannitol production in cell lysate by heat shock treatment of yeast isolate KAY1 when cultured in the medium containing 2% rice starch.

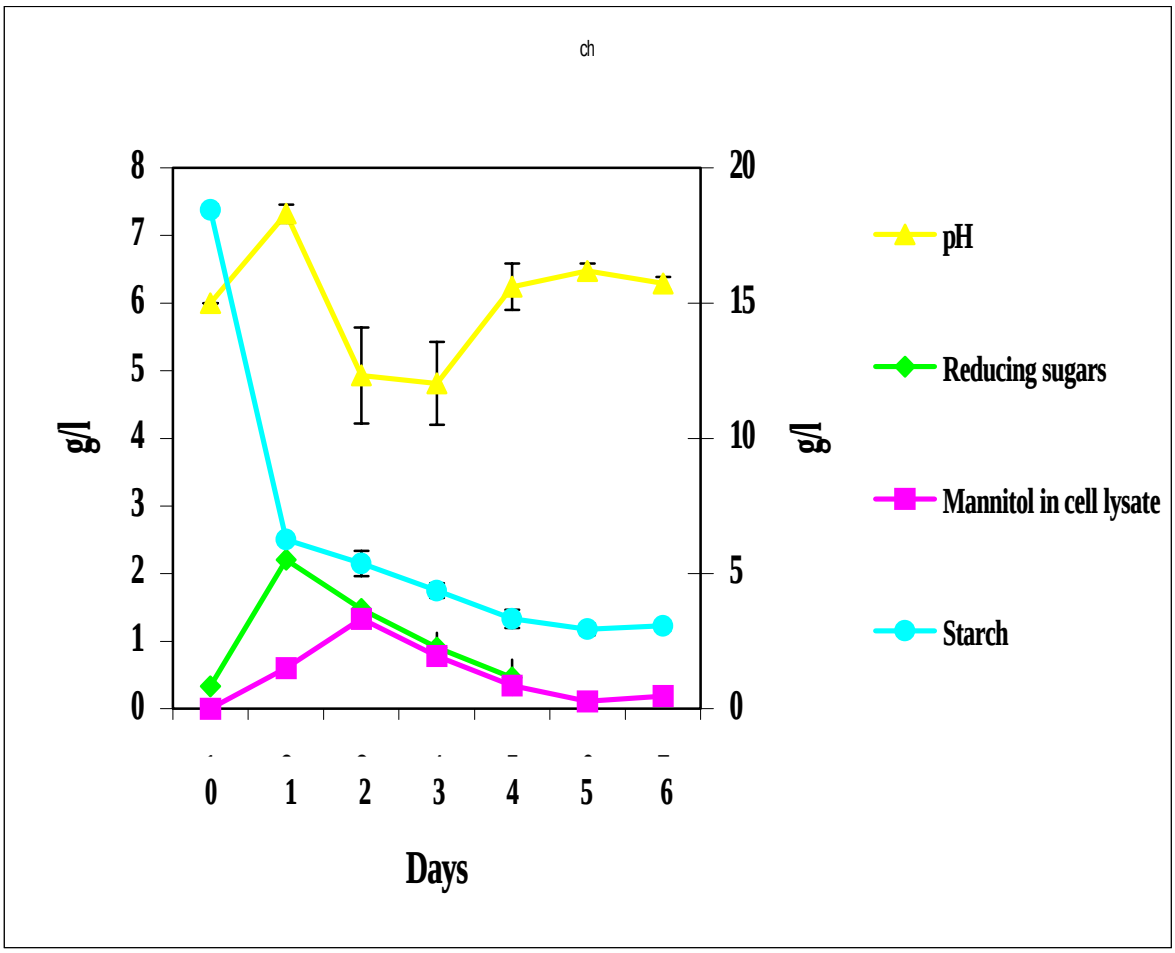


Figure 62. Time courses of mannitol production in cell lysate by heat shock treatment of yeast isolate KAY1 when cultured in the medium containing 2% cassava starch.

explained that the correlation between growth rate and stress sensitivity such that cells growing quickly in a glucose-rich medium are more sensitive to heat (Walker, 1998). Glycerol acted as the compatible solutes in high osmotic pressure condition such as high salt concentration and high sugar concentration. A little amounts of reducing sugars in this experiment was not enough to enhanced the high glycerol production by yeast. Interestingly, the starch was vigorously utilized, it might correlated to the amylase enzyme that released to catch with substrate. Thus, from this study is necessary for further experiment.

Tabel 14. Production of α-amylase, β-amylase, and glucoamylase activity by the yeast isolate KAY1.

Day	Condition	Tatal Protein (mg)	Alpha amylase		Beta amylase		Glucoamylase	
			Total act. (Units)	Specific act. (Units/mg)	Total act. (Units)	Specific act. (Units/mg)	Total act. (Units)	Specific act. (Units/mg)
1	**Heat shock**	0.4516	44.70	98.98	64.51	142.84	123.84	274.22
	Control	0.4004	$46.00^{1.02}$	$114.88^{1.16}$	$91.20^{1.41}$	$227.77^{1.59}$	$131.58^{1.06}$	$328.62^{1.19}$
2	**Heat shock**	0.4496	$88.62^{1.28}$	$197.10^{1.15}$	$130.80^{1.29}$	$290.92^{1.16}$	$204.01^{1.18}$	$453.75^{1.06}$
	Control	0.4029	68.80	170.76	100.67	249.86	171.94	426.75

Soluble starch medium (1% soluble starch, 0.3% yeast extract, 0.05% $MgSo_4 7H_2O$, and 0.1% KH_2PO_4) inoculated with 2% (v/v) of precultured yeast cells (heat shock treatment) (approximately 5.3×10^7) and yeast cells (wihtout heat shock treatment) (1.4×10^8).

U = 1 unit of α, β amylase defined as 1 μmol of maltose that liberated at 30°C pH 6.9 and 4.8 respectively.

U = 1 unit of glucoamylase defined as 1 μmol of glucose that liberated at 30°C pH 4.5.

Yeast isolate KAY1 performed the ability to produced α-amylase, β-amylase and glucoamylase (Table 14). At the first day, the total activity of amylase from without heat shocked cells (control) increased 1.02-to 1.41 fold as compared with that the heat shocked cells and the specific activity of amylase from without heat shocked was higher than the heat shocked cells. In contrast, at the second day the total activity of amylase from heat shocked cells increased 1.18-to 1.29 fold as compared with the control and the specific activity of amylase was also higher than the control. From the results showed that the large amounts of amylase enzyme was produced at the second day. These results compared with the time courses of heat shock treatment (Figure 60, 61 and 62) could explained that the residual starch rapidly decreased at the first day and the second day was

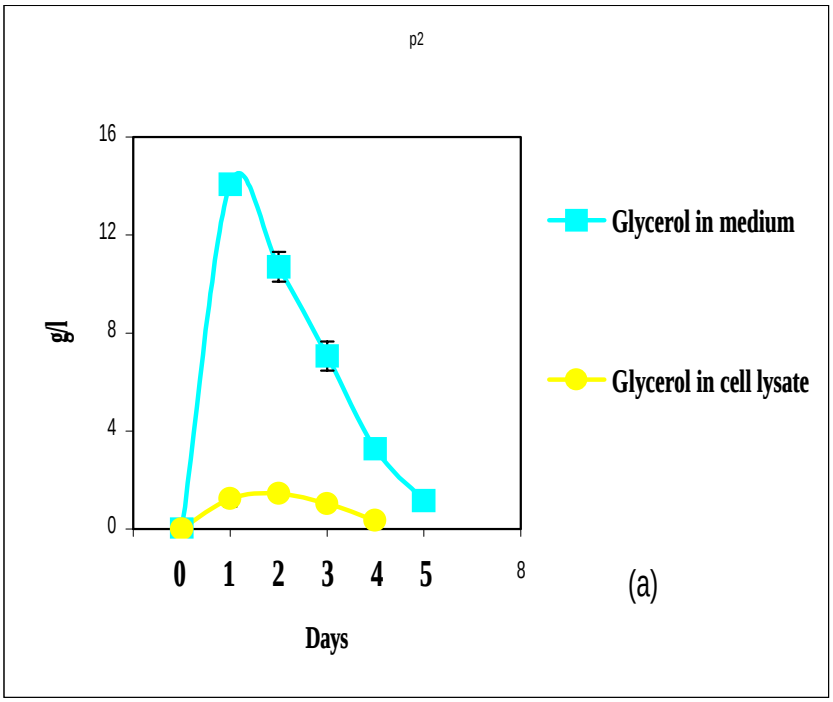
effected by heat shock power which stimulated the yeast cell constructed the high amounts of proteins inside the cell. The amylase enzyme was largely produced to catch the substrate and converted starch into the high amounts of reducing sugars. After that the yeast cells were absorbed reducing sugars into the cell. Consequently, the sugar concentration in the medium stimulated the construction of mannitol in the yeast cell. Because high initial starch concentrations were employed for an economical process, amylase production from yeast should be high to hydrolyzation of the substrate. The results suggested that the heat shock treatment might be an effective and simple method for improving the starch hydrolyzation.

3.8 Cultivation of rhizobia using nutrient sources produced by yeast

The slow-growing *Bradyrhizobium japonicum* USDA 110, and *Bradyrhizobium* spp. THA5, which isolated in Thailand were used in this experiment. The two selected glycerol-producing yeast isolates: PIY2, and PUY4 were able to grow on sucrose as a sole carbon source, converted the sucrose to glycerol. The selected yeast isolate KAY1 was used to change the starch to glycerol, and mannitol. The standard medium for rhizobia cultivation such as G5 and Yeast-mannitol (YM) broth were depicted in Table 15.

3.8.1 Cultivation of *Bradyrhizobium* using medium containing glycerol produced by yeasts

In view of the earlier reports of high glycerol yields by osmophillic yeasts (Spencer and Sallans, 1956; Spencer and Spencer, 1978). A number of *Zygosaccharomyces rouxii* and *Z. bailii* strains were first tested for glycerol production. However, it was found that most of these osmophillic strains were unable to metabolized sucrose (Lie *et al*., 1991). Fortunately, the yeast isolate PIY2, and PUY4 were screened for their ability to produced glycerol by glucose and also produced glycerol by using sucrose as sole source of carbon. Especially, sucrose was cheap and locally available substrate similar to a starch. *Bradyrhizobium*, The symbiont of many tropical legumes, including soybean, cannot use sucrose (Graham, 1964; Elkan and Kwik, 1968) because it lacks invertase, required for hydrolysing the disaccharide sucrose (Martinez-de Drets and Arias, 1972). For fast-growing *Rhizobium* and slow-growing *Bradyrhizobium*, glycerol could supported a very high bacterial density (Ballatti, 1982; Ballatti *et al*., 1987). In this experiment, in the first stage, sucrose was utilized by the



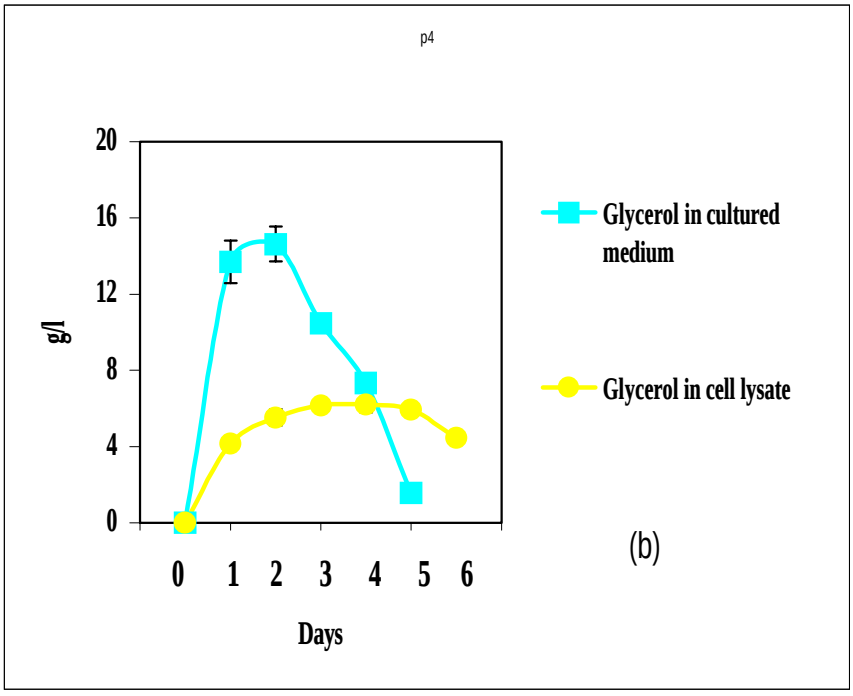


Figure 63. Glycerol production in cultured medium and cell lysates of (a) Isolate PIY2 and (b) Isolate PUY4. The fermentation were performed using YEPD medium containing 5% sucrose for yeast cultivation.

Table 15. The comparison of media for *Bradyrhizobium* cultivation.

	2%starch	5%Sucrose	YMB	G5
Mannitol	-	-	5.0 g	1.0 g
Glycerol	-	-	-	4.0 ml
Yeast extract	3.0 g	10.0 g	0.5 g	1.0 g
$MgSO_4.7H_2O$	0.5 g	-	0.2 g	0.2 g
K_2HPO_4	-	-	0.5 g	0.5 g
KH_2PO_4	1.0 g	-	-	-
NaCl	-	-	0.1 g	0.1 g
Glucose	-	-	-	1.0 g
Arabinose	-	-	-	0.5 g
Tryptone	-	20.0 g	-	-
Sucrose	-	50.0 g	-	-
PVP-40	-	-	-	20.0 g
Fe- EDTA	-	-	-	200 μM (0.073 g/l)
Starch	20.0 g	-	-	-
Distilled water	1000 ml	1000 ml	1000 ml	1000 ml
pH	6.0	5.5	6.8	6.8

ability of yeast to produced glycerol. The cultured medium containing glycerol, can then be used directly for the cultivation of *Bradyrhizobium* (second stage).

In a typical experiment (Figure 63 (a) and (b)) using 5% sucrose, The 5% sucrose fermented by yeast isolate PIY2 gave the maximum yield of glycerol at the first day of cultivation at 14 g/l in cultured medium. However, The yeast isolate PUY4 gave the same level at the second day of cultivation. Relatively, the glycerol in cell lysate were highest at the second day and the third days of fermentation from yeast isolate PIY2 and PUY4, respectively. The yields of glycerol at the second days of fermentation was selected to cultured *Bradyrhizobium*. Before the cultivation, The cultured medium might adjusted. In a representative experiment the ability of *Bradyrhizobium* to grow on fermented sucrose medium was tested. In view of the high amounts of glycerol, the medium was

diluted to provide 0.5% (v/v) glycerol as same as the standard rhizobia culture medium. As depicted at Table 16. G5 medium used as the standard glycerol medium in this experiment. The medium containg 5% sucrose fermented by yeast-isolate PIY2 and PUY4 were adjusted. First steps, diluted to 0.5% (v/v) as same as G5. The amounts of glycerol in adjusted cultured medium was tested by using High Performance Liquid Chromatography. Second steps, added the other substances as same as G5 were 0.1% mannitol, 0.02% $MgSO_4$, $7H_2O$, 0.05% K_2HPO_4, 0.01% NaCl, 0.1% Glucose, 0.05% Arabinose, 200 µM Fe-EDTA, and pH 6.8 at room temperature. Except glycerol and yeast extract which much more for support the bacterial cell in the cultured medium. After heat sterilization, the adjusted medium was inoculated with two strains: *Bradyrhizobium japonicum* USDA 110 and *Bradyrhizobium* spp. THA 5.

Table 16. The comparison of media containing glycerol for *Bradyrhizobium* cultivation**.**

	5%Sucrose	**G5**	**PIY2 medium**	**PUY4 medium**
Mannitol	-	1.0 g	1.0 g	1.0 g
Glycerol	-	4.0 ml	6.0 g	6.0 g
Yeast extract	10.0 g	1.0 g	-	-
$MgSO_4.7H_2O$	-	0.2 g	0.2 g[a]	0.2 g[a]
K_2HPO_4	-	0.5 g	0.5 g[a]	0.5 g[a]
KH_2PO_4	-	-	-	-
NaCl	-	0.1 g	0.1 g[a]	0.1 g[a]
Glucose	-	1.0 g	1.0 g[a]	1.0 g[a]
Arabinose	-	0.5 g	0.5 g[a]	0.5 g[a]
Tryptone	20.0 g	-	-	-
Sucrose	50.0 g	-	-	-
PVP-40	-	20.0 g	-[b]	-[b]
Fe- EDTA	-	200 µM (0.073 g/l)	200 µM (0.073 g/l)	200 µM (0.073 g/l)
Distilled water	1000 ml	1000 ml	1000 ml	1000 ml
pH	5.5	6.8	6.8	6.8

Note that [a] same as standard medium [b] no add

Table 17. Growth of *Bradyrhizobium* on media containing glycerol**.**

	***Bradyrhizobium japonicum* USDA 110**				***Bradyrhizobium* spp.THA5**			
Media	**0 day**	**2 day**	**4 day**	**6 day**	**0 day**	**2 day**	**4 day**	**6 day**
Control	$1.0x10^7$	$1.1x10^7$	$9.0x10^6$	$6.4x10^6$	$1.0x10^7$	$1.1x10^7$	$8.7x10^6$	$8.0x10^6$
G5 (100 ml)	$1.0x10^7$	$7.5x10^8$	$1.9x10^9$	$3.4x10^9$	$1.0x10^7$	$5.3x10^8$	$2.2x10^9$	$5.7x10^9$
G5 (500 ml))	$1.0x10^7$	$8.5x10^8$	$1.4x10^9$	$4.0x10^9$	$1.0x10^7$	$5.9x10^8$	$1.4x10^9$	$6.2x10^9$
YEPD containing 5% sucrose prefermented by isolate PIY2	$1.0x10^7$	$7.0x10^5$	$1.0x10^6$	$6.1x10^6$	$1.0x10^7$	$2.5x10^8$	$2.1x10^9$	$1.0x10^{10}$
YEPD containing 5% sucrose prefermented by isolate PUY4	$1.0x10^7$	$4.4x10^7$	$2.2x10^8$	$3.7x10^8$	$1.0x10^7$	$6.5x10^8$	$1.7x10^9$	$5.0x10^9$

From the Table 17, *B. japonicum* USDA 110 could not grow well in PIY2 and PUY4 cultured medium when compared with G5 medium. In PIY2 cultured medium, rhizobial cells decreased in the second days and slowly increased during the day of cultivation which gave the amounts of cell at 10^6 cell/ml at the sixth day of cultivation. PUY4 cultured medium gave the higher cell at 10^8 cells/ml at the sixth day of cultivation. In contrast, the amounts of cells from G5 medium were 10^9 cells/ml at the forth day and the sixth day of cultivation. The quantitation of G5 medium in the flask was not effect to the bacterial cell growth when compared. For the cultivation of *Bradyrhizobium* spp. THA5. Interestingly, clearly showed that the PIY2 and PUY4 adjusted cultured medium could support the growth of *Bradyrhizobium* spp. THA5. The best results was obtained with the final number of *Bradyrhizobium* cells being almost the same as that found with G5 medium. Especially, PIY2 cultured medium gave high cell density at 10^{10} cells/ml at the sixth days of cultivation. PUY4 cultured medium gave the amounts of cell number similar to G5 medium.

The results presented here clearly demonstrated that sucrose, preferment by yeast, can be used for the cultivation of *Bradyrhizobium*, in particulary, *Bradyrhizobium* spp. THA5. However, PIY2 cultured medium could not enhance the growth of *Bradyrhizobium japunium* USDA 11O, it

might conclude PUY4 cultured medium showed the same results, but it gave the amount of bacterial cell at 10^8 cells/ml when compared with G5 medium.

It might cause of the amounts of ethanol in cultured medium. Lie *et al*., (1991) Reported that no inhibitory effect of 5 g/l ethanol in a glycerol-yeast extract medium were observed. However, growth of *Bradyrhizobium* CB 756 on pure ethanol (0.1, 0.5, 1 and 2.5 g/l) was very poor. The amounts of ethanol in this experiment was not measured or controlled. But the smells of ethanol were observed in the 5% sucrose prefermented by yeast. It was suggested that the amount of ethanol in the medium may effected to the growth of *Bradyrhizobium japonicum* USDA 110, but not effectd for *Bradyrhizobium* spp. THA5 growth.

3.8.2 Cultivation of *Bradyrhizobium* using medium containing mannitol produced by yeasts

A total 6000 ml of starch medium (2000 ml of Khao-klong starch medium + 2000 ml of rice starch medium + 2000 ml of cassava starch medium) were utilized by yeast isolate KAY1. The cultivation product used for the rhizobial carbon sources was mannitol which produced in the yeast cells. After centrifugation, the supernatant was discarded. The yeast cells grown from the three starch cultured medium were pooled into one flask. Cell were lysed by using 4 cycles of freeze-thawing. The initial high mannitol concentration in cell lysate was adjusted by the addition of distilled water. The cell lysate medium was adjusted to provide 0.5% (v/v) as same as YMB standard rhizobial culture medium. The quantitation of mannitol were tested by using High-Performance Liquid Chromatography (HPLC). And adjusted again as depicted in Table 18.

From the Table 18, The cell lysate medium was adjusted. Other substances: 0.05% K_2HPO_4 and 0.01% NaCl were added, and pH adjusted 6.8 at room temperature. After heat sterilization, the medium was inoculated with *B. japonicum* USDA 110 and *Bradyrhizobium* spp. THA5. The results in Table 19, it clearly showed that the cell lysate medium containing mannitol could supported the growth of *B. japonicum* USDA 110 and *Bradyrhizobium* spp. THA5. The best results was obtained with 10^8 cells/ml being almost the same as that found with YMB medium. Mannitol was the traditional carbon and energy source used for small volume cultivation of all

Rhizobium (Stowers, 1985; Elkan, 1987). The 5% inoculum size of 6 days cultured was used in this experiment.

The recommended inoculum level was between 1 and 10%; however, a higher level of inoculum will decrease the time in a given fermentor and was an advantage with the slow-growing rhizobia because it decreased the opportunity for contamination (Elkan, 1987; Vincent, 1982) To check the contamination in the cultured medium, beside to observed the appearance of colony during

Table 18. The comparison of media containing mannitol for *Bradyrhizobium* cultivation**.**

	2%starch medium	**YMB medium**	**KAY1-medium**
Mannitol	-	5.0 g	6.0 g
Glycerol	-	-	0.8 g
Yeast extract	3.0 g	0.5 g	-[b]
$MgSO_4.7H_2O$	0.5 g	0.2 g	-[b]
K_2HPO_4	-	0.5 g	0.5 g[a]
KH_2PO_4	1.0 g	-	-[b]
NaCl	-	0.1 g	0.1 g[a]
Glucose	-	-	-
Arabinose	-	-	-
Tryptone	-	-	-
Sucrose	-	-	-
PVP-40	-	-	-
Fe- EDTA	-	-	-
Starch	20.0 g	-	-
Distilled water	1000 ml	1000 ml	500 ml
pH	6.0	6.8	6.8

Note that [a] same as standard medium

[b] no add

the time incubation on Yeast-mannitol agar at 5-7 days range, the correct method was used. According to Somasegaran and Hoben (1994), bradyrhizobia exhibited these characteristics as follows: rod shape cell morphology and cells accumulate poly-β-hydroxybutyrate (PHB) intracellularly when observed by carbol fuchsin staining; discrete, round colonies varying from flat to domed and even conical shapes on agar surfaces; the color of colonies might be white-opaque, milky to watery-translucent or pink on media containing congo red; colony growth was usually firm with little gum; colonies might be glistening or dull, evenly opaque or translucent, but many colonies develop darker centers of rib-like marking; colony size was about 1 mm; colonies generally did not absorbed congo red whereas the other organisms usually absorbed the red dye.

Table 19. Growth of *Bradyrhizobium* on media containing mannitol.

	***Bradyrhizobium japonicum* USDA 110**				***Bradyrhizobium* spp.THA5**			
Media	**0 day**	**2 day**	**4 day**	**6 day**	**0 day**	**2 day**	**4 day**	**6 day**
Control	$1.0x10^7$	$9.8x10^6$	$6.6x10^6$	$4.4x10^6$	$1.0x10^7$	$9.2x10^6$	$7.5x10^6$	$6.2x10^6$
Yeast-mannitol broth (YMB)	$1.0x10^7$	$1.3x10^7$	$4.6x10^8$	$9.3x10^8$	$1.0x10^7$	$1.3x10^7$	$5.5x10^8$	$1.3x10^9$
2% starch medium prefermented by isolate KAY1	$1.0x10^7$	$1.1x10^7$	$3.5x10^8$	$6.0x10^8$	$1.0x10^7$	$1.1x10^7$	$2.2x10^8$	$9.2x10^8$

The results shown clearly demonstrated that mannitol from the cell lysate of starch-utilizing and sugar alcohol-producing isolate KAY1, could be used for the cultivation of *Bradyrhizobium*. Although, the yeast extract not added into the cell lysate medium, but in the disrupted yeast cell has so many nutrients similar to yeast extracts. Walker (1998) reported that yeasts are rich sources of proteins, nucleic acids, Vitamins, and minerals. These experiments also showed that yeast cells, produced during the utilization of starch, could be used as a replacement for yeast extract in the media for the cultivation of *Bradyrhizobium*.

3.9 Identification of yeasts

From table 20, showed physiological and biochemical properties primarily serve to describe and identify yeast genera and species. The tests most used for routine identification purposes were fermentation of and growth on carbon sources (Figure 64), hydrolysis of urea (Figure 65), growth on nitrogen sources (Figure 66), and growth at various temperatures. There was not a simple standardized method for many of these tests. The results of such test were frequently dependent on the techniques employed, therefore which ever procedure was chosen.

3.9.1 Fermentation and assimilation of carbon compound

Some physiological properties were determined for yeast isolate strains Y69, KAY1, PIY2, PUY4, COY1, LIY2, and FAY2. Type strain *Kluyveromyces marxianus* TISTR 5270 were used as the positive control.

Isolate Y69 showed, glucose, maltose, and sucrose were fermented; cellobiose was very slowly fermented. Negative assimilations were observed for L-rhamnose, L-arabinose, trehalose, salicin, melibiose, melezitose, D-mannitol and D-gluconate. Weak or negative assimilations were detected with D-xylose, cellobiose, and lactose. Positive assimilatitions were observed for D-glucose, D-galactose, sucrose, maltose, raffinose, and starch.

Isolate KAY1, showed negative fermentations. The following carbon compounds were assimilated: glucose, D-xylose, L-arabinose, sucrose, maltose, raffinose, and melezitose. D-ribose, trehalose, and melibiose were weakly assimilated. Negative assimilations were observed for D-galactose, L-rhamnose, cellobiose, salicin, lactose, D-mannitol, and D-gluconate.

Isolate PIY2 showed raffinose was fermented and trehalose was weakly or negative fermented. Negative fermentations were observed for cellobiose, salicin, melibiose, lactose, melezitose, starch, D-mannitol, and raffinose. D-ribose and trehalose were weakly or negative assimilated. Negative assimilations were observed for D-galactose, D-xylose, L-arabinose, L-rhamnose, maltose, cellobiose, salicin, melibiose, lactose, melezitose, starch, D-mannitol, and D-gluconate.

Isolate PUY4 showed the positive fermentations of D-glucose, sucrose, trehalose, melibiose, and lactose. D-galactose, cellobiose, and raffinose were weakly and slowly fermented. Maltose was weakly or negative fermented. Maltose was weakly or negative fermented. Positive

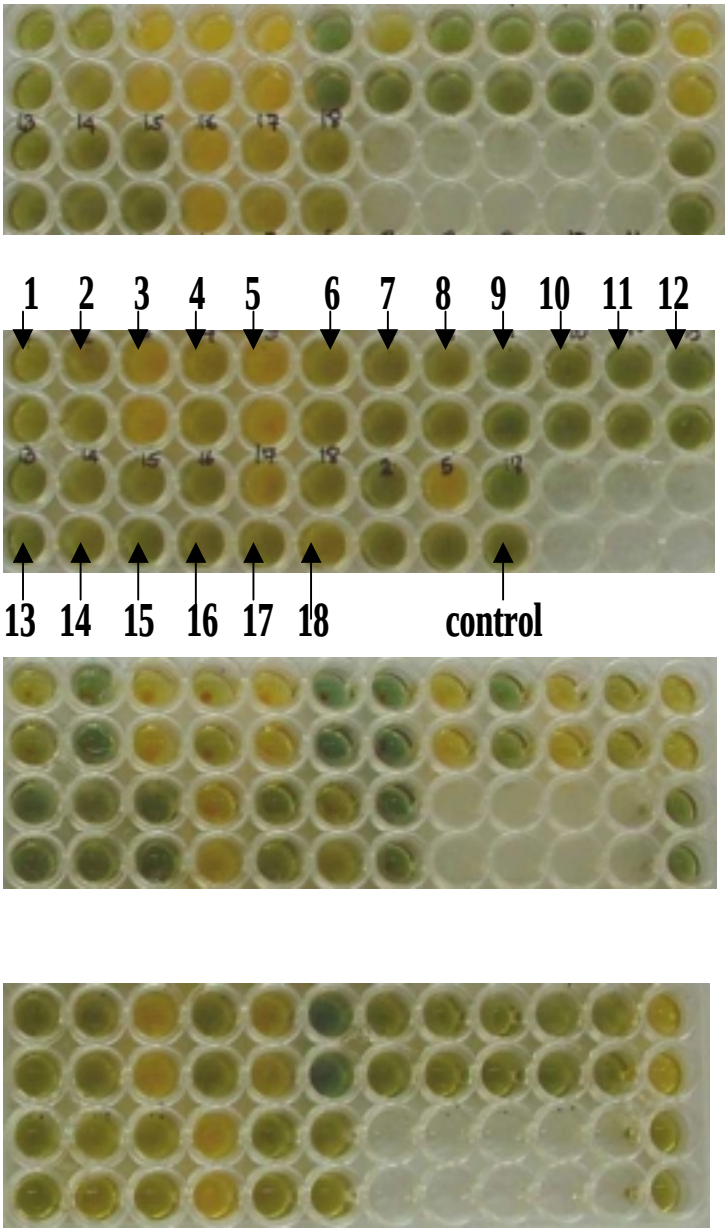


Figure 64. Carbon assimilation of yeast isolates (a) *Kluyveromyces marxianus*, (b) KAY1, (c) PIY2, and (d) PUY4. The tests were applied in ELISA plate. Each well of a set containing the various carbon sources in a basal medium was inoculated with one drop of a suspension of cells from a young active growing culture. Wells: 1, L-arabinose; 2, Cellobiose; 3, Fructose; 4, D-galactose; 5, D-glucose; 6, D-gluconate; 7, Lactose; 8, Maltose; 9, D-mannitol; 10, Melezitose; 11, Melibiose; 12, Raffinose; 13, L-rhamnose; 14, D-ribose; 15, Salicin; 16, Sucrose; 17, α,α-trehalose; and18, D-xylose.

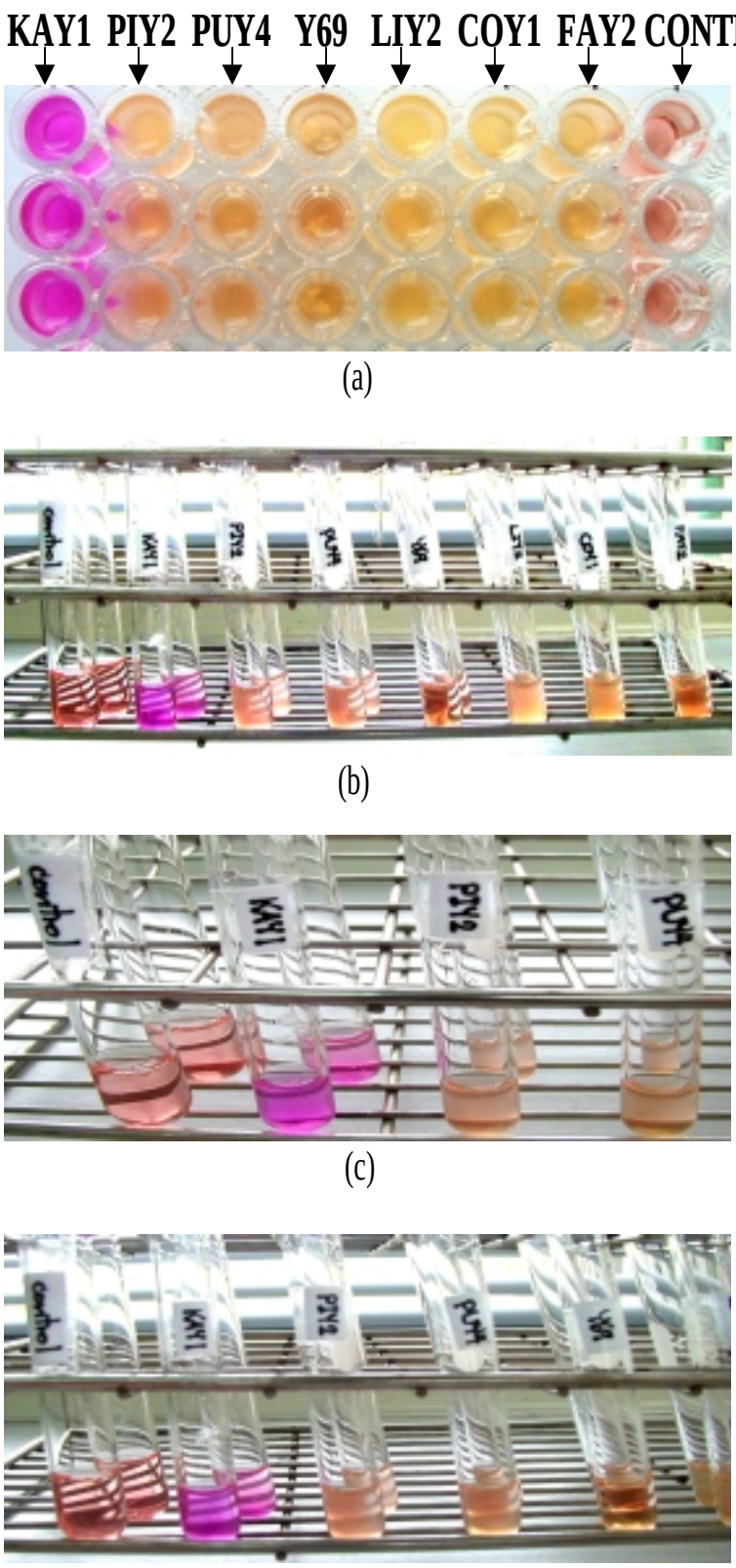
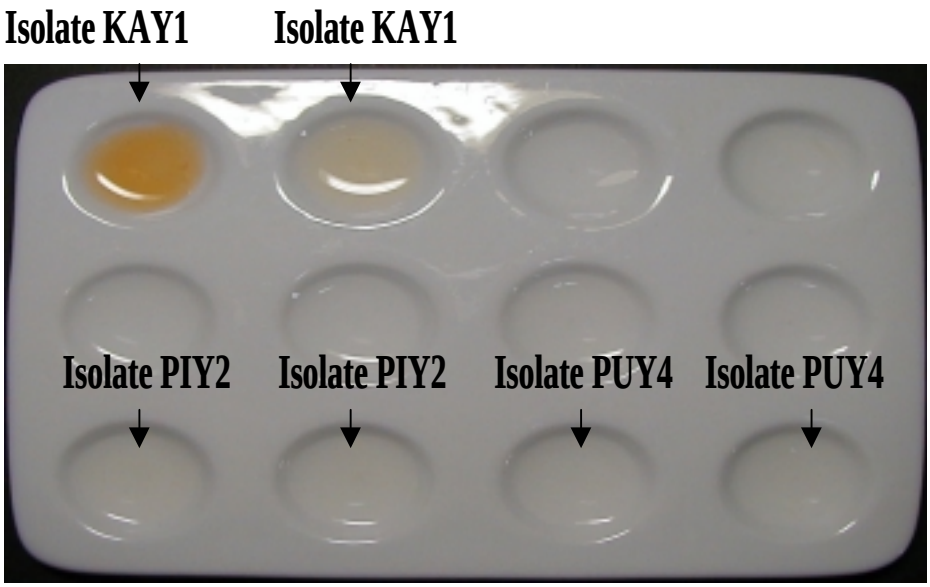
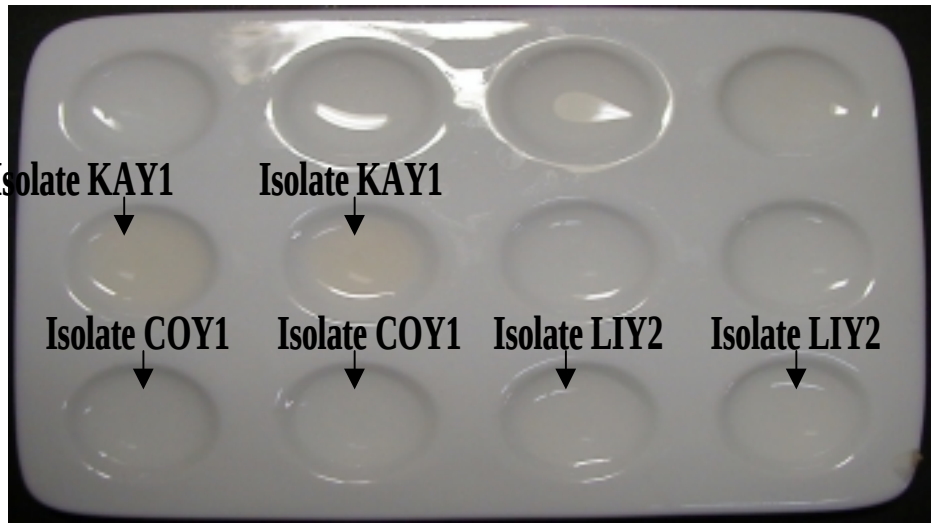


Figure 65. Urea hydrolysis test. (a) Various yeast isolates to be tested were inoculated with a needle on to a plate of a Urea R broth. (b), (c), and (d) The tests were examined every half for a change of color to red, which indicated hydrolysis of urea.



(a)



(b)

Figure 66. Nitrate assimilation of yeast isolates (a) KAY1, PIY2, and PUY4 and (b) KAY1, COY1, and LIY2. A few drops of reagent 1 and reagent 2 were added to test the culture. The development of a distinct pink or red color indicated the presence of nitrate produced as a result of the partial utilization of nitrate.

assimilations were observed for D-glucose, D-galactose, sucrose, trehalose, cellobiose, and raffinose. Weakly or negative assimilations were detected with D-xylose and maltose. Negative assimilations were D-ribose, L-arabinose, L-rhamnose, salicin, melibiose, lactose, melezitose, starch, D-mannitol, and D-gluconate.

Isolate LIY2, D-glucose was fermented and maltose was very weakly fermented. Positive assimilations was D-glucose and weak assimilations were detected with D-galactose, D-ribose, L-arabinose and cellobiose. Negative assimilations were observed for D-xylose, L-rhamnose, sucrose, maltose, trehalose, salicin, melibiose, lactose, raffinose, melezitose, D-mannitol and D-gluconate.

Isolate COY1, positive and weakly fermentations were observed for D-glucose. Assimilations of D-glucose, D-xylose, sucrose, maltose, salicin, melezitose, and D-mannitol were positive. Weak assimilations were detected with D-ribose, and L-rhamnose. D-galactose, L-arabinose, trehalose, cellobiose, melibiose, raffinose, starch, and D-gluconate were negative assimilation.

Isolate FAY2 showed D-glucose was weakly fermented. Weakly or negative fermentations were maltose and sucrose. Fermentations of D-galactose, trehalose, melibiose, lactose, cellobiose, raffinose, and D-xylose were negative. The following carbon compounds were assimilated: D-glucose, D-ribose, D-xylose, and trehalose. Sucrose and salicin were weakly or negative assimilated. Negative assimilations were observed for D-galactose, L-arabinose, L-rhamnose, maltose, cellobiose, melibiose, lactose, raffinose, melezitose, starch, D-mannitol, and D-gluconate.

There appear to be no exceptions to the rule that when a yeast strain ferments a carbohydrate it was also able to grow on it. However, the reverse does not hold true: many yeast grow aerobically on sugars they could not fermented. It was essential that only pure, high-grade carbohydrates were used in the preparation of the media used in these tests (Kurtzman *et al*., 1998). Yeasts vary in their ability to fermented sugars as measured by the production of carbon dioxide. Yeasts of the genera *Kluyveromyces*, *Saccharomyces*, and *Zygosaccharomyces* for example, ferment, at least, glucose vigorously, whereas others, such as *Rhodosporidium* and *Sterigmatomyces*, did not noticeably ferment any sugars. Species ranging from non-fermentative to strongly fermentative were found in other genera.

Table 20. Physiological and biochemical characteristics of yeast isolates.

			Yeast isolates								Data from Kurtzman *et al.* (1998)							
15. *Pichia kluyverri* var. *eremophila*	16. *Pichia membranifaciens*	Yeast strains / Methods	1. *Kluyveromyces maxianus* TISTR 5270 (control)	2. Y69	3. KAY1	4. PIY2	5. PUY4	6. LIY2	7. COY1	8. FAY2	9. *Kluyveromyces maxianus*	10. *Saccharomycopsis fibuligera*	11. *Rhodotorula glutinis*	12. *Zygosaccharomyces bailii*	13. *Candida guilliermondii*	14. *Pichia cactophilla*	17. *Pichia toletana*	18. *Pichia pastoris*
		Fermentation (C-source)																
+	w/-	**D-glucose**	+	+/w	-	+	+	+/w	+/w	+/w	+	+/w	-	+	+	w/-	ws	+
-	-	**D-galactose**	+	-	-	-	ws	-	-	-	+	-	-	-	+/w	-	-	-
-	-	**Maltose**	-	+	-	-	w/-	w/-	-	w/-	-	+/w	-	-	w/-	-	-	-
-	-	**Sucrose**	+	+	-	s	+	-	-	w/-	+	+/w	-	V	+	-	-	-
-	-	**α, α-trehalose**	-	-	-	-	+	-	-	-	-	-	-	w/-	w/-	-	-	-
N	N	**Melibiose**	-	-	-	-	+	-	-	-	N	N	N	N	N	N	N	N
-	-	**Lactose**	-	-	-	-	+	-	-	-	V	-	-	-	-	-	-	-
N	N	**Cellobiose**	-	s	-	-	ws	-	-	-	N	N	N	N	N	N	N	N
-	-	**Raffinose**	-	-	-	-	ws	-	-	-	+	w/-	-	-	+	-	-	-
N	N	**D-xylose**	-	-	-	-	-	-	-	-	N	N	N	-	N	N	N	N
		Assimilation (C- source)																
+	+	**D-glucose**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 20. (continued).

			Yeast isolates								Data from Kurtzman *et al.* (1998)							
15. *Pichia kluyerri* var. *eremophila*	16. *Pichia membranifaciens*	Yeast strains / Methods	1. *Kluyveramyces maxianus* TISTR 5270 (control)	2. Y69	3. KAY1	4. PIY2	5. PUY4	6. LIY2	7. COY1	8. FAY2	9. *Kluyveromyces maxianus*	10. *Saccharomycopsis fibuligera*	11. *Rhodotorula glutinis*	12. *Zygosaccharomyces bailii*	13. *Candida guiliermondii*	14. *Pichia cactophilla*	17. *Pichia toletana*	18. *Pichia pastoris*
-	-	D-galactose	+	+	-	-	+	w/-	-	-	+	-	V	V	+	-	-	-
-	-	D-ribose	w/-	-	w/-	w/-	-	w/-	w/-	+	-	-	V	-	V	-	-	-
V	V	D-xylose	w/-	w/-	+	-	w/-	-	+	+	+	-	V	-	-	+/w	+	V
-	-	L-arabinose	+	-	+	-	-	w/-	-	-	V	-	V	-	-	-	-	-
-	-	L-rhamnose	-	-	-	-	-	-	w/-	-	-	-	V	-	-	-	V	+
-	-	Sucrose	+	+	+	+	+	-	+	w/-	+	+	+	V	+	-	+	-
-	-	Maltose	-	+	+	-	w/-	-	+	-	-	+	+	-	+	-	+	-
		Assimilation (C-source)																
-	-	α, α-trehalose	-	-	w/-	w/-	+	-	-	+	V	V	+	+/w	+	-	+	+
-	-	Celllobiose	+	w/-	-	-	+	w/-	-	-	V	+	V	-	+	-	+	-
-	-	Salicin	w/-	-	-	-	-	-	+	w/-	V	+	+/w	-	+	-	+	-
-	-	Melibiose	-	-	w/-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	Lactose	+	w/-	-	-	-	-	-	-	v	-	-	-	-	-	-	-
-	-	Raffinose	+	+	+	+	+	-	-	-	+	V	V	-	+	-	-	-
-	-	Melezitose	-	-	+	-	-	-	+	-	-	V	+	-	-	-	V	-
-	-	Starch	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-

Table 20. (continued).

			Yeast isolates								Data from Kurtzman *et al.* (1998)							
15. *Pichia kluyerri* var. *eremophila*	16. *Pichia membranifaciens*	Yeast strains / Methods	1. *Kluyveramyces maxianus* TISTR 5270 (control)	2. Y69	3. KAY1	4. PIY2	5. PUY4	6. LIY2	7. COY1	8. FAY2	9. *Kluyveromyces maxianus*	10. *Saccharomycopsis fibuligera*	11. *Rhodotorula glutinis*	12. *Zygosaccharomyces bailii*	13. *Candida guiliermondii*	14. *Pichia cactophilla*	17. *Pichia toletana*	18. *Pichia pastoris*
-	-	D-mannitol	-	-	-	-	-	-	+	-	V	V	V	+	+	-	+	+
-	-	D-gluconate	-	-	-	-	-	-	-	-	-	+/w	+	-	V	-	V	-
		Assimilation (N-source)																
-	-	Nitrate	ND	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-
-	-	Urea hydrolysis	ND	-	+	-	-	-	-	-	N	N	+	-	-	-	-	-
		Other characteristics																
N	N	Growth at 25 °C	ND	+	+	+	+	+	+	+	N	N	N	N	N	N	N	N
N	N	Growth at 30 °C	ND	+	+	+	+	+	+	+	N	N	N	N	N	N	N	N
N	N	Growth at 35 °C	ND	+	+	+	+	+	+	+	N	N	N	N	N	N	N	N
+	V	Growth at 37 °C	ND	+	+	+	+	+	+	+	+	+/w	+	V	+	+	-	X
V	N	Growth at 40 °C	ND	+	-	+/w	+/w	+	+/w	+/w	N	N	N	N	N	N	-	N

Table 20. (continued).

			Yeast isolates								Data from Kurtzman *et al.* (1998)							
15. *Pichia kluyerri* var. *eremophila*	**16. *Pichia membranifaciens***	**Yeast strains / Methods**	**1. *Kluyveramyces maxianus* TISTR 5270 (control)**	**2. Y69**	**3. KAY1**	**4. PIY2**	**5. PUY4**	**6. LIY2**	**7. COY1**	**8. FAY2**	**9. *Kluyveromyces maxianus***	**10. *Saccharomycopsis fibuligera***	**11. *Rhodotorula glutinis***	**12. *Zygosaccharomyces bailii***	**13. *Candida guiliermondii***	**14. *Pichia cactophilla***	**17. *Pichia toletana***	**18. *Pichia pastoris***
		Other characteristics																
N	N	**Sediment**	+	-	+	+	+	+	+	+	+	+	+	N	+	N	N	N
+	+	**Pellicle**	-	-	-	ND	+	+	+	+	+	V	-	-	+	+	+	-
+	+	**Pseudomycelium**	-	+	-	-	+	+	+	-	+	N	+	-	+	+	+	-
-	-	**True hyphae**	-	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-
+	+	**Ascospore**	ND	+	-	+	ND	+	+	+	+	+	-	+	+	+	+	+

Symbols:

+ = positive
- = negative
X = positive or weak
N = no data
+/w = positive or weak
w/- = weak or negative
V = variable
ws = weak and slow
ND = not determined

3.9.2 Assimilation of nitrogen compound

Positive of nitrate assimilations were observed for yeast isolate KAY1. Negative assimilations were observed for nitrate, which were used as nitrogen sources. Y69, PIY2, PUY4, LIY2, COY1, and FAY2 were negative. The ability to utilize nitrate was an important criterion in defining some genera, for instance the genus *Pichia* was distinguished from the former genus *Hansenula* by its inability to grow with nitrate as the sole source of nitrogen. Yeasts, which grow with nitrate as the sole source of nitrogen, were also able to grow on nitrite but the reverse did not always apply. For instance, some strains of *Debaryomyces hansenii* utilize nitrite but not nitrate. Nitrite could be toxic to the yeast because nitrous acid was formed at pH values below 6, therefore media should be adjusted initially to pH 6.5, and nitrite used in low concentration. (van der Walt, 1970).

3.9.3 Hydrolysis of urea

Isolate KAY1 could grow on Urea R broth, and negative were determined for yeast isolates Y69, PIY2, PUY4, LIY2, COY1, and FAY2. Urea hydrolysis was generally absented in ascogenous species, whereas it was marked in the basidiomycetous genera such as *Cryptococcus* and *Rhodotorula*. (Barnett *et al*., 1990). The ascogenous yeasts *Schizosaccharomyces pombe*, *Yarrowia lipolytica*, and *Lipomyces* species were exceptions. Urease catalyses the hydrolysis of urea, giving ammonia and carbamate which, itself, hydrolyses to form carbonic acid and another molecule of ammonia. The overall reaction leads to an increase in pH (Kurtzman *et al*., 1998).

3.9.4 Effect of temperature on cell growth

Vigorous growth was observed at 25, 30, 35, 37, and 40°C for yeast isolate Y69 and LIY2. Weakly growth at 40°C for yeast isolates PIY2, PUY4, COY1, and FAY2. Negative growth at 40°C for yeast isolate KAY1. The strains of most yeast species grow best at temperature between 20 and 28°C. However, some yeast, and particularly those from restricted and specific habitats, require higher or lower temperatures. Yeasts from polar regions grow poorly at 20°C but grow well between 4°C and 15°C (Sinclair and Stokes, 1965). In contrast, yeasts from mammals, such as *Cyniclomyces guttulatus*, required temperature of 35-37°C to grow well, and 30-35°C was reported to be the optimum for *Malassezia furfur* (Leeming and Notman, 1987).

3.9.5 Ascospore formation

The yeast isolates were assayed for ascospore formation. The vegetative cells were observed for asci after 3 days and 5 weeks of incubation at 30°C in sporulation medium. Isolate Y69 (Figure 67) showed asci are unconjugated, attached to hyphae or free. The asci produce 1-4, ascospores that might be spheroidal or ellipsoidal, and might have one or move ledges. Surfaces might be smooth or roughed.

Isolate KAY1 (Figure 68) yeast cells are ovoidal to elongate, and have polar budding. Hyphae and pseudohyphae were presented. No ascospores were observed for isolate KAY1.

Isolate PIY2 (Figure 69) showed the multilateral budding. Asci were persistent and might be unconjugated or showed conjugation between a cell and its bud. Ascospores are smooth, spheroidal, with 1-4 per ascus.

Isolate PUY4 (Figure 70) showed monopolar budding, cells were ellipsoidal or cylindroidal. Psueudohyphae were formed. Ascospores not formed.

Isolate LIY2 (Figure 71) showed monopolar budding, pseudohyphae were presented. Asci might be conjugated or unconjugated, and persistant. The asci produced 1-4, ascospores were produced that might be spheroidal.

Isolate COY1 (Figure 72) showed multilateral budding. Psueudohyphae were presented. Asci might be unconjugated, and persistent. Form 1-4, spheroidal ascospores.

Isolate FAY2 (Figure 73) budding was multilateral. Asci were unconjugated, and produced 1-4 spheroidal ascospores.

Ascosporulation was generally induced under conditions which restricted vegetative growth. However, it was sometimes important that cells should be well nourished and growing vigorously on a rich medium when transferred to such conditions, though some strains sporulate without any special preparation. A variety of media have been specially formulated to induced sporulation, probably the most commonly used were: malt agar, acetate agar, Gorodkowa agar, V8 vegetable juice agar. YM agar, corn meal agar, and carrot wedges. Most of these media did not contained much carbohydrate and as a consequence they supported little vegetative growth.

Some genera appear to sporulate best on a particular medium: acetate agar has been recommended for *Saccharomyces* (Kurztman, 1998; Lodder, 1970), and dilute V8 agar for

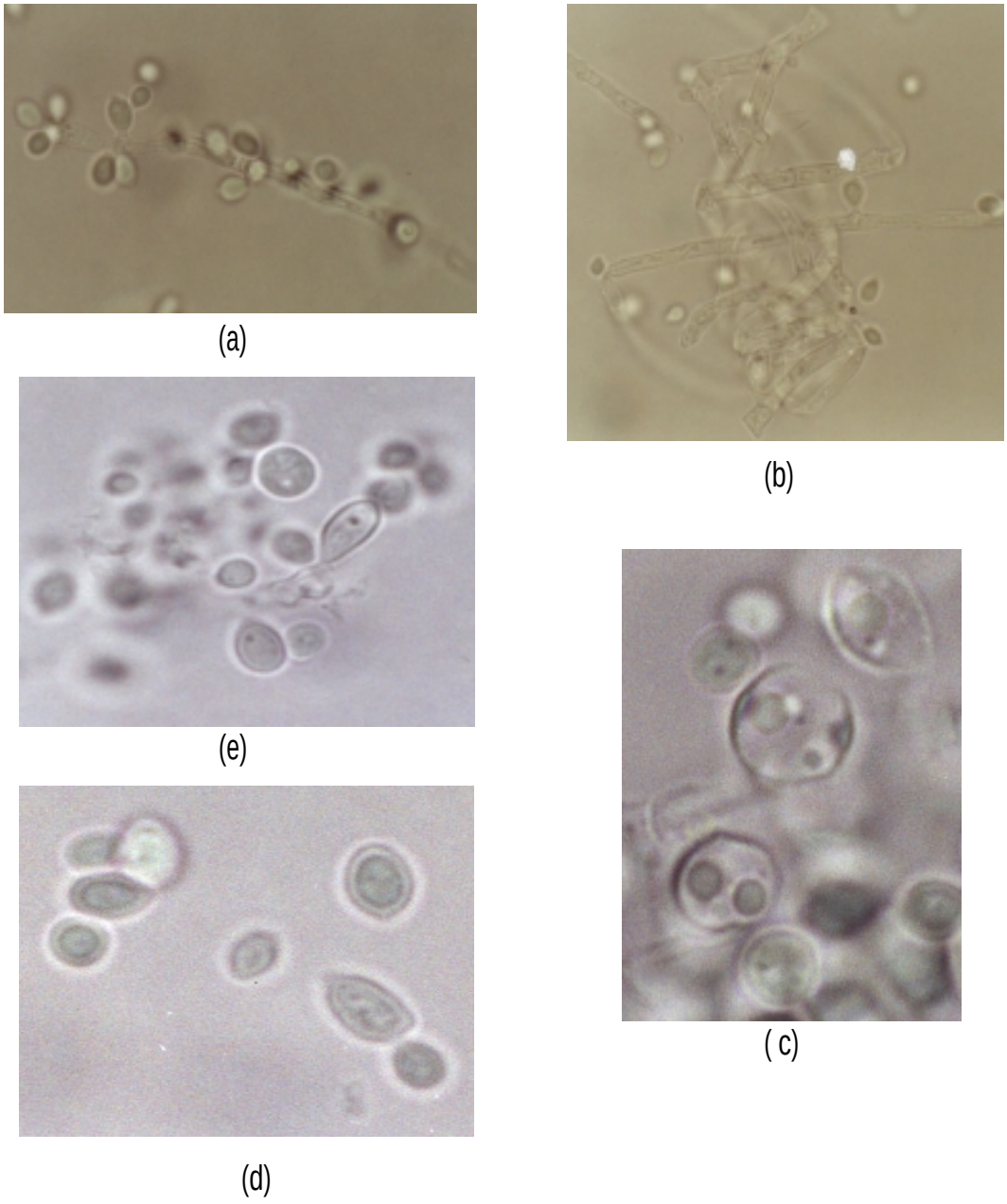


Figure 67. Cell morphology of yeast isolate Y69 (1000x). (a) and (b) Budding cells and hyphae on MY agar after 3-day cultivation at 25°C, (c) vegetative cells and asci with ascospores after 5-week cultivation on Acetate agar at 25°C, and (d) vegetative cells after 5-week cultivation on MY agar at 25°C, and (e) vegetative cells after 5-week cultivation on GPY agar at 25°C.

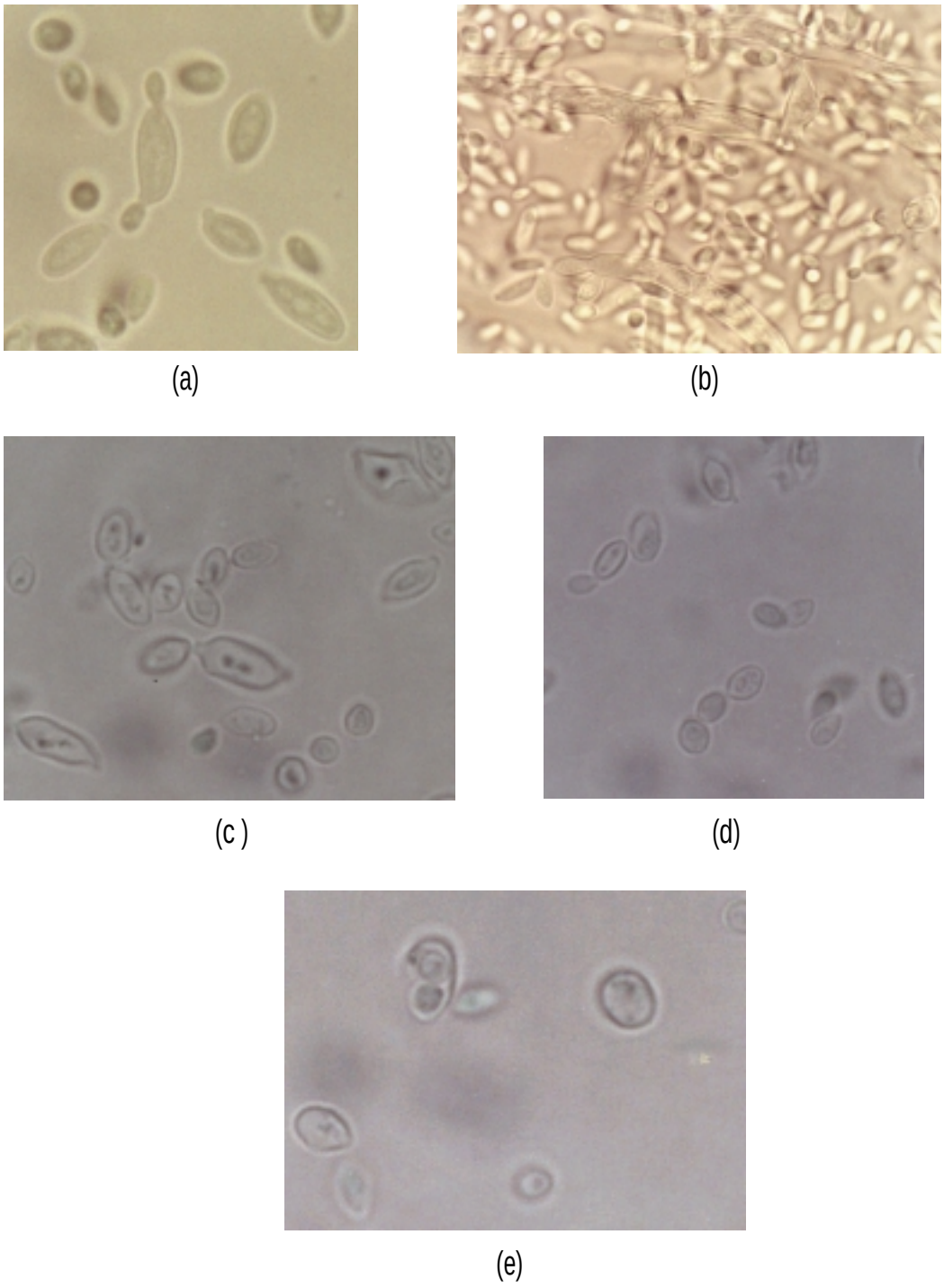


Figure 68. Cell morphology of yeast isolate KAY1 (1000x). (a) Vegetative cells after 3-day cultivation on MY agar at 25°C, and (b) budding cells and hyphae on MY agar after 3-day cultivation at 25°C, (c) on Acetate agar after 5-week cultivation at 25°C, (d) on MY agar after 5-week cultivation at 25°C, and (e) vegetative cells after 5-week cultivation on GPY agar at 25°C.

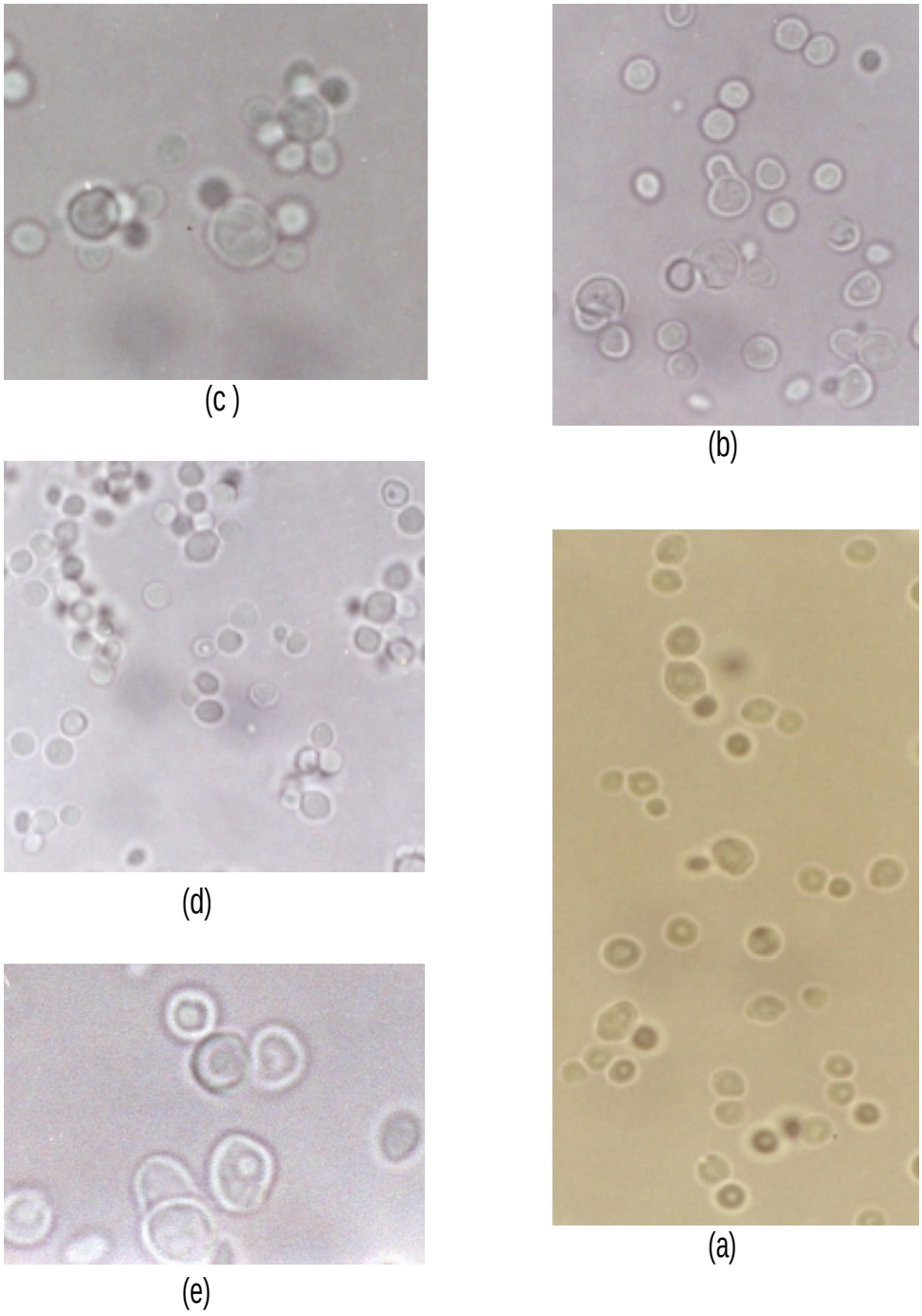


Figure 69. Cell morphology of yeast isolate PIY2 (1000x). (a) After 3-day cultivation on MY agar at 25°C, (b) after 5-week cultivation on GPY agar at 25°C, (c) after 5-week cultivation on Acetate agar at 25°C, (d) after 5-week cultivation on MY agar at 25°C, and (e) after 5-week cultivation on GPY agar at 25 °C.

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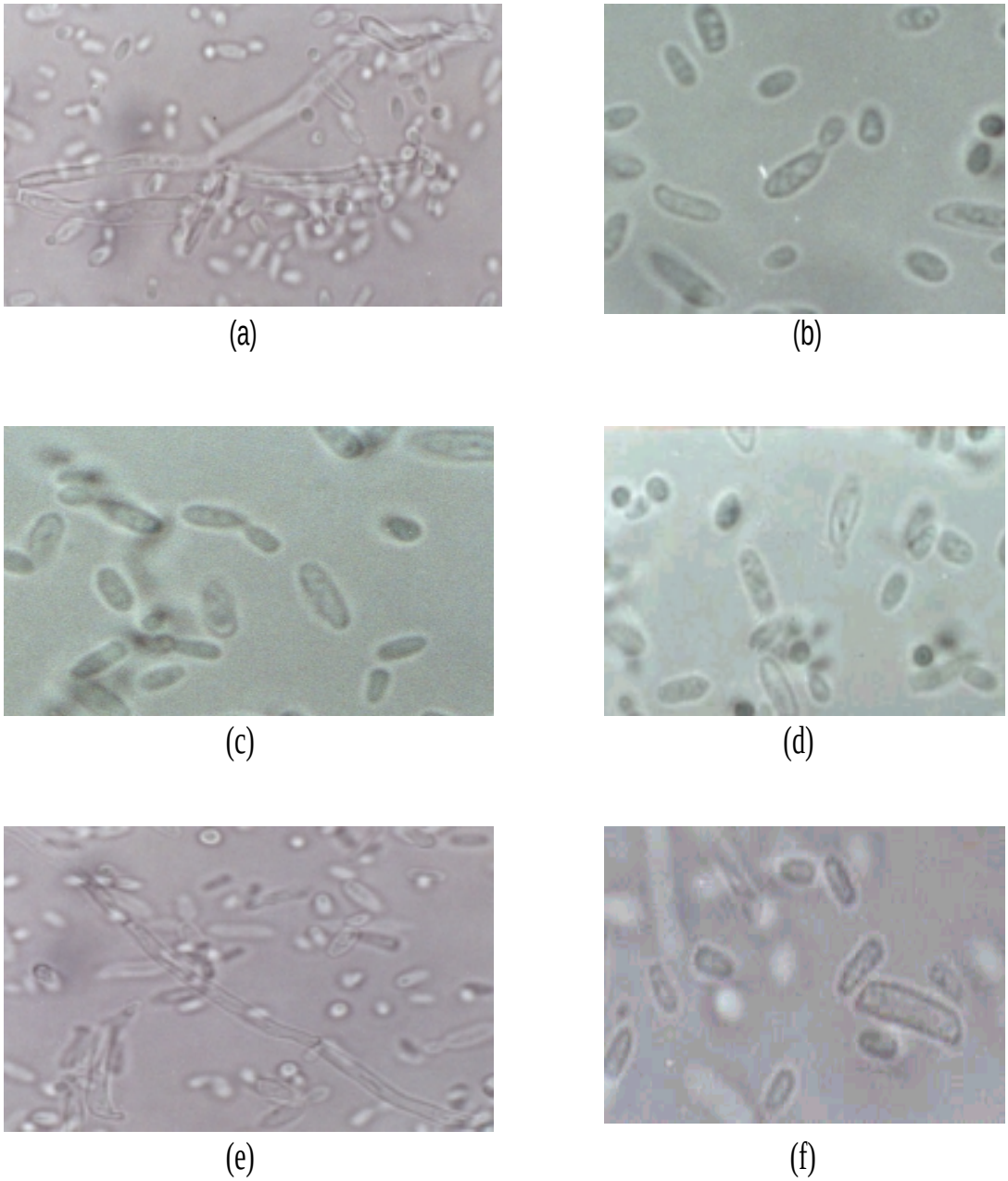
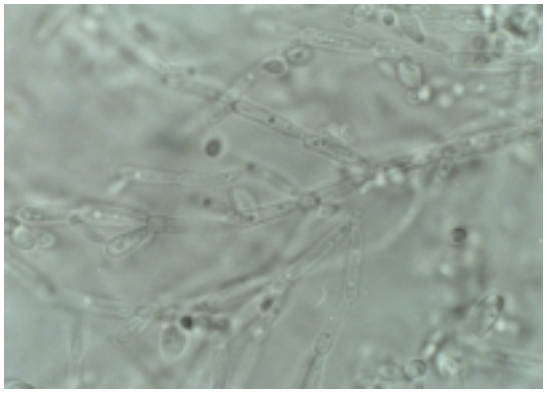
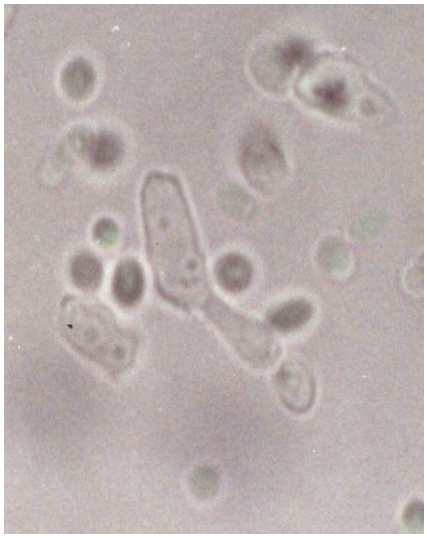


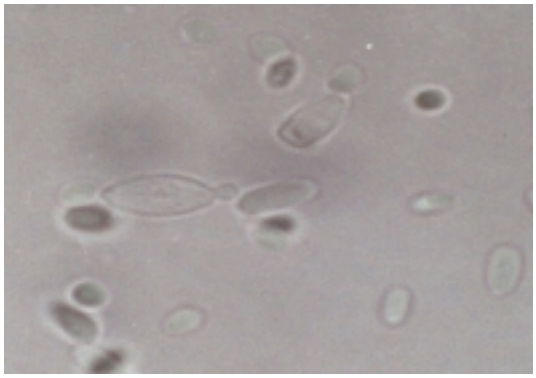
Figure 70. Cell morphology of yeast isolate PUY4 (1000x). (a) After 3-day cultivation on MY agar at 25°C, (b), (c) and (d) vegetative cells after 5-week cultivation on Acetate agar at 25°C, (e) and (f) budding cells and pseudohyphae after 5-week cultivation on MY agar at 25°C.



(a)



(b)



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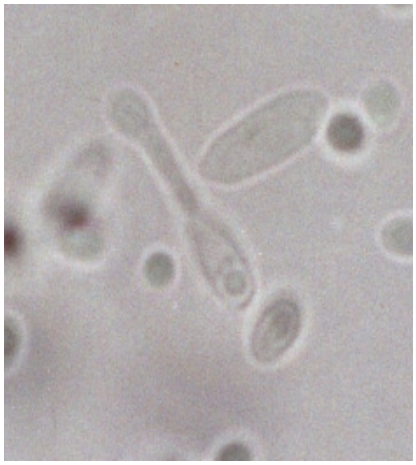
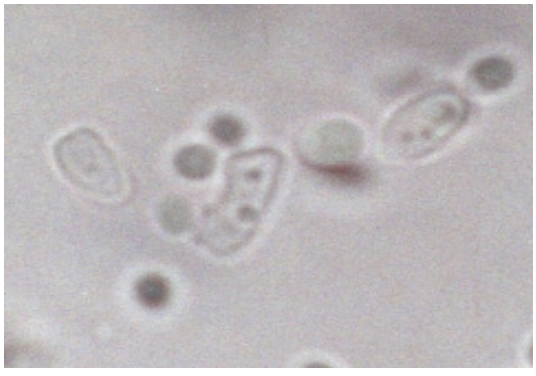
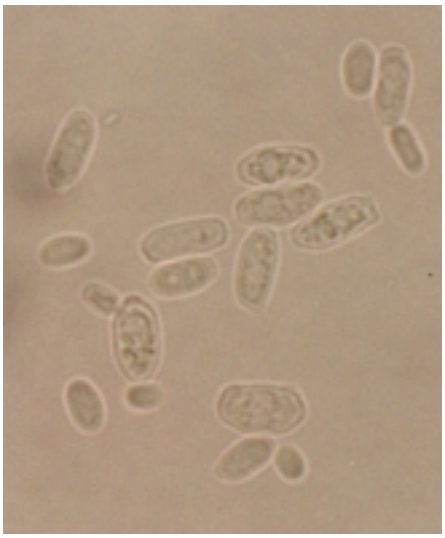


(e)

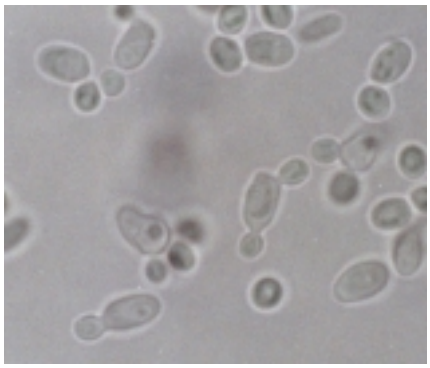


(d)

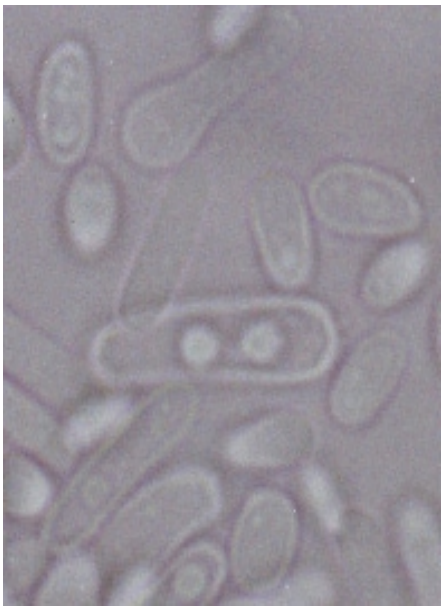
Figure 71. Cell morphology of yeast isolate LIY2 (1000x). (a) Pseudohyphae on GPY agar after 5-week cultivation at 25°C, (b) ascosporogenous culture after 5-week cultivation on Acetate agar at 25°C, (c), (d), and (e) ascosporogenous culture after 5-week cultivation on Acetate agar at 25°C.



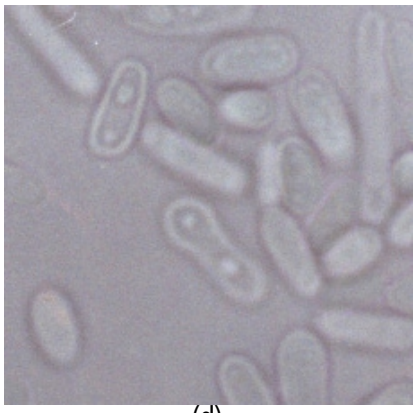
(a)



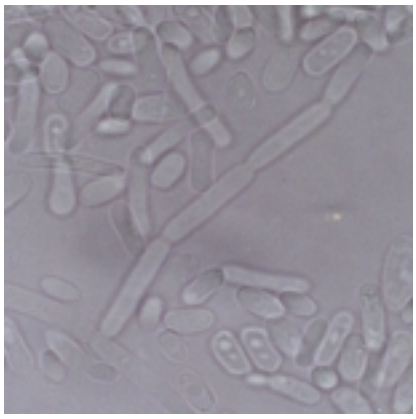
(b)



(c)



(d)



(e)

Figure 72. Cell morphology of yeast isolate COY1 (1000x). (a) On MY agar after 3-day cultivation at 25°C, (b) vegetative cells after 5-week cultivation on Acetate agar at 25°C, (c) and (d) asci with ascospores on GPY agar after 5-week cultivation at 25°C, and (e) budding cells and pseudohyphae on GPY agar after 5-week cultivation at 25°C.

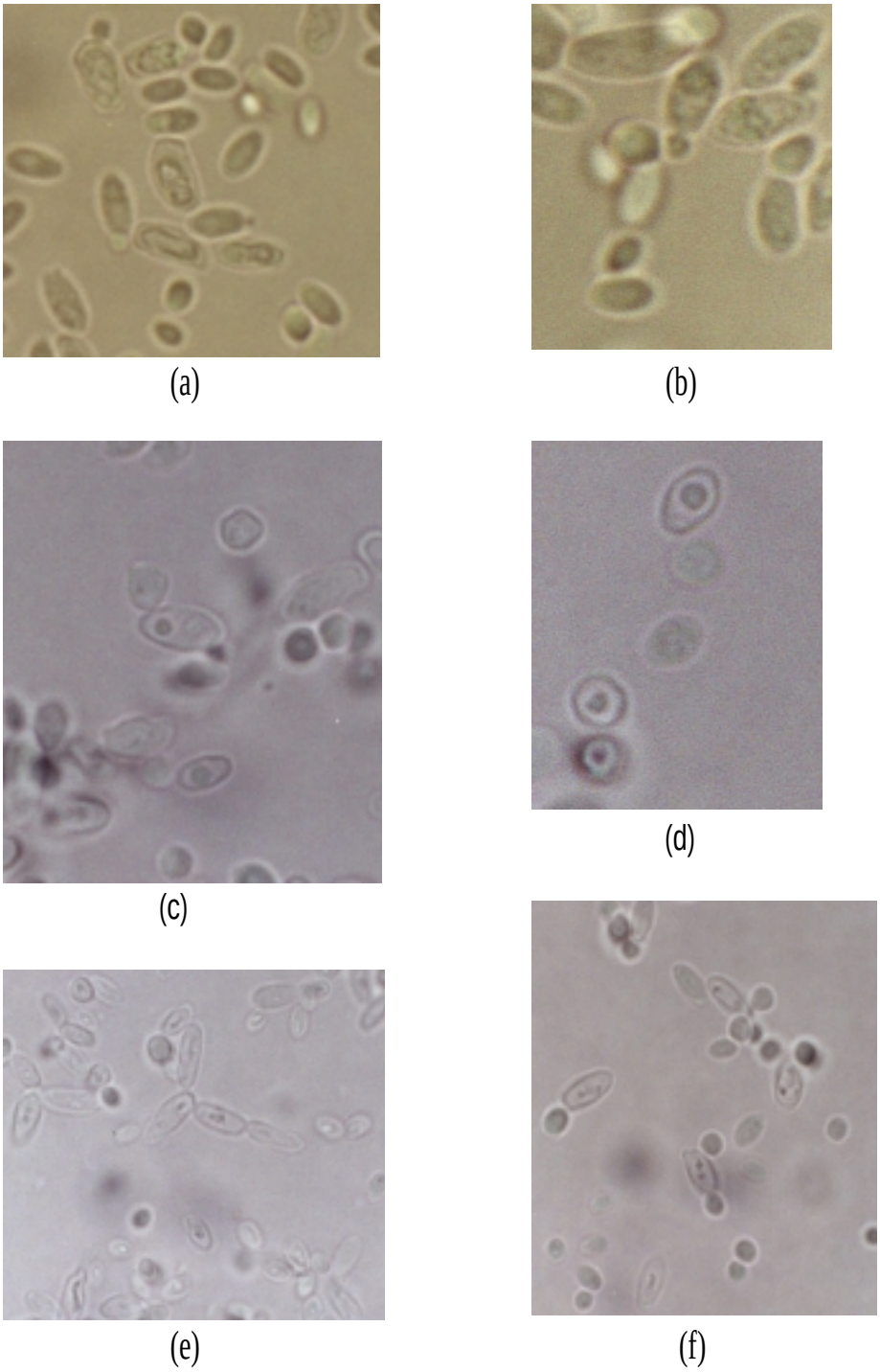


Figure 73. Cell morphology of yeast isolate FAY2 (1000x). (a) and (b) After 3-day cultivation on MY agar at 25°C, (c) and (d) ascosporogenous culture after 5-week cultivation on GPY agar at 25°C, (e) ascosporogenous culture after 5-week cultivation on MY agar at 25°C, and (f) ascosporogenous culture after 5-week cultivation on Acetate agar at 25°C.

Metshnikowia (Pitt and Miller, 1968); many strains of *Pichia* sporulate on malt agar. Some strains of *Zygosaccharomyces rouxii* are reported to sporulate best on media containing 2% sodium chloride (Kreger-van Rig, 1984)). Sporulation of many strains of the genus *Lipomyces* was favored by dilute media at low temperature (15-20°C). Temperature could affected ascus formation markedly. Temperatures between 20 and 25 °C were suitable for most yeasts. Nevertheless, strains of *Debaryomyces hansenii* generally sporulate best at 20°C or slightly lower, and many strains of the genus *Metschnikowia* required temperatures between 12 and 17 °C (Kurztman and Fell, 1998).

Some yeasts sporulate rapidly, i.e. within 48 hours, especially when first isolated others might required much longer, up to 6 weeks or more. The ability to sporulate sometimes declines when a strain was maintained in the laboratory, and might even be lost altogether. This occurs rapidly in some isolates, perhaps after 2 or 3 subcultures, whereas other strains might be kept for many years without any apparent decline in their ability to form spores.

Seven yeast isolates, Y69, KAY1, PIY4, PUY4, LIY2, COY1, and FAY2 were characterized by morphological, physiological, and biochemical. Based on those data their genus or species was predicted (Table 21). The yeast isolate Y69 showed the data most likely to species *Saccharomycopsis fibuligera*, KAY1 belonged to the species *Rhodotorula glutinis*, PIY2 most likely to species *Zygosaccharomyces bailii*, PUY4 most likely to species *Candida guiliermondii* var. *membranifaciens*. Three yeast isolates LIY2, COY1, and FAY2 has the characters of the genus *Pichia* in physiological. Chemo-taxonomic properties also suggested that two isolates were LIY2 and COY1 belonged to the species *Pichia cactophilla* and *Pichia pastoris*, respectively. Isolate COY1 most likely to three species, *Pichia kluyveri* var. *eremophila*, *Pichia membranifaciens*, and *Pichia toletana*.

Table 20. Identification results of yeast isolates.

Isolated number	**Posibility of genus and species**
Y69	*Saccharomycopsis fibuligera*
KAY1	*Rhodotorula glutinis*
PIY2	*Zygosaccharomyces bailii*

Table 21. (continued).

Isolated number	Posibility of genus and species
PUY4	*Candida guiliermondii var. membranaefaciens*
LIY2	*Pichia cactophilla*
COY1	*Pichia kluyveri* var. *eremophila* *Pichia membranifaciens* *Pichia toletana*
FAY2	*Pichia pastoris*

CHAPTER IV

CONCLUSION

This study exhibited that a total of 147 yeast strains isolated from various sources and 15 type strains were screened for their sugar alcohol-producing and starch-utilizing abilities. When using the medium containing high glucose concentration (YEPD with the addion of 5% glucose) in the primary screening, one hundred and seventeen isolates produced glycerol found only in their cultured broth, and thirteen isolates produced mannitol accumulated in cells. From the last screening step for sugar alcohol production ability using starch and sucrose as carbon sources, 22 isolates could utilized starch when testing with cassava starch. It was found that the yeast isolate KAY1 could utilize starch and produced mannitol detected in only cell lysate. Isolates PIY2 and PUY4 were also produced glycerol when cultured in the medium containing sucrose.

For the carbon source, Khao-klong starch, rice starch, and cassava starch to produced glycerol and mannitol, yeast isolate KAY1 could produced mannitol at the similar concentrations which were around 1.2-1.5 g/l in cell lysate. The maximum amounts of mannitol could be found at the first day of incubation. The rice starch medium gave the highest yield of mannitol at the second day and cassava starch medium was proved at the third day of fermentation. However, the composition of starch were investigated, 2% starch concentration was suitable substrate for sugar alcohol production. The concentration of yeast extract which achieved for supported the growth and reducing sugars exhaustion was the level of 0.3% for Khao-klong starch or cassava starch medium and 0.4% for rice starch medium. The suitable inoculum size for 2% Khao-klong starch medium was 6%. For 2% rice starch medium, the maximum amounts of mannitol in cell lysate was gave from 2% inoculum size and 4% inoculum size enhanced the highest amounts of mannitol in 2% cassava starch medium.

In this experiment, heat shock treatment was performed at 45°C for 20 min, a novel method was used for enhanced the sugar alcohol production. It was considered that the method supported the yeast isolate KAY1 to produced a large amounts protein of amylase to converted the starch to high amounts of reducing sugars. Consequently, a large amounts of reducing sugars was uptake into the yeast cell and the high amounts of mannitol were constructed. A residual starch concentration was rapidly decreased, at the same time the maximum yield of sugar alcohol production was appeared.

When the cultivation of rhizobia were tested. The adjusted cell lysate medium containing 0.5% mannitol obtained from yeast isolate KAY1 were gave the final number of *Bradyrhizobium japonicum* USDA 110 and *Bradyrhizobium* spp. THA 5 cell at 10^8 cells/ml at the sixth day, being almost the same as that found with YMB the standard rhizobia culture medium. In separate experiments, the ability of *Bradyrhizobium* to grow on fermented sucrose medium was also tested. The good result was obtained with 0.5% glycerol diluted from 5% sucrose prefermented by yeast isolate PIY2 and PUY4 at the second day of fermentation, gave the amounts of bacterial cells at 10^8-10^9 cells/ml at the sixth day. In contrast, with the *Bradyrhizobium japonicum* USDA 110, the number of viable cells gave lower than *Bradyrhizobium japonicum* spp. THA 5 when cultured in the same medium and compared with the G5 acted as glycerol standard medium. The results presented here clearly demonstrated that mannitol-yeast cell from starch hydrolyzation and glycerol in cell-free sucrose fermentation, can be used for the cultivation of *Bradyrhizobium*.

The identification of yeast isolates were investigated. It was found that the yeast isolate KAY1 most likely belongs to the genus *Rhodotorula*. Isolates PIY2 and PUY4 were classified in the genera *Zygosaccharomyces* and *Pichia*, respectively.

However, some fermentation conditions have not been investigated for example pH control, phosphate concentration, effect of aeration, or effect of temperature, and other various nitrogen sources. This study apparently presented rather simple and without operation control.

In the future research work, for the isolates KAY1. Increased the large scale production, optimization, and strain improvement should lead to even higher product concentration in the final fermentation broth in the future. In addition, the raw material utilization such as starch fermentation technology which were study in this experiments offered the advantages of being simple and requiring

only a low capital investment. Therefore, by using the utilization of expensive hexoses or mannitol and yeast extract, especially in developing countries, can be reduced.

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APPENDICES

A. Reagents

1. Lactophenol-cotton blue

Lactic acid	20.0	ml
Phenol crystal	20.0	g
Glycerol	40.0	ml
Distilled Water	20.0	ml

Keep in brown bottle and add 0.05 g of cotton blue or methylene blue.

2. Malachite green (spore straining)

Malachite green	5.0	g
Distilled water	100.0	ml

3. Carbol fuchsin (Ziehl's)

Basic fuchsin	0.3	g
Ethanol 95%	10.0	ml

Added with Phenol 5% aqueous solution 100.0 ml.

4. Silver nitrate-Sodium hydroxide reagent

Preparation of reagent

Dipping solution I	Make 1 ml of a saturated aqueous silver nitrate solution up to 200 ml with acetone. Redissolve the resulting precipitate by adding 5 ml of water and shaking.
Dipping solution II	Dissolve 2 g of sodium hydroxide pellets in 2 ml of water with heating, and make up to 100 ml with methanol.
Storage	Dipping solution I should be prepared freshly for using each time, dipping solution II may be stored for several days.
Substances	Silver nitrate Sodium hydroxide pellets

	Acetone
	Methanol
Solvent for mobile phase	1-Butanol-Acetone-Water (5:4:1)
Chromatographic plates	Commercial plates of 20x20 cm Silica gel 60 F 254 (MERCK Art. 5554.) were used. The plates were impregnated by soaking in buffered methanol solution and allowing them to dry at 110°C for 30 min.
Buffered methanol solution	Chloroform-Methanol (50:50)

Reaction

The ionic silver in the reagent is reduced to metallic silver by reducing carbohydrates.

Method

The chromatograms are freed from mobile phase in a stream of warm air, immersed in solution I for 1 second or sprayed evenly with it, then dried in a stream of cold air, then immersed in solution II for 1 second or sprayed with it, and finally heated to 100°C for 1-2 min.

5. Iodine solution

Potassium iodide	6.6	g
Iodine	0.66	g
Distilled water	165.0	ml

Dissolved 6.6 g of potassium iodide and 0.66 g of iodine in 165 ml of distilled water.

6. Dinitrosalicylic acid reagent

Distilled water	1414.0	ml
3,5-Dinitrosalicylic acid (DNS)	10.0	g
Sodium hydroxide (NaOH)	19.0	g
Potassium sodium tartate	306.0	g
Phenol (dissolved at 50°C)	7.6	g

Solution I: Dissolved 10 g of DNS and 19 g of NaOH in 1,416 ml of distilled water with warming and vigorously stirring. Solution II: Dissolved 306 g of sodium-potassium tartate in 500 ml of distilled water

containing 7.6 g of phenol and 8.3 g of sodium metabisulfite. The two solution were mixed and kept in brown bottles.

7. Nitrite test reagents

Reagent I Dissolve 8 g of sulfanilic acid in either 1 liter of 5 N acetic acid (1 part glacial acetic acid to 2.5 parts of water) or 1 liter of dilute sulfuric acid (1 part concentrated acid to 20 parts of water).

Reagent II Dissolved 5 g of α-naphthylamine in 1 liter of either 5 N acetic acid or of diluted sulfuric acid (1:20), or dissolve 6 ml of dimethyl-α-napthylamine in 1 liter of 5 N acetic acid.

8. Lowry's Procedure for quantitation of proteins

Reagents

A 2% sodium carbonate in 0.1 N NaOH (sodium carbonate 21.2 g/L; 10 ml/L NaOH 10 N)

B 0.5% $CuSO_4.5H_2O$ in 1% sodium citrate (sodium citrate in stead of tartrate, modified by Leggeti-Bailey, 1967)

C 1 N Folin phenol reagent (Folin phenol comes in 2 N and should be diluted immediately before use)

D 1 ml Reagent B+ 50 ml Reagent A (or similar ratio) (make up immediately before use, good for one day) Standard, Bovine serum albumin at concentration of 1 mg/ml sample volume total of 100 μl.

Procedure

1) Add 1 ml Reagent D to each of the standards and unknown tubes. Vortex immediately. Incubate precisely 10 min at room temperature.
2) Add 0.1 ml Reagent C (dilute 1:1 with deionized water). Vortex immediately. Incubate 30 min . (sample incubated longer than 60 min should be discarded.).
4) Read absorbance at 750 nm.
5) Plot standard curve and calculate the unknown.

Standard : BSA standard at concentration of the 1000 μg/ml, 700 μg/ml, 500 μg/ml,

300 μg/ml, 200 μg/ml, and 100 μg/ml.

B. Culture media

1. Malt yeast extract (MY) broth

Malt extract	3.0	g
Yeast extract	3.0	g
Peptone	5.0	g
Glucose	10.0	g
Distilled water	1000.0	ml

The compositions were suspended in 1 liter of distilled or deionized water and heated to boil until complete dissolving. Final pH 7.0 at room temperature and sterilized by autoclaving for 15 min at 15 lbs pressure (121°C).

2. Malt yeast extract (MY) Agar

Malt extract	3.0	g
Yeast extract	3.0	g
Peptone	5.0	g
Glucose	10.0	g
Agar	15.0	g
Distilled water	1000.0	ml

The compositions were suspended in 1 liter of distilled or deionized water and heated to boil until complete dissolving. Final pH 7.0 at room temperature and sterilized by autoclaving for 15 min at 15 lbs pressure (121°C).

3. Yeast extract-peptone-dextrose (YEPD) containing 5% Glucose

Glucose	50.0	g
Tryptone	20.0	g
Yeast extract	10.0	g
Distilled water	1000.0	ml

The compositions were suspended in 1 liter of distilled or deionized water and heated to boil until complete dissolving. Final pH 5.5 at room temperature and sterilized by autoclaving for 15 min at 15 lbs pressure (121°C).

4. Yeast extract-peptone-dextrose (YEPD) containing 2% Glucose

Glucose	20.0	g
Tryptone	20.0	g
Yeast extract	10.0	g
Distilled water	1000.0	ml

The compositions were suspended in 1 liter of distilled or deionized water and heated to boil until complete dissolving. Final pH 5.5 at room temperature and sterilized by autoclaving for 15 min at 15 lbs pressure (121°C).

5. Starch agar medium for amylase activity

Yeast extract	3.0	g
$(NH_4)_2SO_4$	5.0	g
$MgSO_4.7H_2O$	0.5	g
KH_2PO_4	1.0	g
Agar	15.0	g
Starch	20.0	g
Distilled water	1000.0	ml

The compositions were suspended in 1 liter of distilled or deionized water and heated to boil until complete dissolving. Agricultural products such as cassava starch, rice starch, and rice starch (khao klong) were use as the main carbon sources for a fermentation of yeast. Final pH 6.0 at room temperature and sterilized by autoclaving for 15 min at 15 lbs pressure (121°C).

6. Soluble starch medium

Yeast extract	3.0	g
$(NH_4)_2SO_4$	5.0	g
$MgSO_4.7H_2O$	0.5	g
KH_2PO_4	1.0	g
Agar	15.0	g

Soluble Starch	20.0	g
Distilled water	1000.0	ml

The compositions were suspended in 1 liter of distilled or deionized water and heated to boil until complete dissolving. Final pH 6.0 at room temperature and sterilized by autoclaving for 15 min at 15 lbs pressure (121°C).

7. Starch broth

Yeast extract	3.0	g
$(NH_4)_2SO_4$	5.0	g
$MgSO_4.7H_2O$	0.5	g
KH_2PO_4	1.0	g
Starch	20.0	g
Distilled water	1000.0	ml

The compositions were suspended in 1 liter of distilled or deionized water and heated to boil until complete dissolving. Agricultural products such as cassava starch, rice starch, and rice starch (khao klong) were use as the main carbon sources for a fermentation of yeast. Final pH 6.0 at room temperature. Sterilized by autoclaving for 15 min at 15 lbs pressure (121°C).

8. Urea R broth

Yeast extract	0.1	g
Monopotassium phosphate	0.091	g
Disodium phosphate	0.095	g
Urea	20.0	g
Phenol red	0.01	g
Distilled water	1000.0	ml

Final pH 6.0 at room temperature.

9. Fermentation basal medium (Wickerham, 1951).

Yeast extract	4.5	g
Peptone	7.5	g
Distilled water	1000.0	ml

Add Bromothymol blue to give a sufficiently dark green color. Bromothymol blue stock solution contains 50 mg in 75ml of distilled water. Add 4 ml of stock solution per 100 ml fermentation basal medium. Sterilized by autoclaving for 15 min at 15 lbs pressure (121°C).

10. Glucose-peptone-yeast extract broth (2% (w/v))

Glucose	20.0	g
Yeast extract	5.0	g
Peptone	10.0	g
Distilled water	1000.0	ml

The compositions were suspended in 1 liter of distilled or deionized water and heated to boil until complete dissolving. Sterilized by autoclaving for 15 min at 15 lbs pressure (121°C).

11. Glucose-peptone-yeast extract agar (GPY agar)

Glucose	40.0	g
Peptone	10.0	g
Agar	20.0	g
Distilled water	1000.0	ml

Add 500 ml of yeast infusion to 500 ml of demineralized water and dissolve. Alternatively, 5 g of yeast extract in 500 ml of demineralized water can be substituted for the yeast infusion. Sterilized by autoclaving for 15 min at 15 lbs pressure (121°C).

12. Acetate agar 2 (McClary *et al.*, 1959)

Glucose	1.0	g
Potassium chloride	1.8	g
Sodium acetate trihydrate	8.2	g
Yeast extract	2.5	g
Agar	15.0	g
Distilled water	1000.0	ml

The compositions were suspended in 1 liter of distilled or deionized water and heated to boil until complete dissolving. Sterilized by autoclaving for 15 min at 15 lbs pressure (121°C).

13. Nitrate broth

KNO_3	1.0	g
Glucose	5.0	g
K_2HPO_4	0.5	g
$CaCl_2$	0.5	g
$MgCO_3$	0.2	g
Distilled water	1000.0	ml

The compositions were suspended in 1 liter of distilled or deionized water and heated to boil until complete dissolving. Sterilized by autoclaving for 15 min at 15 lbs pressure (121°C).

14. Yeast-mannitol broth (YMB)

Mannitol	5.0	g
Yeast extract	0.5	g
$MgSO_4.7H_2O$	0.2	g
K_2HPO_4	0.5	g
NaCl	0.1	g
Distilled water	1000	ml

The compositions were suspended in 1 liter of distilled or deionized water and heated to boil until complete dissolving. Final pH 6.8 at room temperature and sterilized by autoclaving for 15 min at 15 lbs pressure (121°C).

15. G5 medium

Mannitol	1.0	g
Glycerol	4.0	ml
Yeast extract	1.0	g
$MgSO_4.7H_2O$	0.2	g
K_2HPO_4	0.5	g
NaCl	0.1	g
Glucose	1.0	g
Arabinose	0.5	g
PVP-40	20.0	g
Fe- EDTA	200	μM (0.073 g/L)

Distilled water 1000 ml

The compositions were suspended in 1 liter of distilled or deionized water and heated to boil until complete dissolving. Final pH 6.8 at room temperature and sterilized by autoclaving for 15 min at 15 lbs pressure (121°C).

C. Instruments

1. A typical counting chamber (haemocytometer) (Singleton, 1992) (Figure 1C)

The instrument, seen from one side at (a) consists of rectangular glass block in which the central plateau lies precisely 0.1 mm below the level of the shoulders on either side. The central plateau is separated from each shoulder by a trough, and is itself divided into two parts by a shallow trough (seen at (b)). On the surface of each part of the central plateau is an etched grid (c) consisting of a square which is divided into 400 small squares, each 1/400 mm^2. A glass cover-slip is positioned as shown at (b) and is pressed firmly onto the shoulders of the chamber; to achieve proper contact it is necessary, while pressing, to move the cover-slip (slightly) against the surface of the shoulders. Proper (close) contact is indicated by the appearance of a pattern of color lines (Newton′s rings), shown in black and white at (b).

1.1 Using the chamber. A small volume of a bacterial suspension is picked up in a Pasteur pipette by capillary attraction; the thread of liquid in the pipette should not be more than 10 mm. The is then placed as shown in (b), i. e. with the opening of the pipette in contact with the central plateau, and the side of the pipette against the cover-slip. With the pipette in this position, liquid is automatically drawn by capillary attraction into the space bounded by the cover-slip and part of the central plateau; *the liquid should not overflow into the trough*. (It is sometimes necessary to tap the end of the pipette, *slightly*, against the central plateau to encourage the liquid to enter the chamber). A second sample can be examined, if required, in the other half of the counting chamber. The chamber is left for 30 minutes to allow the cells to settle, and counting is the carried out under a high power of the microscope-which is focused on the grid
of the chamber. Since the volume between grid and cover-slip is accurately known, the count of cells per unit volume can be calculated.

1.2 A work example. Each small square in the grid is 1/400 mm^2. As the distance between grid and cover-slip is 1/10 mm, the volume of liquid over each small square is $1/4000^3$mm-i. e. ¼,000,000 ml.

Suppose, for example, that on scanning all 400 small squares, 500 cells were counted; this would give an average of 500/400 (=1.25) cells per small square, i. e. 1.25 cells per ¼,000,000 ml. The sample therefore contains 1.25*4,000,000 cells/ml, i. e. $5*10^6$ cells/ml.

If the sample had been diluted before examination (because it was too concentrated), the count obtained must be multiplied by the dilution factor; for example, if diluted 1-in-10, the count should be multiplied by 10.

N. B. The chamber described above is the *Thomas chamber*; in a *Helber chamber* the distance between central plateau and cover-slip is 0.02 mm.

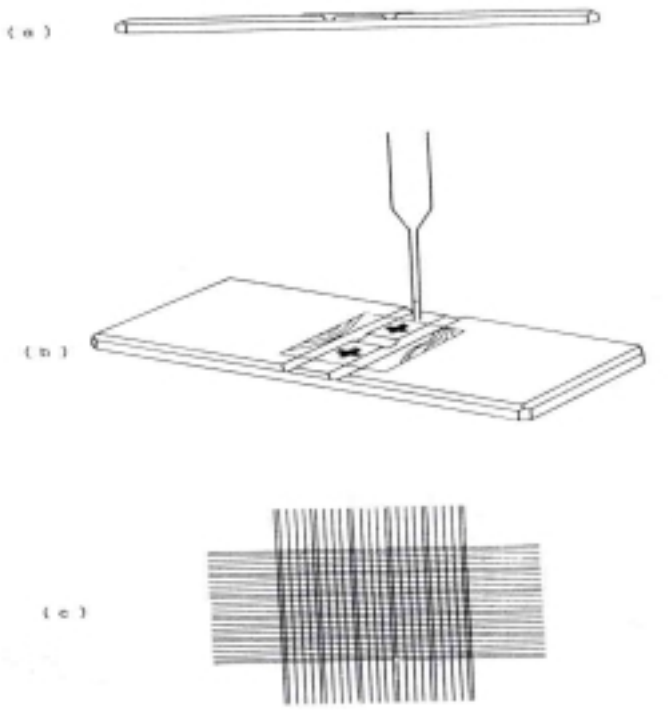


Figure 1C. A typical counting chamber (haemocytometer).
Source: Singleton (1992).

D. Additional tables

Table 1D. Characteristics of yeast isolates from various sources when cultured in Malt-yeast extract agar for 3 days at 30°C.

Source of yeast isolates	No.	Isolate number	Colony morphology																	Colony diam. (mm)	Cell size μm	Colony color	Vegetative reproduction	Vegetative cell
			Form				Elevation						Margin					Surface							
			Punciform	Circular	Irregular	Filamentous	Flat	Raised	Convex	Pulvinate	Umbonate	Crateriform	Entire	Undulate	Lobate	Filamentous	Fringed	Glistening	Dull					
1. Look-pang	1	HUY1		\		\					\		\			\			\	2-3	5*5-7	White	Multilateral budding	Short ellipsoids
	2	HUY2	\						\				\					\		2.5-3	4*4-5	White	Multilateral budding	Short ellipsoids
	3	HUY3		\					\				\					ND	ND	2-3	3-4*5-7	ND	ND	ND
	4	HUY4		\								\					\	ND	ND	ND	5-7*5-7	ND	ND	ND
2. Khao-mak	5	RIY1		\					\				\					\		2-2.5	3-5*3-5	White	Multilateral budding	Spherical
	6	RIY2		\					\				\					\		2-2.5	3-5*3-5	White	Multilateral budding	Spherical
	7	RIY3		\					\				\					\		2-2.5	3-5*3-5	White	Multilateral budding	Spherical
	8	RIY4		\					\				\					\		2-2.5	3-5*3-5	White	Multilateral budding	Spherical
	9	RIY5		\					\				\					\		2-2.5	3-5*3-5	White	Multilateral budding	Spherical
	10	RIY6		\					\				\					\		2-2.5	3-5*3-5	White	Multilateral budding	Spherical

Table 1D. (continued).

Source of yeast isolates	No.	Isolate number	Colony morphology																	Colony diam. (mm)	Cell size µm	Colony color	Vegetative reproduction	Vegetative cell
			Form				Elevation						Margin					Surface						
			Punciform	Circular	Irregular	Filamentous	Flat	Raised	Convex	Pulvinate	Umbonate	Crateriform	Entire	Undulate	Lobate	Filamentous	Fringed	Glistening	Dull					
	11	RIY7		\					\				\					\		2-2.5	5*5-6	White	Multilateral budding	Short ellipsoids
3. Sake cake	12	SAY1	\	\					\				\					\		1-1.5	5*5	White	Multilateral budding	Spherical
4.Solid waste from food factory	13	WAY1		\						\			\					\		0.5-0.8	6*6	White	Multilateral budding	Spherical
	14	WAY2		\						\			\					\		0.5-0.8	6*6	White	Multilateral budding	Spherical
	15	WAY 3		\						\			\					\		0.5-0.8	5*5	White	Multilateral budding	Spherical
	16	WAY4		\				\						\					\	3-4	4-5*4-5	White	Multilateral budding	Spherical
	17	WAY5		\					\		\							\		1.5-2	3-4*3-4	White	Multilateral budding	Spherical
	18	WAY6				\							\			\			\	2-3	ND	White	Arthroconidia	Elongated
5.Sugar-cane pulp	19	CAY2		\					\				\					\		2.5-3	1-2*3-5	White	Bipolar budding	Ovoidal
6. Fruit: orange	20	ORY1		\					\				\					\		2-2.5	2.5*4-7	White	Bipolar budding	Ovoidal
	21	ORY2		\					\					\					\	2.5 - 3	4*5	White	Multilateral budding	Ellipsoidal
	22	ORY3		\				\							\				\	4-5	2*5-10	White	Multilateral budding	Elongated
	23	ORY4		\				\					\					ND	ND	3-4	1.5-2*4-5	White, cream	Bipolar budding	Ogival

Table 1D. (continued).

Source of yeast isolates	No.	Isolate number	Colony morphology																	Colony diam. (mm)	Cell size µm	Colony color	Vegetative reproduction	Vegetative cell
			Form				Elevation						Margin					Surface						
			Punciform	Circular	Irregular	Filamentous	Flat	Raised	Convex	Pulvinate	Umbonate	Crateriform	Entire	Undulate	Lobate	Filamentous	Fringed	Glistening	Dull					
7. Fruit: pineapple	24	PIY1			\			\									\		\	2-2.5	2-3*4-5	White	Multilateral budding	Ellipsoidal
	25	PIY2		\				\					\					\		1	3-4*3-4	White	Multilateral budding	Spherical
	26	PIY3		\				\					\					\		2-3	1.5-2*5-8	Slightly orange	Bipolar budding	Ogival
8. Fruit: red grape	27	GRY1				\			\							\		\		2-.25	3-4*7-9	Red, pink	Bipolar budding	Ellipsoidal
	28	GRY2		\					\				\			\		\		2-3	4-4.5*4-5	White	Multilateral budding	Spherical
	29	GRY3		\					\				\					\		2	1-1.5*1-2	White	Multilateral budding	Spherical
10. Fruit: passion fruit	30	PAY1		\					\				\					\		2-2.5	4-5*5-7	White, yellow	Multilateral budding	Ellipsoidal
	31	PAY2			\			\						\					\	3-3.5	2-2.5*5-7	White	Monopolar budding	Ellipsoidal
	32	PAY3				\						\				\			\	5-6	4-5*7-8	White	Arthroconidia	Elongated
	33	PAY4		\				\						\					\	1-2	2-3*5-8	White, cream	Monopolar budding	Ellipsoidal
11. Fruit: cherry	34	CHY1			\			\							\			\		1.5-2	2-3*10-15	White, cream	Monopolar budding	Elongated

Table 1D. (continued).

Source of yeast isolates	No.	Isolate number	Colony morphology																	Colony diam. (mm)	Cell size μm	Colony color	Vegetative reproduction	Vegetative cell
			Form				Elevation						Margin					Surface						
			Punciform	Circular	Irregular	Filamentous	Flat	Raised	Convex	Pulvinate	Umbonate	Crateriform	Entire	Undulate	Lobate	Filamentous	Fringed	Glistening	Dull					
12. Fruit: apple	35	APY1		\								\					\		\	2.5-3	3-4*3-5	White	Multilateral budding	Spherical
	36	APY2		\					\				\					\		3	2*4-6	White, yellow	Monopolar budding	Ovoidal
	37	APY3		\					\				\					\		2	2*3-4	White, cream	Multilateral budding	Spherical
	38	APY4			\		\							\					\	2	ND	White	Multilateral budding	Elongated
	39	APY5			\			\									\		\	4-5	2*5-6	White, cream	Monopolar budding	Elongated
	40	APY6			\		\										\		\	2-3	ND	White, cream	Monopolar budding	Ellipsoidal
13. Yan bean	41	LIY1				\						\				\			\	1-2	1.5-2*3-5	White	Monopolar budding	Elongated
	42	LIY2			\			\						\				\		4-5	2-3*5-6	White	Monopolar budding	Ellipsoidal
	43	LIY3		\					\				\					\		1-1.5	2-3*2-3	White, cream	Multilateral budding	ND
	44	LIY4			\		\							\					\	3-5	2-3*5-7	White, cream	Multilateral budding	Ellipsoidal
	45	LIY5		\					\				\					\		1-2	5*5	White, cream	Multilateral budding	ND

Table 1D. (continued).

Source of yeast isolates	No.	Isolate number	Colony morphology																	Colony diam. (mm)	Cell size µm	Colony color	Vegetative reproduction	Vegetative cell
			Form				Elevation						Margin					Surface							
			Punciform	Circular	Irregular	Filamentous	Flat	Raised	Convex	Pulvinate	Umbonate	Crateriform	Entire	Undulate	Lobate	Filamentous	Fringed	Glistening	Dull					
14. Oat meal	46	OAY1			\							\		\					\	3-3.5	ND	White	Arthroconidia	Elongated
	47	OAY2		\				\					\						\	2-3	3-4*4-6	White, cream	Multilateral budding	Ellipsoidal
	48	OAY3			\		\										\		\	2-4	2-3*4-7	White, cream	Monopolar budding	Ellipsoidal
	49	OAY4				\						\					\		\	6-7	ND	White	Arthroconidia	Elongated
15. Fruit: Makampom	50	SOY1		\			\						\					\		4-5	5*6	White, cream	Multilateral budding	Ellipsoidal
	51	SOY2		\				\					\	\					\	3-5	3-4*5-6	White	Multilateral budding	Ellipsoidal
	52	SOY3		\			\						\					\		2-3	1-2*3-4	White, cream	Bipolar budding	Ovoidal
	53	SOY4		\			\						\					\		2-3	1-2*3-4	White, cream	Bipolar budding	Ovoidal
	54	SOY5		\					\				\						\	2-3	3-4*5-6	White	Multilateral budding	Spherical
	55	SOY6		\								\	\						\	1.5-2	1-1.5*6-9	White	Monopolar budding	Filamentous
	56	SOY7		\				\					\						\	4-5	ND	White	ND	Filamentous

Table 1D. (continued).

Source of yeast isolates	No.	Isolate number	Colony morphology																	Colony diam. (mm)	Cell size µm	Colony color	Vegetative reproduction	Vegetative cell
			Form				Elevation						Margin					Surface							
			Punciform	Circular	Irregular	Filamentous	Flat	Raised	Convex	Pulvinate	Umbonate	Crateriform	Entire	Undulate	Lobate	Filamentous	Fringed	Glistening	Dull					
16. Fruit: rambutan	57	TAY1			\							\		\	\				\	3-4	1-2*2-3	White, cream	Monopolar budding	ND
	58	TAY2		\				\					\					\		3-4	2-3*2-3	White	Multilateral budding	Ellipsoidal
	59	TAY3		\								\					\		\	3-4	2-4*4-6	White	Multilateral budding	Ellipsoidal
	60	TAY4		\				\					\					\		2-2.5	1*2-3	White, cream	Monopolar budding	ND
	61	TAY5		\				\					\						\	3	1-1.5*2-3	White, cream	Monopolar budding	Ellipsoidal
	62	TAY6			\							\		\	\				\	2	1*2-3	White	Monopolar budding	ND
	63	TAY7			\							\			\				\	2-3	2-3*2-3	White	Multilateral budding	Ellipsoidal
	64	TAY8		\					\				\						\	2-3	4-5*5-6	White	Multilateral budding	Ellipsoidal
	65	TAY9			\							\		\						4-5	2-3*3-4	ND	ND	ND
17. Fruit: Water-melon	66	MEY1		\					\				\					\		1.5-2	2-3*2-3	White, cream	Multilateral budding	Spherical
	67	MEY2			\				\				\						\	3-4	1-3*3-5	White, cream	Monopolar budding	Ellipsoidal

Table 1D. (continued).

Source of yeast isolates	No.	Isolate number	Colony morphology																	Colony diam. (mm)	Cell size µm	Colony color	Vegetative reproduction	Vegetative cell
			Form				Elevation						Margin					Surface						
			Punciform	Circular	Irregular	Filamentous	Flat	Raised	Convex	Pulvinate	Umbonate	Crateriform	Entire	Undulate	Lobate	Filamentous	Fringed	Glistening	Dull					
	68	MEY3		\				\					\						\	2-3	1-2*4-6	White	Monopolar budding	Elongated
	69	MEY4			\		\							\					\	3-4	3*5-6	White	Multilateral budding	Spherical
18. Fruit: Phut-sa	70	PUY1			\			\						\					\	4-5	1-2*4-5	White, cream	Multilateral budding	Ellipsoidal
	71	PUY2		\					\				\						\	3-4	1-1.5*2-4	White	Multilateral budding	Ellipsoidal
	72	PUY3		\					\				\					\		1-2	1*3-4	White	Monopolar budding	Ellipsoidal
	73	PUY4		\								\	\					\		1	1-1.5*4-6	White, cream	Monopolar budding	Ellipsoidal
19. Lemon	74	CIY1			\		\						\						\	1-2	2-2.5*4-5	White	Monopolar budding	Ellipsoidal
20. Fruit: Chilli	75	LLY1		\					\				\					\		2-2.5	3-4*3-5	White, cream	Multilateral budding	Ellipsoidal
	76	LLY2		\								\	\						\	1-2	3-5*3-6	White, cream	Multilateral budding	Ellipsoidal
	77	LLY3		\								\		\					\	3-4	ND	White, cream	ND	ND
	78	LLY4			\							\		\					\	2-3	ND	White, cream	Pseudohyphae	Filamentous

Table 1D. (continued).

Source of yeast isolates	No.	Isolate number	Colony morphology																	Colony diam. (mm)	Cell size μm	Colony color	Vegetative reproduction	Vegetative cell
			Form				Elevation						Margin					Surface						
			Punciform	Circular	Irregular	Filamentous	Flat	Raised	Convex	Pulvinate	Umbonate	Crateriform	Entire	Undulate	Lobate	Filamentous	Fringed	Glistening	Dull					
21. Fruit: dragon fruit	79	DRY1			\							\		\						3-4	ND	ND	Pseudohyphae	Filamentous
22. Fruit: Strawberry	80	STY1			\			\						\					\	3	1-1.5*3-4	White, cream	Monopolar budding	Ellipsoidal
	81	STY2		\								\		\					\	2-3	ND	White	ND	ND
	82	STY3		\			\							\					\	1-2	ND	White, cream	ND	ND
	83	STY4		\				\					\					\		2-3	ND	White, cream	ND	ND
	84	STY5			\			\						\				\		3-5	ND	White, cream	ND	ND
	85	STY6			\							\		\					\	1-2	ND	White	ND	ND
23. Fruit: Banana	86	BAY1			\							\		\					\	1-2	ND	White	ND	ND
	87	BAY2		\				\					\					\		1-1.5	ND	White, cream	ND	ND
	88	BAY3		\					\				\					\		1	ND	White, cream	ND	ND
24. Fruit: rozelle	89	KAY1				\										\		\		3	2-3*2-3	Red, pink	Monopolar budding	Ogival

Table 1D. (continued).

Source of yeast isolates	No.	Isolate number	Colony morphology																	Colony diam. (mm)	Cell size µm	Colony color	Vegetative reproduction	Vegetative cell
			Form				Elevation						Margin					Surface						
			Punciform	Circular	Irregular	Filamentous	Flat	Raised	Convex	Pulvinate	Umbonate	Crateriform	Entire	Undulate	Lobate	Filamentous	Fringed	Glistening	Dull					
25. Fermented fruits	90	MAY1			\						\			\					\	2-3	1.5-2*5-7	White	ND	Elongated
	91	MAY2			\			\						\					\	3-4	2-3*4-6	White	Monopolar budding	Elongated
	92	AFY1			\									\					\	4-4.5	1-1.5*6-8	White	Monopolar budding	Elongated
	93	AFY2		\										\					\	2-2.5	2-3*6-8	White	Monopolar budding	Elongated
	94	AFY3		\									\					\		1-2	3-4*3-4	White	Multilateral budding	Spherical
	95	AFY4		\										\					\	2	2-3*2-3	White	Arthroconidia	Elongated
	96	AFY5		\										\					\	1-2	2-3*3	White	Arthroconidia	Elongated
	97	GFY1			\			\					\						\	3-5	2-2.5*5-6	White	Monopolar budding	ND
25. Fermenting tomatoes	98	POY1				\										\			\	5-7	5*7	White, cream	Multilateral budding	Ellipsoidal
	99	POY2		\					\				\						\	1-2	2-3*4-5	White, cream	Monopolar budding	Ogival
	100	POY3			\			\						\					\	3-4	1-2*3-5	White, cream	Bipolar budding	Ellipsoidal

Table 1D. (continued).

Source of yeast isolates	No.	Isolate number	Colony morphology																	Colony diam. (mm)	Cell size µm	Colony color	Vegetative reproduction	Vegetative cell
			Form				Elevation						Margin					Surface							
			Punciform	Circular	Irregular	Filamentous	Flat	Raised	Convex	Pulvinate	Umbonate	Crateriform	Entire	Undulate	Lobate	Filamentous	Fringed	Glistening	Dull					
26. Fruit: sapodilla	101	LAY1			\			\						\					\	2-2.5	2-2.5*5-6	White, cream	Monopolar budding	Elongated
	102	LAY2		\					\				\					\		2	3-4*5-6	White, cream	Multilateral budding	Ellipsoidal
27. Fruit: guava	103	FAY1		\								\	\						\	2	2*2-3	White	Multilateral budding	Spherical
	104	FAY2		\								\		\					\	2-2.5	3*4-5	White	Monopolar budding	Ellipsoidal
	105	FAY3			\		\										\		\	3-4	2*5-8	White, cream	Monopolar budding	Elongated
	106	FAY4		\			\						\						\	2	2-3*3-4	Cream, yellow	Multilateral budding	Ellipsoidal
28. Fruit: Indian-plum	107	SUY1		\				\					\					\		2	3*5	White, cream	Multilateral budding	Spherical
	108	SUY2		\				\							\				\	2	1-1.5*3-4	White, cream	Monopolar budding	Ellipsoidal
29. Pickle	109	VEY1		\					\				\					\		1-2	ND	White	ND	ND
30. Fermented milk products	110	MIY1		\				\						\					\	2-2.5	2-3*8-10	White	Monopolar budding	Elongated
	111	YOY1		\					\				\					\		1-1.5	4*4-4.5	White	Multilateral budding	Ellipsoidal
	112	MLY1		\				\					\						\	3-5	2-4*5-7	White, cream	Multilateral budding	Ellipsoidal

Table 1D. (continued).

Source of yeast isolates	No.	Isolate number	Colony morphology																	Colony diam. (mm)	Cell size µm	Colony color	Vegetative reproduction	Vegetative cell
			Form				Elevation						Margin					Surface							
			Punciform	Circular	Irregular	Filamentous	Flat	Raised	Convex	Pulvinate	Umbonate	Crateriform	Entire	Undulate	Lobate	Filamentous	Fringed	Glistening	Dull					
	113	MLY2		\								\			\				\	4-6	ND	White	ND	ND
	114	MLY3		\			\										\	\		2-3	1-2*5-7	White, cream	Multilateral budding	Elongated
	115	COY1			\			\						\					\	3-3.5	1-2*5-6	White	Monopolar budding	Elongated
	116	CNY1		\				\					\						\	2-3	1*2-3	White, cream	Multilateral budding	Ellipsoidal
	117	CNY2		\					\				\					\		1-2	1-2*3-5	White, cream	Multilateral budding	Ellipsoidal
	118	CRY1		\			\						\					\		2-3	2-3*2-3	White, cream	Multilateral budding	Spherical
	119	CRY2		\			\						\						\	4-5	ND	White, cream	Fission	Elongated
31. Soya milk	120	SMY1			ND						ND					ND				ND	ND	ND	ND	ND
	121	SMY2			ND						ND					ND				ND	ND	ND	ND	ND
	122	SMY3			ND						ND					ND				ND	ND	ND	ND	ND
32. Bluberry jam	123	BLY1			\				\					\					\	4-5	ND	White	Arthroconidia	Filamentous
	124	BLY2		\					\				\					\		2-2.5	2*4-5	White, cream	Monopolar budding	Ovoidal

Table 1D. (continued).

Source of yeast isolates	No.	Isolate number	Colony morphology																	Colony diam. (mm)	Cell size µm	Colony color	Vegetative reproduction	Vegetative cell
			Form				Elevation						Margin					Surface						
			Punciform	Circular	Irregular	Filamentous	Flat	Raised	Convex	Pulvinate	Umbonate	Crateriform	Entire	Undulate	Lobate	Filamentous	Fringed	Glistening	Dull					
	125	BLY3		\									\						\	3	4-5*5-7	White, cream	Multilateral budding	Spherical
	126	BLY4			\		\							\					\	4	2*5-7	White, cream	Monopolar budding	Ellipsoidal
33. Mamalade	127	AMY1			\			\									\		\	1.5-2	2*4-10	White, cream	Monopolar budding	Elongated
	128	AMY2		\				\					\						\	2	3-4*5-6	White, cream	Multilateral budding	Spherical
34. Confectionery	129	SWY1			\			\						\					\	5	2*6-10	White, cream	Monopolar budding	Elongated
	130	SWY2		\					\				\						\	2	4-5*5-6	White, cream	Multilateral budding	Spherical
	131	SWY3		\				\					\					\		2-2.5	1-2*5-7	White, cream	Bipolar budding	Ellipsoidal
	132	SWY4		\				\					\						\	2-3	1-2*3-4	White, cream	Multilateral budding	Spherical
35. Tomatoes source	133	SPY1			\									\					\	2-3	2-3*4-5	White, cream	Monopolar budding	ND
	134	SPY2		\				\					\						\	1.5-2	3-4*4-6	White	Multilateral budding	Ellipsoidal
36. Chilli source	135	SCY1			\			\					\						\	3-4	2*6	White, cream	Monopolar budding	Ellipsoidal
	136	SCY2			\		\							\					\	4-5	3-4*3-4	White	Multilateral budding	Spherical
	137	SCY3		\			\						\						\	1-2	4-5*4-5	White, cream	Multilateral budding	Spherical

Table 1D. (continued).

Source of yeast isolates	No.	Isolate number	Colony morphology																	Colony diam. (mm)	Cell size µm	Colony color	Vegetative reproduction	Vegetative cell
			Form				Elevation						Margin					Surface							
			Punciform	Circular	Irregular	Filamentous	Flat	Raised	Convex	Pulvinate	Umbonate	Crateriform	Entire	Undulate	Lobate	Filamentous	Fringed	Glistening	Dull					
37. Syrup	138	SYY1			\			\						\					\	3-4	2-3*4-6	White, cream	Monopolar budding	Ellipsoidal
	139	SYY2		\				\					\						\	1-2	ND	White	Multilateral budding	Ellipsoidal
	140	SYY3		\				\					\						\	1-2	ND	White	Multilateral budding	Ellipsoidal
38. Salad	141	SLY1			\							\		\					\	2-4	3*2-3	White	Multilateral budding	Spherical
	142	SLY2			\							\		\					\	1-2	2-3*3-4	White	Multilateral budding	Spherical
39. Chicken meat	143	CMY1		\					\				\					\		1-2	3-5*3-5	White, cream	Multilateral budding	Spherical
	145	Y60				\						\		\					\	2-3	2-3*3-4	White	ND	ND
	146	Y64		\							\		\					\		2.5	5-6*5-6	White	Multilateral budding	Spherical
	147	Y69				\										\			\	3-4	ND	White	Pseudohyphae	Filamentous
40. Cassava roots	144	Y24				\						\				\			\	3-3.5	3*3	White	Bipolar budding	ND

Table 1D. (continued).

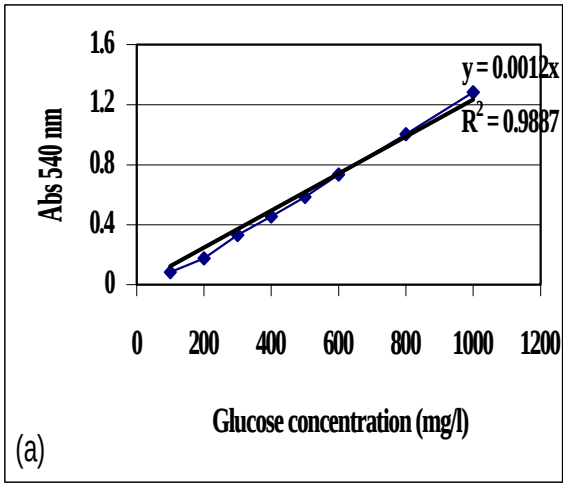
Source of yeast isolates	No.	Isolate number	Colony morphology																	Colony diam. (mm)	Cell size µm	Colony color	Vegetative reproduction	Vegetative cell
			Form				Elevation						Margin					Surface						
			Punciform	Circular	Irregular	Filamentous	Flat	Raised	Convex	Pulvinate	Umbonate	Crateriform	Entire	Undulate	Lobate	Filamentous	Fringed	Glistening	Dull					
41. Type strains *Saccharomycopsis fibuligera* TISTR 5033	1					\											\		\	4	ND	White	Pseudohyphae	Filamentous
Rhodotorula rubra	2			\					\				\					\		1-1.5	5.5-6*6	Red	Monopolar budding	Ogival
TISTR 5067																								
***Hansenula anomala* TISTR 5082**	3			\					\				\						\	1.5-2	3-5*3-5	White	Multilateral budding	ND
***Hansenula anomala* TISTR 5113**	4			\					\				\	\					\	1	3*3-7	White	Monopolar budding	ND
***Kluyveromyces maxianus* TISTR 5270**	5			\					\				\					\		1-1.5	2*3-4	White	Monopolar budding	ND
Sacharomyces cerevisiae burgandi	6			\					\				\					\		2-2.5	4-5*4-5	White, cream	Multilateral budding	Spherical

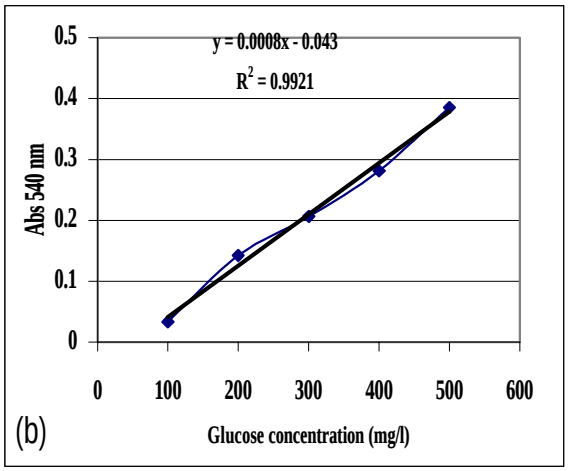
Table 1D. (continued).

Source of yeast isolates	No.	Isolate number	Colony morphology																	Colony diam. (mm)	Cell size µm	Colony color	Vegetative reproduction	Vegetative cell
			Form				Elevation						Margin					Surface						
			Punciform	Circular	Irregular	Filamentous	Flat	Raised	Convex	Pulvinate	Umbonate	Crateriform	Entire	Undulate	Lobate	Filamentous	Fringed	Glistening	Dull					
Sacharomyces cerevisiae champaign	7			\					\				\					\		2-2.5	4-5*4-5	White, cream	Multilateral budding	Spherical
Sacharomyces cerevisiae	8			ND					ND				ND					ND		ND	ND	ND	ND	ND
Sacharomyces bayanus	9			ND					ND				ND					ND		ND	ND	ND	ND	ND
Geotricum candidum	10					\											\		\	5-7	3-5*10-15	White	ND	ND
Candida utilis	11			\					\				\					\		2.5-3	2-3*6-7	White, cream	ND	ND
Candida krusei	12			ND					ND				ND					ND		ND	ND	ND	ND	ND
Candida famata	13			ND					ND				ND					ND		ND	ND	ND	ND	ND
Candida tropicalis	14			ND					ND				ND					ND		ND	ND	ND	ND	ND
Endomycopsis fibuligera	15			ND					ND				ND					ND		ND	ND	ND	ND	ND

Note. ND= not detected

E. Additional figures





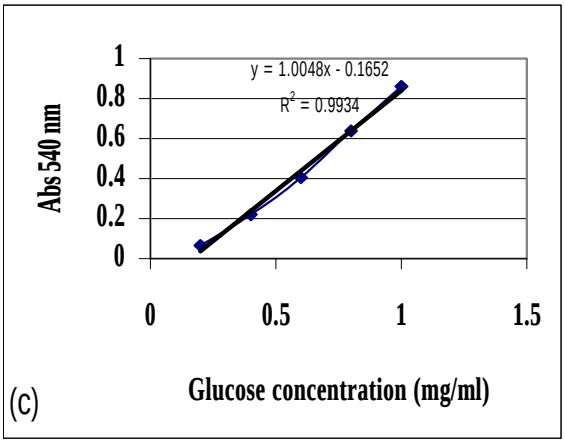
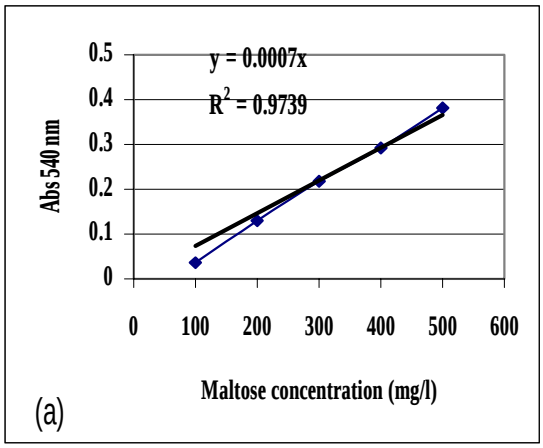
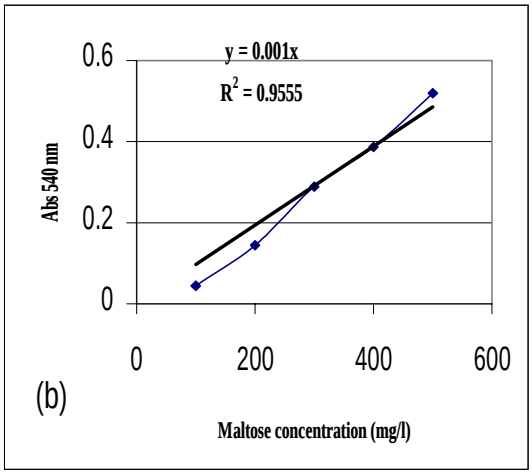


Figure 1E. Standard curves of reducing sugars (a), (b), and (c).





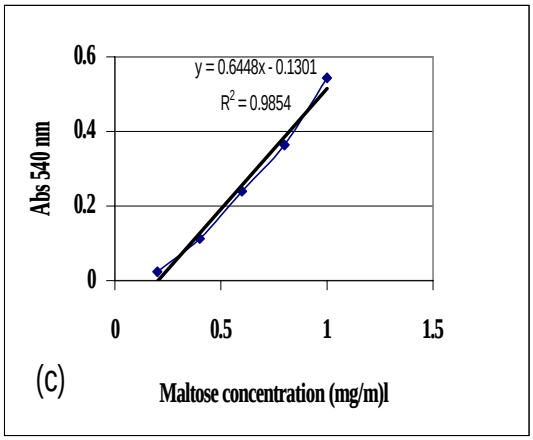


Figure 2E. Standard curves of maltose (a), (b), and (c).

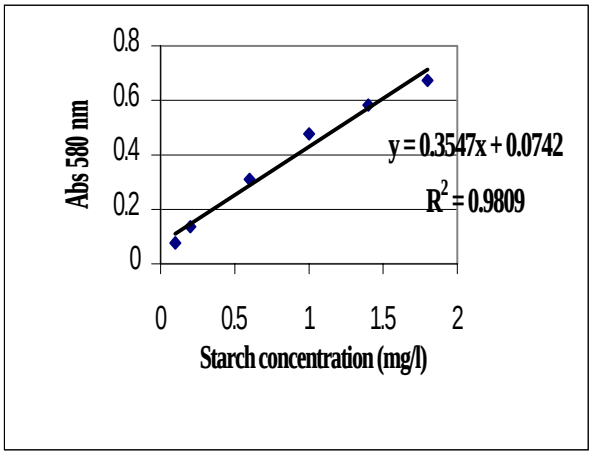


Figure 3E. Standard curve of starch (Khao-klong starch).

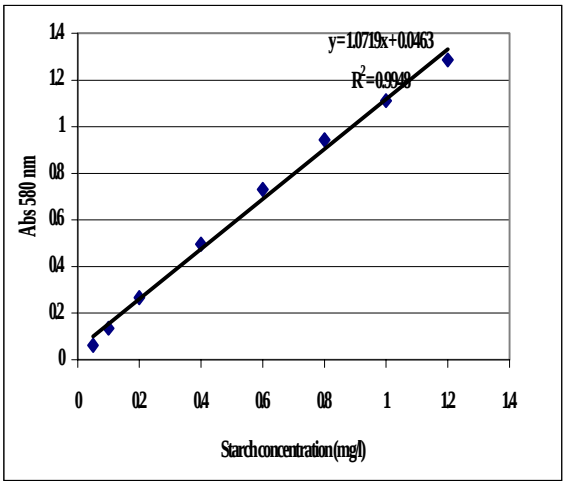


Figure 4E. Standard curve of starch (rice starch).

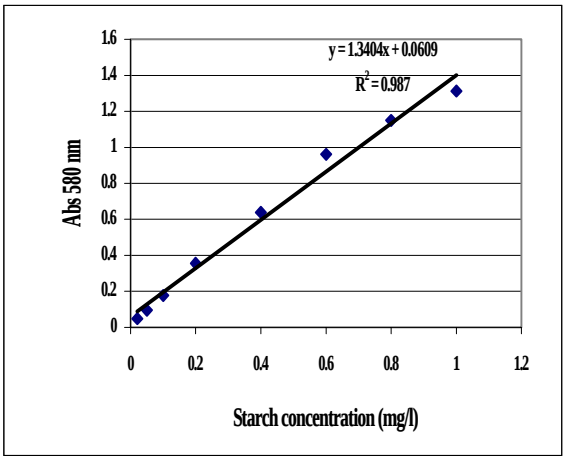


Figure 5E. Standard curve of starch (cassava starch).

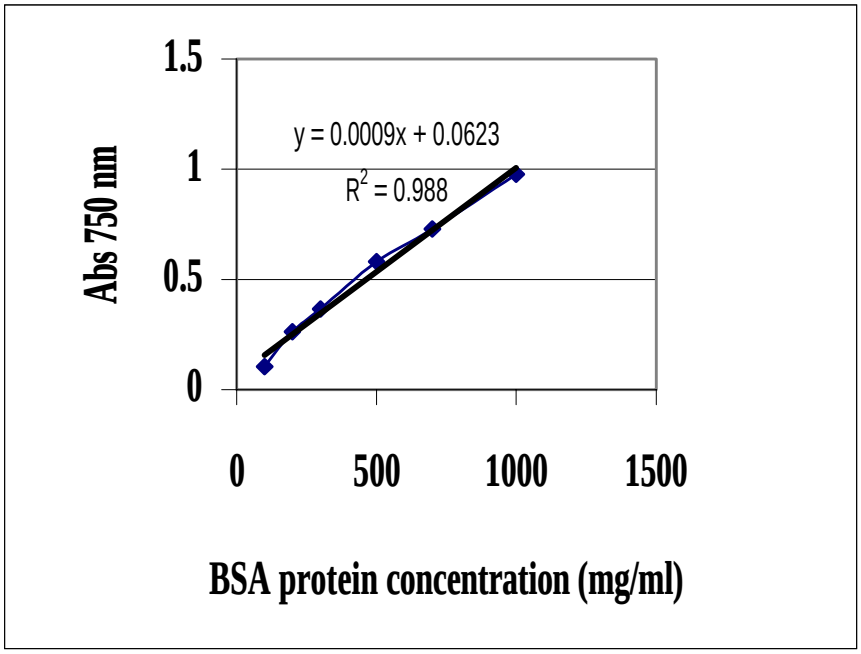
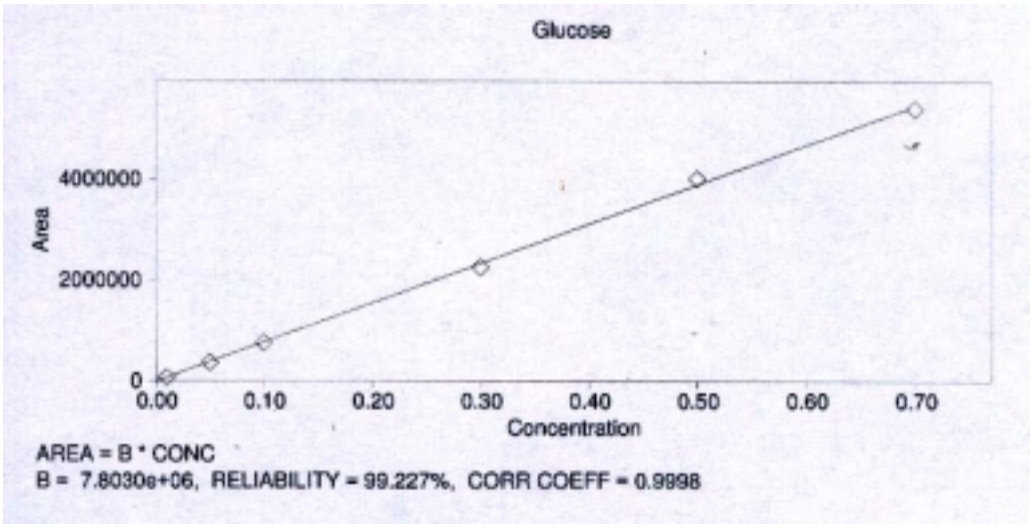


Figure 6E. Standard curve of protein**.**



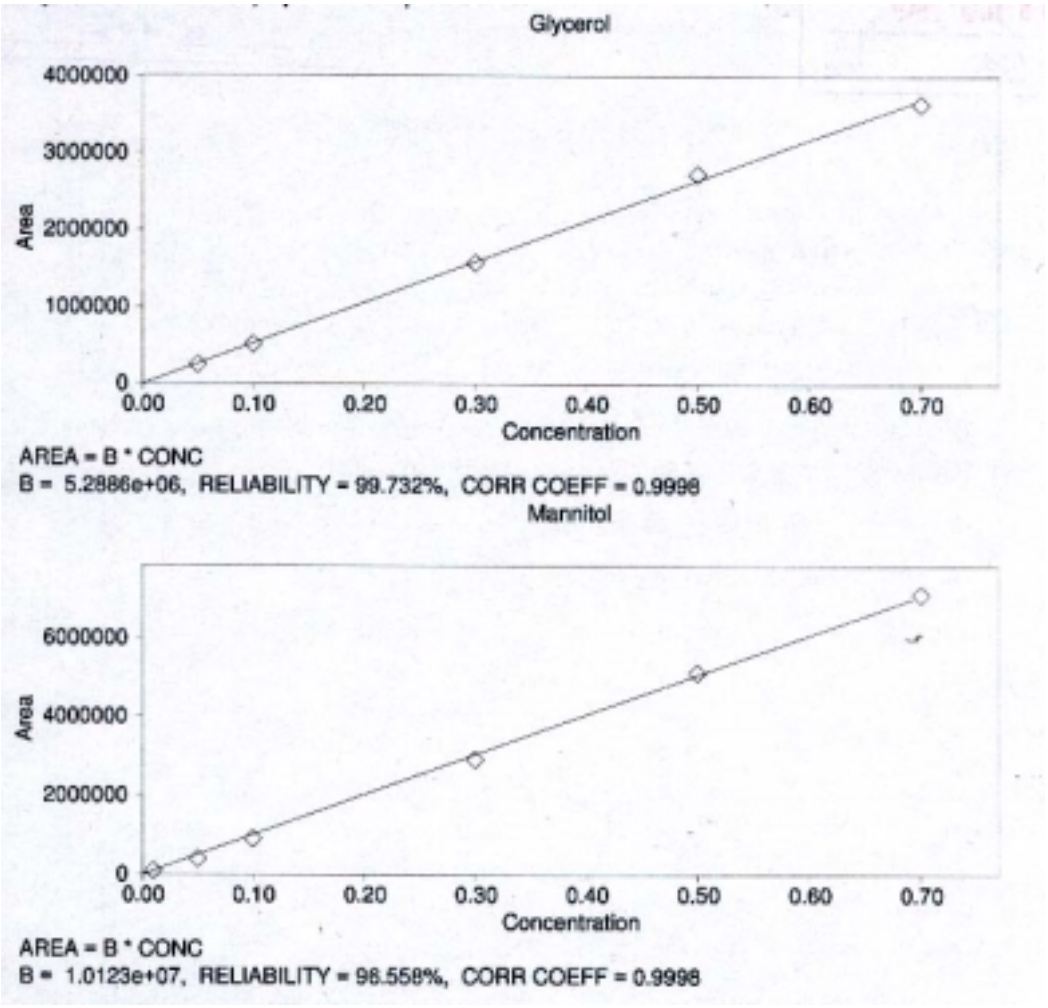
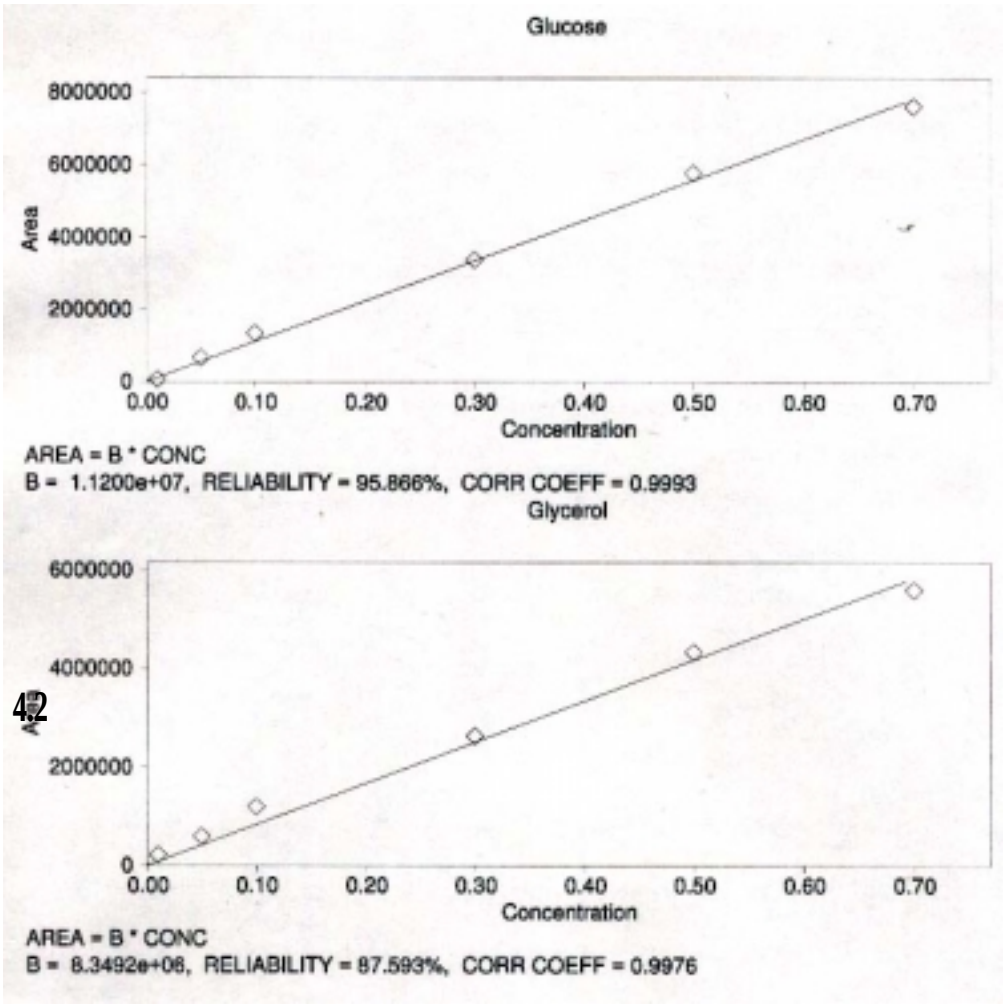


Figure 7E. Calibration curves for High-Performance Liquid Chromatography analysis by using refractive index detector.



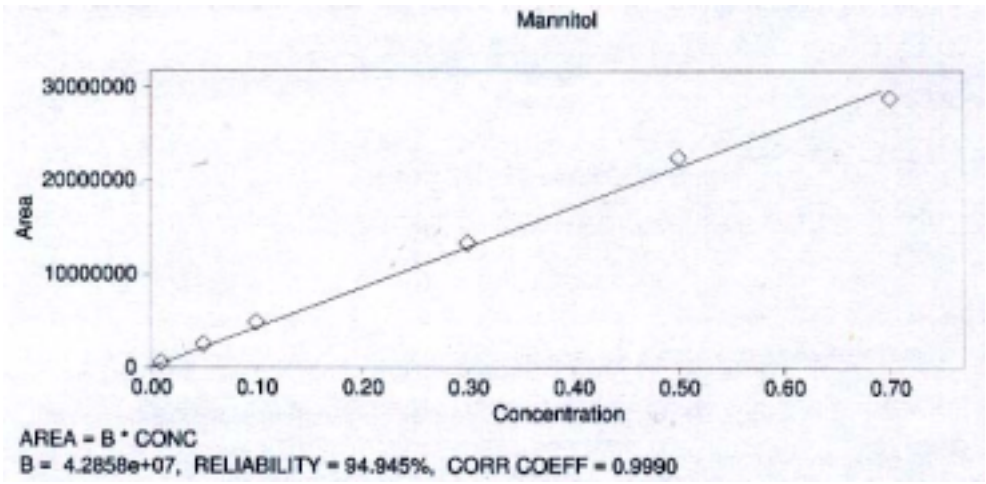
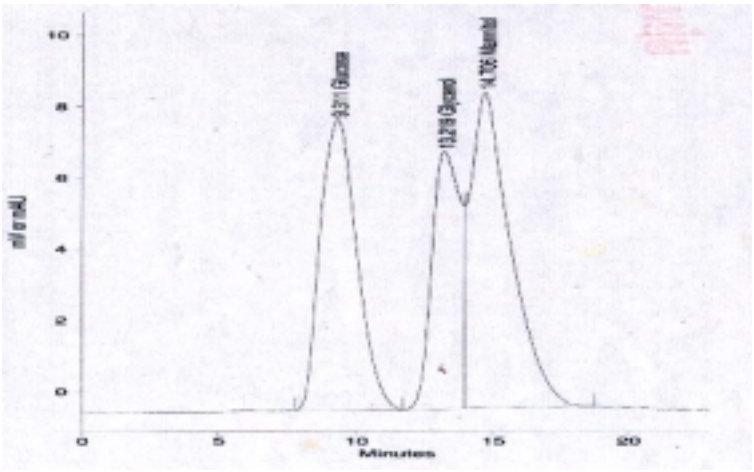
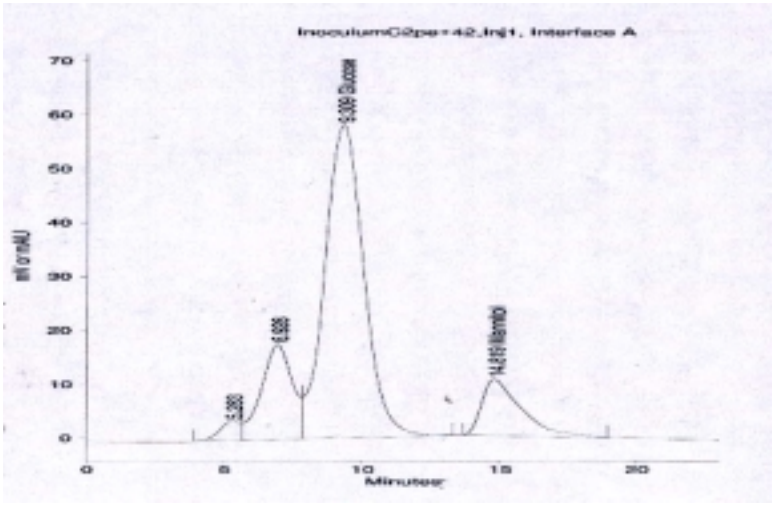


Figure 8E. Calibration curves for High Performance Liquid Chromatography analysis by using UV detector.





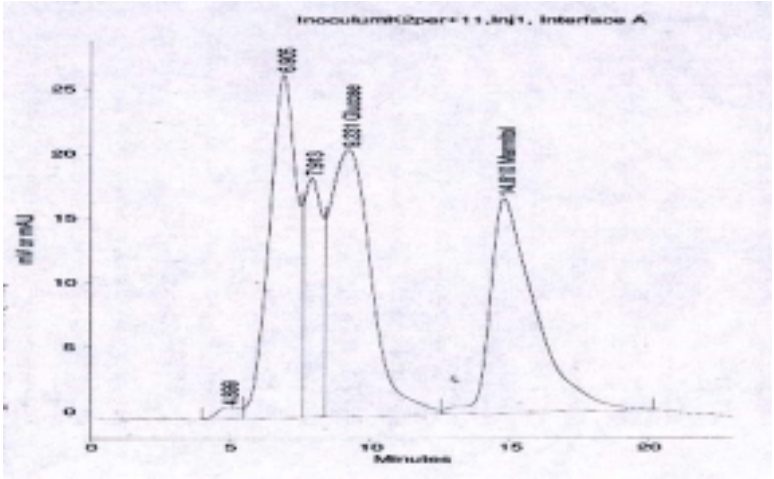


Figure 9E. Example of HPLC chromatogram.

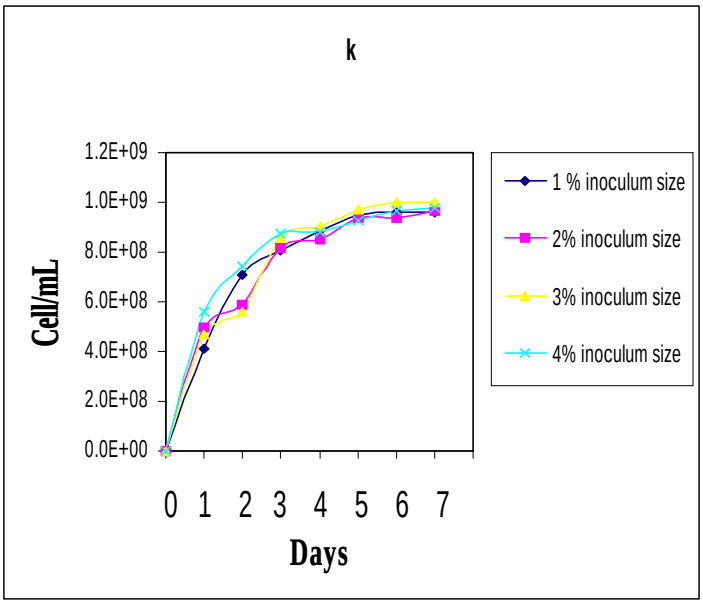


Figure 10E. Growth curve of yeast isolate KAY1 when cultured in starch medium containing 2% Khao-klong starch.

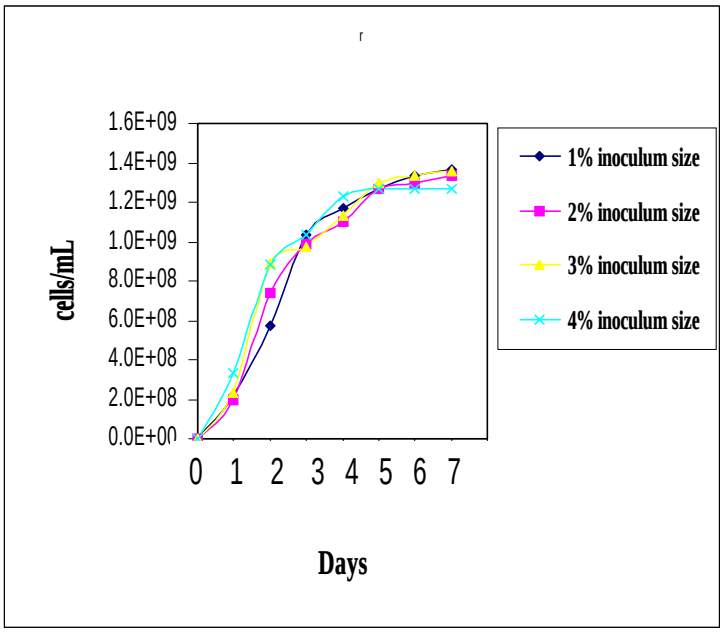


Figure 11E. Growth curve of yeast isolate KAY1 when cultured in starch medium containing 2% rice starch.

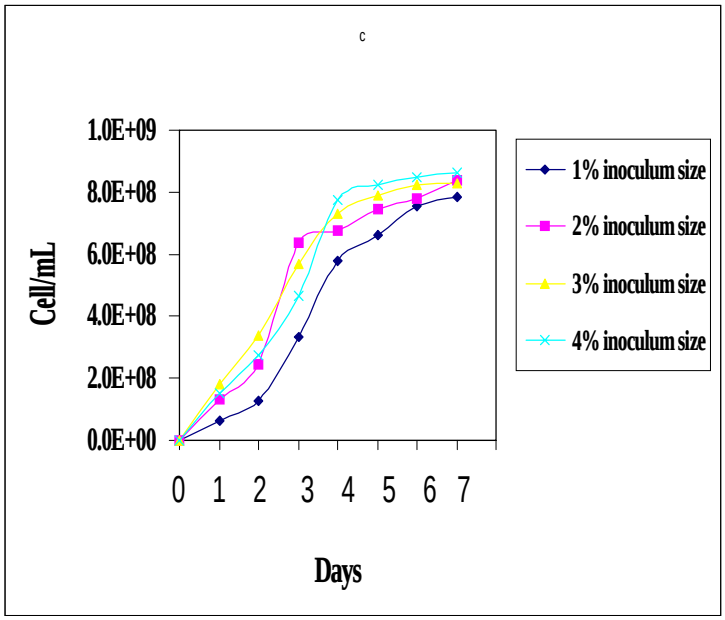


Figure 12E. Growth curve of yeast isolate KAY1 when cultured in starch medium containing 2% cassava starch.

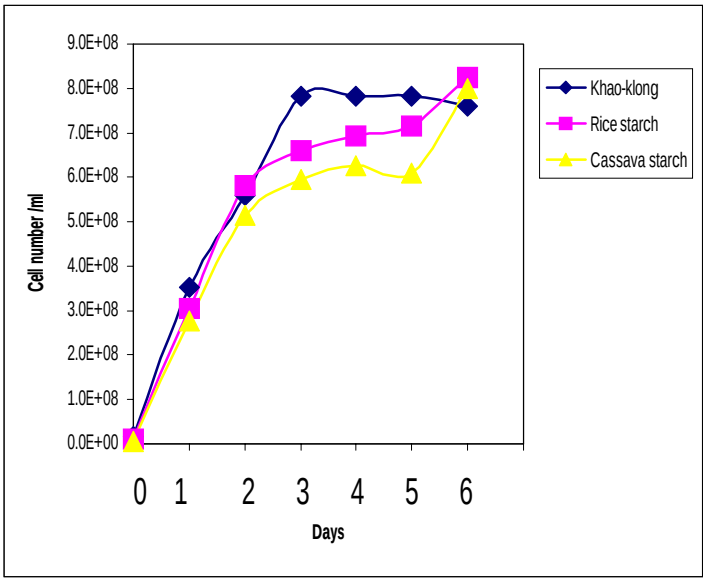


Figure 13E. Growth curve of yeast isolate KAY1 when cultured in starch medium containing 2% starch of either Khao-klong-, or rice-, or cassava-starch (Heat shock treatment at 45°C for 20 min).