ISOLATION AND CHARACTERIZATION OF THERMOTOLERANT YEAST FOR ETHANOL PRODUCTION

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A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Master of Science in Biotechnology

Suranaree University of Technology

Academic Year 2006

การคัดแยกและแสดงลักษณะเฉพาะของยีสต์ทนร้อนเพื่อผลิตเอทานอล

นางสาวอภิรดี ศรีภิรมย์รักษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
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ปีการศึกษา 2549

ISOLATION AND CHARACTERIZATION OF THERMOTOLERANT YEAST FOR ETHANOL PRODUCTION

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Master's Degree.

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อภิรดี ศรีภิรมย์รักษ์ : การคัดแยกและแสดงลักษณะเฉพาะของยีสต์ทนร้อนเพื่อผลิตเอทานอล (ISOLATION AND CHARACTERIZATION OF THERMOTOLERANT YEAST FOR ETHANOL PRODUCTION)
อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.โชคชัย วนภู, 98 หน้า.

การศึกษายีสต์ทนร้อนเพื่อการผลิตเอทานอลเป็นไปอย่างแพร่หลาย เนื่องจากยีสต์ทนร้อนสามารถเจริญและทำให้เกิดกระบวนการหมักได้ดีในประเทศเขตร้อน วัตถุประสงค์ของการศึกษาครั้งนี้คือเพื่อคัดแยกยีสต์ทนร้อนเพื่อผลิตเอทานอลและจัดจำแนกชนิดของยีสต์ที่คัดแยกได้ โดยสามารถคัดแยกยีสต์ทนร้อนสายพันธุ์ S1 ได้จากหญ้าหมักจากฟาร์มมหาวิทยาลัยเทคโนโลยีสุรนารี เมื่อศึกษาลักษณะทางสัณฐานวิทยาและสมบัติทางชีวเคมี จากการเลี้ยงเซลล์บนอาหาร yeast extract peptone dextrose (YPD) ที่อุณหภูมิ 40 องศาเซลเซียส เป็นเวลา 3 วัน พบว่าเซลล์มีรูปร่างเป็นรูปไข่จนถึงรูปท่อนปลายมน ขนาดเซลล์ประมาณ 2.7-4.2 x 5.6-10.1 ไมโครเมตร การเรียงตัวของเซลล์ทั้งเดี่ยวและคู่ พบการแตกหน่อของเซลล์ และมีการพัฒนาของเส้นใยเทียม สำหรับลักษณะการเจริญในอาหารเหลว เป็นแผ่นแห้งและหนาสีขาวเกาะอยู่บนบริเวณผิวหน้าของอาหารเหลว การเจริญบนอาหารแข็งโคโลนีมีความหนืดคล้ายเนยเหลว และมีสีครีม ยีสต์ทนร้อนที่คัดเลือกได้นี้ไม่สามารถสร้างสารพิษต้านทานต่อยีสต์สายพันธุ์ *Saccharomyces cerevisiae* EC 1118 แบคทีเรีย *Escherichia coli* ATCC 25922 และ *Bacillus subtilis* ATCC 6633 จากการวิเคราะห์สารพันธุกรรม โดยศึกษา 18S rDNA พบว่ายีสต์ทนร้อนสายพันธุ์ S1 มีความใกล้เคียงกับ *Issatchenkia* sp. 99% จากการศึกษาแหล่งไนโตรเจน แหล่งคาร์บอน (น้ำตาลกลูโคส) และค่าพีเอช (pH) ที่เหมาะสมต่อการผลิตเอทานอลที่อุณหภูมิ 40 องศาเซลเซียส ในอาหาร M9 minimal medium ปริมาตร 125 มิลลิลิตร ในฟลาสก์พบว่ายีสต์ทนร้อนสายพันธุ์ S1 ให้ปริมาณเอทานอลสูงสุด 74.58% โดยทฤษฎี ที่ค่าพีเอช 4.0 เมื่อใช้แอมโมเนียม ซัลเฟต และ น้ำตาลกลูโคส ความเข้มข้น 5% โดยปริมาตร เป็นแหล่งไนโตรเจน และ แหล่งคาร์บอน ตามลำดับ จากนั้นศึกษาการผลิตเอทานอลในถังหมักขนาด 2 ลิตรที่มีปริมาตรอาหาร 1.5 ลิตรโดยใช้สภาวะที่เหมาะสมต่อการผลิตเอทานอลที่ได้จากการศึกษาขั้นต้น พบว่ายีสต์ทนร้อนสายพันธุ์ S1ให้น้ำหนักเซลล์แห้งและผลิตเอทานอลได้มากที่สุดภายในเวลา 24 ชั่วโมง และการผลิตเอทานอลลดลงเมื่อให้อากาศตลอดกระบวนการหมักเป็นเวลา 72 ชั่วโมง ในทางตรงกันข้าม เมื่อให้อากาศ ภายใน 24 ชั่วโมงการผลิตเอทานอลจะคงที่ โดยให้ปริมาณเอทานอลสูงสุด 76.06% โดยทฤษฎี อย่างไรก็ตามการผลิตเอทานอลในถังหมักให้ผลการผลิตน้อยกว่าในระดับขวดฟลาสก์ เมื่อทดสอบการผลิตกรดอินทรีย์บางชนิดในอาหาร M9 minimal medium ด้วยวิธี High performance liquid chromatography (HPLC) พบว่า มีสารชนิดหนึ่งที่ไม่สามารถจัดจำแนกได้เกิดขึ้นในช่วงเวลาที่

13.5 นาที ควบคู่ไปกับการเจริญและการผลิต เอทานอลของยีสต์ทนร้อนสายพันธุ์ S1 ดังนั้นควรวิเคราะห์สารชนิดนี้ เนื่องจากยีสต์จะผลิตในปริมาณสูงมีความเข้มข้นประมาณ 40 เท่าของ กรดอะซีติก ซึ่งอาจเป็นผลมาจากเมทาบอลิซึมบางอย่างของยีสต์ในระหว่างกระบวนการหมัก

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

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ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

APIRADEE SRIPIROMRAK : ISOLATION AND CHARACTERIZATION OF THERMOTOLERANT YEAST FOR ETHANOL PRODUCTION.
THESIS ADVISOR : ASST. PROF. CHOKCHAI WANAPU, Ph.D. 98 PP.

THERMOTOLERANT YEAST/ETHANOL PRODUCTION

Ethanol production by thermotolerant yeasts have been extensively studied, because thermotolerant yeasts are capable of growth and fermentation during the summer months in non-tropical countries as well as under tropical climates. The aim of this study was to isolate and characterize thermotolerant yeast to produce ethanol. Thermotolerant yeast strain S1 was isolated from silage sample in Suranee University of Technology farm. According to the morphological and biochemical characterization, morphology of the thermotolerant yeast strain S1 cells are ovoidal to elongate, 2.7-4.2 x 5.6-10.1 µm, single or in pairs, budding cell are present and pseudomycelium are developed. The features of the appearance of cultures when cells grown in liquid medium after 3 days at 40 °C, heavy, dry climbing pellicles are formed on the surface of liquid medium and the growth is butyrous and light cream colored on agar medium. It can grow and ferment when glucose was used as a carbon source. This yeast was not producing killer toxin against with *Saccharomyces cerevisiae* EC 1118, *Escherichia coli* ATCC 25922 and *Bacillus subtilis* ATCC 6633. Genetic analysis was determined on the basis of 18S rDNA sequence, the results showed that the S1 strain had 99% similarity to *Issatchenkia* sp. The optimization of nitrogen and carbon (glucose) sources, and pH for ethanol production at 40 °C was observed using M9 minimal medium. In shake flask experiment, the thermotolerant yeast strain S1 gave the highest ethanol concentration (74.58% theoretical yield) at pH

4.0 when ammonium sulphate (1%, w/v) and glucose (5%, w/v) were used as nitrogen and carbon sources, respectively. The optimum condition for ethanol production in shake flask experiment was also conducted in 2 L fermenter. The yeast gave the highest cell dry weight and ethanol yield within 24 hours and the ethanol production was decreased within 72 hours. In contrast to aeration for 24 hours, ethanol production was stable about 76.06% of theoretical yield. However, the ethanol production was lower than that of shake flask experiment. Additionally, the production of some organic acids using HPLC technique was investigated. High amount of an unknown compound produced during the yeast growth and its ethanol production was also detected at the retention time of 13.5 minutes. Therefore, ethanol production by thermotolerant yeast strain S1 should be considered because this yeast produces an unknown compound (amount 40x of acetic acid), this might involve in some metabolisms of the yeast during fermentation.

School of Biotechnology

Academic Year 2006

Student's Signature_____

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ACKNOWLEDGEMENT

First of all, I would like to thank my family and Mr. Chalongchai Duangmung for the love, warm and support, especially my parents for their patience and sacrifice in making me what I am today.

I would like to express my gratitude to Asst. Prof. Dr. Chokchai Wanapu who gave me the inspiring, thoughtful guidance, stimulating suggestions and encouragement in all the time of research, and my deep appreciation is also expressed to Asst. Prof. Dr. Sureelak Rodtong and Assoc. Prof. Dr. Neung Teaumroong, for all advice and guidance.

I wish to express my gratitude to the officers in F1, F2 and F3 building for their kind and gave me the comfortable to do the experiments.

I would like to extend the special thanks to p'nuch, sang noy, dang, koy, kul, and tadam for their encouragements, technical, patience, power support, and friendliness. I also want to thanks my colleagues in School of Biotechnology at Suranaree University of Technology.

Finally, thank for destiny and all of my inspiration.

Apiradee Sripiromrak

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LIST OF ABBREVIATIONS

ATP	=	adenosine triphosphate
°C	=	degree celcius
DNA	=	deoxyribonucleic acid
dNTPs	=	deoxynucleosine 5’ phosphate
GC	=	gas chromatography
g	=	gram
g/L	=	gram per liter
h	=	hour
HPLC	=	high performance liquid chromatography
ITS	=	internal transcribed spacer
L	=	liter
µl	=	microliter
µm	=	micrometer
mM	=	millimolar
M	=	molarity
nm	=	nanometer
nM	=	nanomolar
N	=	normality
NADH	=	nicotinamide adenine dinucleotide
OD	=	optical density

LIST OF ABBREVIATIONS (Continued)

PCR	=	polymerase chain reaction
psi	=	pound per square inch
rpm	=	round per minute
rDNA	=	ribosomal deoxyribonucleic acid
rRNA	=	ribosomal ribonucleic acid
SEM	=	scanning electron microscope
SSF	=	simultaneous saccharification and fermentation
SDS	=	sodium dodecyl sulfate
T_{max}	=	maximum temperature
T_m	=	melting temperature
T_{min}	=	minimum temperature
T_{opt}	=	optimum temperature
tsp	=	thermo separation product
tRNA	=	transfer ribonucleic acid
U	=	unit
v/v	=	volume per volume
vvm	=	volumes of air per volume per minute
w/v	=	weight per volume
YPD	=	yeast extract peptone dextrose
YM	=	yeast extract-malt extract

CHAPTER I

INTRODUCTION

1.1 Significance of the study

The production of pure ethanol apparently begins in the 12-14th century along with improvements in the art of distillation permitting the condensation of vapors of lowers boiling liquids. During the middle ages, alcohol was not only mainly used for the production or as a constituent of medical drugs, but also for the manufacture of painting pigments and other chemical industries. And it was only in the 19th century that this trade became an industry with enormous production, due to economic improvements of the distilling process (Roehr, 2001). Now, ethanol is an important industrial chemical with emerging potential as a biofuel to replace vanishing fossil fuels (Alfenore *et al*., 2002).

Ethanol may be produced commercially by chemical synthesis or biosynthesis. Chemical synthesis is by hydration of ethylene (C_2H_4). For the biosynthesis, in the fermentation process, yeast uses monosaccharides as a carbon source and then converts to ethanol via glycosis under anaerobic conditions. The overall reaction can be summarized as follow:

$$\underset{\text{Glucose}}{C_6H_{12}O_6} + 2ADP + 2Pi \xrightarrow{\text{Yeast}} \underset{\text{Ethanol}}{2\ C_2H_5OH} + 2\ CO_2 + 2ATP + 2H_2O$$

At the beginning of the 20th century, several kinds of raw materials were exploited for ethanol production, such as molasses or agricultural production, and the possibility of hydrolyzing lignocellulosic materials was investigated (Roehr, 2001). Carbohydrate-rich raw materials suitable for ethanol production can be classified into three groups of agricultural products: which all sugar, starch and lignocellulose. The first raw material group, sugar refers to sugar-beet as well as sugarcane and molasses. The second group, starch from such crops as cassava, cereals and potatoes. The last group, lignocellulose, covers waste materials from the harvesting of agricultural crops such as rice straw, corn cob and sugarcane waste (Mogg, 2004).

In general, industrial yeast strains are able to grow and efficiently ferment ethanol at pH values of 3.5-6.0 and temperatures of 28-30° C, with efficiency dropping off rapidly at higher temperature. According to Roehr (2001), there are several potential benefits of thermotolerant yeast for using in the production of industrial alcohol as follows:

a. Thermotolerant yeast exhibits rapid metabolic activity and a high fermentation rate with high product output.

b. The solubility of oxygen and other gases in the fermentation broth decreases with increasing temperature. This phenomenon supports the establishment and long-term maintenance of anaerobic conditions.

c. The viscosity of the fermentation broth decreases with increasing temperature. Therefore, the energy required to maintain proper agitation of the growth media is reduced.

d. The metabolic activity of microbes and frictional effects of agitation serves to generate large amounts of heat. Thus, additional energy to maintain the vessels at

the desired temperature as well as the cooling requirements after sterilization is reduced.

e. The chances of contamination are minimized.

In this, there is insufficient evidence for the existence of a thermophilic yeast. This thesis will deal with thermotolerant yeast, defined here as those yeasts reported to grow at temperatures $\geq 40\,^{\circ}C$ following Slapack *et al*. (1987).

1.2 Research objectives

The purposes of this study are as follows:

2.1 To isolate and select thermotolerant yeast(s) for ethanol production

2.2 To study effects of pH and nutrients on ethanol production by selected thermotolerant yeast(s)

2.3 To identify the selected thermotolerant yeast

2.4 To apply the selected thermotolerant yeast for ethanol production

1.3 Research hypothesis

The thermotolerant yeast could promote high yield of ethanol at high temperature. Also, the metabolic activity of microbes and frictional effects of agitation serve to generate large amounts of heat. Thus, additional energy to maintain the vessels at the desired temperature as well as the cooling requirements after sterilization should be reduced.

1.4 Scope and limitation of the study

Thermotolerant yeasts were isolated from hot spring, wastewater from sugar and starch industries, silage, etc. All isolates of yeasts were screened and selected by considering the viability and ethanol fermentation at 40 °C. The optimization of some ethanol production conditions was investigated. Selected thermotolerant yeast isolate was identified using morphological, physiological and biochemical characteristics. Genomic analysis of the selected isolated was also performed.

1.5 Expected results

The high efficient thermotolerant yeast strain for ethanol production would be obtained. The yeast strain could be useful for ethanol industry.

CHAPTER II

LITERATURE REVIEW

2.1 Yeasts and thermotolerant yeasts

2.1.1 Yeasts

Yeasts are ascomycetous or basidiomycetous fungi that reproduce vegetatively by budding or fission, and that form sexual states which are not enclosed in a fruiting body (Boekhout and Kurtzman, 1996). The yeast species are all characterized by a similar set of features, both morphological and physiological. This type of description, in which physiological characters are important, distinguishes yeast taxonomy from other fungal taxonomy (Kreger-van Rij, 1984).

Identification of yeast genera can often be achieved by morphological tests supplemented with a few physiological tests. With regard to the latter, sole carbon and nitrogen source assimilation by yeasts may be determined by auxanography which nowadays can be conveniently carried out using commercially available kits: for example, Analytical Profile Index (API) strips (BioMérieux, France) or the automated/computerized BCCM/Allev 2.00 system (Louvain-la-Neuve, Belgium). Sugar assimilation and fermentation tests are commonly accomplished using glucose, galactose, maltose, sucrose, lactose, raffinose, trehalose and xylose. With regard to fermentation of these sugars, Scheffers (1987) has argued that the anaerobic liberation of CO_2 into Durham tubes is not very accurate for detecting slowly fermenting yeast

species. Ethanol production assays are deemed to be more appropriate determinants of sugar fermentation by yeasts (Walker, 1998).

Yeasts are used in many industrial processes, such as the production of alcoholic beverages, biomass and various metabolic products. The last category includes enzymes, vitamins, capsular polysaccharides, carotenoids, polyhydric alcohols, lipids, glycolipids, citric acid, ethanol, carbon dioxide and compounds synthesized by the introduction of recombinants DNA into yeasts. Some of these products are produced commercially while others are potentially valuable in biotechnology (Kurtzman and Fell, 1997). Some yeast species have potential to be uses in food, beverage and fermentation industries (Jacobson and Jolly, 1989) (Table 2.1).

Table 2.1 Some present and potential uses of yeasts in the food, beverage and fermentation industries (Jacobson and Jolly, 1989)

Application	**Yeast species**
Ale fermentation	*Saccharomyces cerevisiae*
Bread and dough leavening	*S. cerevisiae, S. exiguus, S. rosei*
D-Arabitol (sweetener)	*Candida diddensiae*
Emulsifier	*C. lipolytica*
Ethanol fermentation	*S. cerevisiae*
Fish and poultry feeds (astaxanthin)	*Phaffia rhodozyma*
Fodder and single cell protein	*C. utilis*
Lactose and milk fermentation	*C. pseudotropicalis, Kluyveromyces fragilis, K. lactis*

Table 2.1 (Continued)

Application	**Yeast species**
Lager beer fermentation	*S. carlsbergensis*
Mannitol (humectant)	*Torulopsis manitofaciens*
Shoyu, Miso	*Zygosaccharomyces rouxii*
Wine fermentation	*S. cerevisiae*
Xylitol (sweetener)	*T. candida*
D-xylose fermentation	*C. shehatae, Pachysolen tannophilus, Pichia stipitis, P. segobiensis*

Yeasts occur widely in nature and have been recovered from widely differing terrestrial as well as marine sources. Certain yeasts are more or less ubiquitous while others appear to be restricted to very specific habitats. Yeasts seldom occur in the absence of either molds or bacteria (Kreger-van Rij, 1984).

2.1.2 Thermotolerant yeasts

Temperature is one of the most important environmental factors affecting microbial activity. Microorgnisms have generally been divided into three groups: psychophiles, mesophiles and thermophiles depending on their range of temperature for growth. The range of temperature consists of minimum (T_{min}), optimum (T_{opt}) and maximum (T_{max}) temperatures. Microorganisms, which fall into overlapping categories, have been classified as thermotolerant mesophiles or as thermophiles, depending on the point of view of the investigation. In many cases, researchers have reported the highest temperature at which growth is detected (Slapack *et al*., 1987).

Arthur and Watson (1976) defined psychrophilic yeast, temperature limits of growth, 2 to 20 °C; mesophilic yeast, temperature limits of growth, 5 to 35 °C; thermophilic yeast, temperature limits of growth, 28 to 45 °C; and thermotolerant, temperature limits of growth, 8 to 42 °C. The term is usually used to describe yeast, which grows at temperature slightly above the T_{max} of most yeasts but very little consistency in the literature as to how the optimum temperature should be defined. The optimum temperature can be defined as the temperature at which growth rate, fermentation rate, or the cellular yield is the highest. Travassos and Cury (1966) defined thermophilic yeasts as those growing optimally at ≥ 37 °C. Some literature refers to thermophilic yeasts, where the organisms can only grow up to 37 °C, while yeasts, which grow at temperatures exceeding 45 °C, are refered to as thermotolerant by other investigators. McCracken and Gong (1982) defined thermotolerant yeasts as those with a T_{min}, T_{opt}, and T_{max} of 20 °C to 26 °C, 26 °C to 35 °C, and 37 °C to 45 °C, respectively, while mesophiic yeasts had a T_{min} of 5 °C to10 °C, T_{opt} of 24 °C to 30 °C, and T_{max} of 35 °C to 40 °C. However, it becomes evident that these schemes are not appropriate for many yeasts, e.g., *Candida macedoniensis* (T_{min} 5 °C, T_{max} 45 °C) and *Saccharomycopsis guttulata* (T_{min} 34 °C, T_{max} 42 °C). The highlighting will be placed on yeasts capable of ethanol production at ≥ 40 °C and/or ≥ 45 °C as described in Table 2.2.

Table 2.2 Thermotolerance of yeast genera tested to grow and produce ethanol at 37 °C to 45 °C (Hacking *et al*., 1984)

Genus	Number of species			
	Total test at 37 °C	**Above 50% ethanol yield at 37 °C**	**Above 50% ethanol yield at 40 °C**	**Growth and ethanol production at 45 °C**
Candida	15	5	4	1
Hansenula	7	1	0	0
Kluyveromyces	12	8	5	5
Pichia	4	0	0	0
Saccharomyces	14	13	3	0
Schizosaccharomyces	2	1	0	0
Torulopsis	1	0	0	0

2.1.3 Effect of high temperature on cellular components of thermotolerant yeast

The ability of microorganisms to adapt to different temperature environments has attracted considerable attention, but the mechanism underlying this phenomenon is not well understood (Arthur and Watson 1976). In order for growth and/or fermentation to occur at high temperature, all cell constituents must be stable and functional. Thermophiles appear to encompass a range of molecular mechanisms and do not appear to be due to any single factor. Table 2.3 presents a brief overview on some of various cell components (Slapack *et al*., 1987).

Table 2.3 Possible physiological basis of thermostability of cellular components (Slapack *et al*., 1987)

Component of protein synthesis
Higher GC content and melting temperature (T_m) of nucleic acid
DNA repair
Rapid resynthesis (mRNA)
Unique bases (tRNA-thiolated nucleotides, ribose methylations)
Increase of thermostability of enzymes required for protein synthesis (tRNA syntheases, polymerases
Higher T_m of ribosome (especially 50S subunit)
Interaction between ribosomes, ribosomal proteins, nucleic acids, and possibly membranes, leading conformation changes
Proteins
Amino acid composition and sequence (especially hydrophobic ones)
Type of bonding (hydrophobic, ionic, hydrogen) directs protein folding and conformations
Association with membranes
Rapid protein resynthesis
Activation or denaturation energy required
Ionic strength
Presence of cations (Ca^{+2}, Mg^{+2}, K^{+}, NH_4^{+})
Heat shock proteins, glycosylated proteins
Salt bridges and aggregation
Unique stabilizing factors
Polyamines
Polyols (glycerol)
Trehalose
Membranes and lipids
Composition of phospholipids (cardiolipin) and class of neutral lipids
Shift in fatty acids with higher T_m (increased chain length, degree of branching, molecular geometry)
Increase degree of saturation of fatty acids and/or sterols for more rigid membrane
Unique lipids
Increase protein-lipid ratio
Reversible gel to liquid—crystalline phase transition
Interaction of membrane proteins and lipids

The thermopile yeasts were reported to the heat stability of intracellular membranes. When these membranes are heat labile the yeasts are not thermophilic. An important component of membranes in temperature adaptation is unsaturated fatty acid. Psychrophilic yeasts have a high content of unsaturated fatty acids (Kocková-Kratochvílová, 1990). Some of fatty acid compositions of yeasts examples are shown in Table 2.4.

Arthur and Watson (1976) concluded that a knowledge of the properties of the biological membrane in fundamental to an understanding of the ability of a microorganism to grow and reproduce in different temperature environments.

Table 2.4 Fatty acid composition of psychrophilic, mesophilic, thermotolerant and thermophilic yeasts (Arthur and Watson, 1976)

Thermal domain	Yeasts	Percentage of total fatty acids								
		Saturated				Unsaturated [a]				
		$< C_{14}$	C_{14}	C_{16}	C_{18}	$C_{14:1}$	$C_{16:1}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
Psychrophile	*Lucosporidium frigidum*	1±1[b]	2±1	6±1	Tr[c]	Tr	Tr	11±1	27±2	53±2
Mesophile	*C. lipolytica*	2±1	3±1	6±1	1±1	3±1	20±2	44±5	21±4	Tr
Thermo-	*C. parapsilosis*	2±1	2±1	18±1	6±1	Tr	3±1	53±2	16±1	Tr
tolerance	*S. telluris*	3±1	3±1	12±1	3±1	Tr	3±1	58±2	17±1	Tr
Themophile	*Torulopsis bovina*	3±1	16±1	9±1	4±1	3±1	42±1	24±3	-	-
	C. sloofii	4±1	26±1	6±1	4±1	3±1	34±1	23±3	-	-

[a] Degree of unsaturation (percentage of monoene+2[percentage of diene]+3[percentage of triene])/100.
[b] ±Standard deviation for four to six values.
[c] Tr, Trace, less than 1%.

Thermotolerance may be defined as the transient ability of cells subjected to high temperatures to survive subsequent lethal exposures to elevated temperatures

(Laszlo, 1988). Yeast cells exhibit a rapid molecular response when exposed to elevated temperature. This is called the heat-shock response and is a ubiquitous regulatory phenomenon in all living cells. Sub-lethal heat-shock treatment of yeast leads to the induction of synthesis of a specific set of proteins, the highly conserved "heat-shock proteins" (Hsps) (Walker, 1998). The known functions of the major classes of Hsps in *S. cerevisiae* are summarized in Table 2.5.

Table 2.5 Major heat-shock proteins of *S. cerevisiae* (Watson, 1990; Mager and Moradas-Ferreira, 1993; Parsell and Lindquist, 1994; Mager and De Kruijff, 1995; Tsiomenko and Tuymetova, 1995)

Heat-shock protein	**Proposed physiological function**
Hsp104	Acquisition of stress tolerance, constitutively expressed in respiring, not fermenting cells and on entry in to stationary phase
Hsp83	Chaperon function
Hsp70 family	Interact with denatured, aggregated proteins and assists in solubilizing them with simultaneous refolding (i.e. chaperon function), also involved in post-translation import pathways
Hsp60	Similar to Hsp70, this chaperonin famil facilitate post-translational assembly of proteins
Small Hsps	
Hsp30	Cellular role still elusive, but may be involved in entry into stationary phase and the induction of sporulation, Hsp30 may regulate plasma membrane ATPase
Hsp26	
Hsp12	
Others	
Ubiquitin	Responsible for much of the turnover of stress-damaged proteins
Catalase	Antioxidant defence
GP400 and P150	Secretory heat-shock proteins (unknown function)

Several Hsps have been shown to perform molecular 'chaperoning' functions in the yeast cell, while others are implicated in conferment of thermotolerance, glycolysis and polyubiquitination of proteins (Parsell and Lindquis, 1994). Hsps functioning as chaperons (e.g. Hsp60, chaperonin) prevent protein aggregation and the accumulation of aberrant proteins. Hsps may also assist in the degradation of stress-damaged proteins by enhancing the flow of substrates through proteolytic pathways. Recently, eukaryotic chaperon function has been studied genetically in yeast, and *S. cerevisiae* chaperonon Cct (chaperonin-containin-tailless complex polypeptide) was shown to be required for the *in vivo* assembly of cytoskeletal elements (Stoldt *et al*., 1996). The heat-shock response in *S. cerevisiae* is one of the best molecularly characterized responses of eukaryotic cells and has been widely reviewed (Watson, 1990; Piper, 1993; Parsell and Lindquist, 1994; Mager and De Kruijff, 1995; Ruis and Schüller, 1995). Hsp gene expression basically involves increased transcription of genes containing the promoters of the heat shock element (HSE) which occurs in the presence of heat due to the activation of the heat shock transcription factor (HSF). In *S. cerevisiae,* the HSE is unresponsive to other stresses (osmotic, oxidative, DNA damage, glucose repression, etc.) and is exclusively induced by a sub-lethal heat shock. The HSF in yeast, which is an essential protein involved in normal growth, is not required for induced tolerance against severe stress, but its activation by heat shock is required for growth at high temperatures (Ruis and Schüller, 1995). Although several authors have implicated Hsps in yeast thermotolerance (and ethanol tolerance), the functional significance of these proteins in stress tolerance is still unresolved. Indeed, an obligatory role for Hsps in thermotolernace of *S. cerevisiae* has recently been questioned by Gross and Watson (1996) who showed that non-

fermenting cells acquired thermotolerance in the absence of protein synthesis. Nevertheless, a few specific Hsps are recognized to exert a protective role against thermal stress in *S. cerevisiae* (Piper, 1993). Lindquist and Kim (1996) have provided compelling evidence that the synthesis of one particular Hsp, namely Hsp104, is required to confer thermotolerance in respiratory (but not fermentative) cultures of *S. cerevisiae* .

2.2 Classification and identification of yeasts

Yeasts are classified on the basis of the microscopic appearance of the cells, the mode of sexual reproduction, certain physiological features (especially metabolic capabilities and nutritional requirements) and biochemical features.

The physiological features, that distinguish different yeasts, include the range of carbohydrates (mono-, di-, tri-, and polysaccharides) that a given organism can use as a source of carbon and energy under semi-anaerobic and aerobic condition, the relative ability to grow in the presence of 50-60% (w/v) D-glucose or 10% (w/v) sodium chloride plus 5% (w/v) glucose (a measure of osmotolerance) and the relative ability to hydrolyze and utilize lipids. These properties help investigators determine which yeast strains merit investigation for a particular application (Glazer and Nikaido, 1995).

Yeasts, which form one of the important subclasses of fungi, are rather more complex and usually larger than bacteria. They are distinguished from most fungi by their usual existence as single ovoid cells about 8 μm long and 5 μm in diameter, doubling every 1-3 hours in favorable media (Wayman and Parekh, 1990).

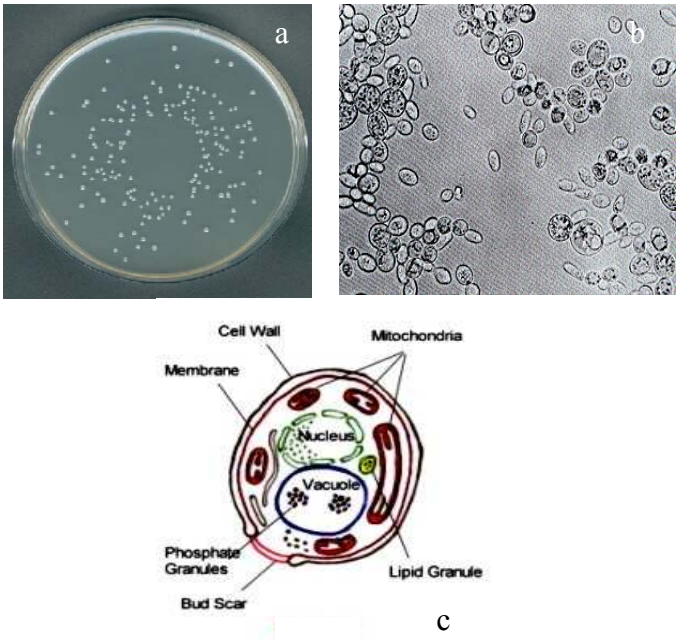


Figure 2.1 Yeast cell; a) colonies of *S. cerevisiae* on agar plate (www, 2005), b) *S. cerevisiae* under microscrope (400x) (www, 2006) and c) Yeast cell composition (www, 2002)

Individually yeast cells appear colorless, but when grown on artificial solid media they produce colonies which may be white, cream colored, or tinged with brownish pigments. Colony characteristics are useful in the taxonomy of yeasts, very difficult group to classify (Figure 2.1). Physiological characteristics are also used to a great extent in determining yeast species (Alexopoulos, 1962). Yeasts may reproduce asexually or sexually (Wayman and Parekh, 1990).

a) Asexual reproduction

Alexopoulos (1962) classified yeasts into the budding yeasts and the fission yeasts, depending on their types of asexual reproduction. The budding yeasts reproduce by budding, in this process the protoplasm of the cell, covered by a thin

membrane, pushes out of the cell wall in the form of a bud and forms a daughter cells (Figure 2.2). The bud enlarges until it is separated from the mother cell by a constriction at the base. Under some conditions, buds do not separate from the mother cell and a branched chain of cells called a pseudomycelium forms (Figure 2.3).

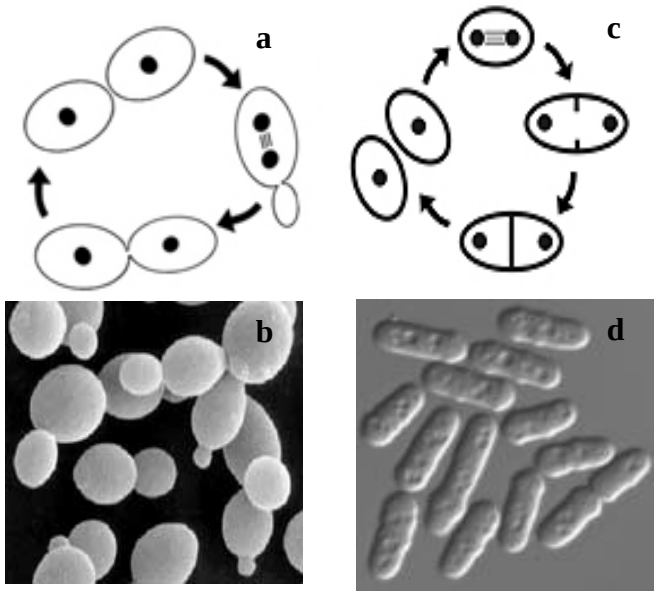


Figure 2.2 Asexual reproduction; a) budding yeast. Formation of new cell begins with blowing out of a new cell, at the pole of the cell. Mitosis follows with migration of one nucleus to the new cell. New wall material is then laid down in the passage between the two cells and separation of the cells will occur (www, 2005), b) shows *S. cerevisiae* cells reproducing asexually by budding (www, 2003) and c) fission yeast. Mitosis of the nucleus occurs and, follows by elongation of the cell and formation of a cell wall that divides the cell in half, and separates the two nuclei (www, 2005); d) shows *Schizosaccharomyces pombe* cells reproducing asexually by fission (www, 2003)

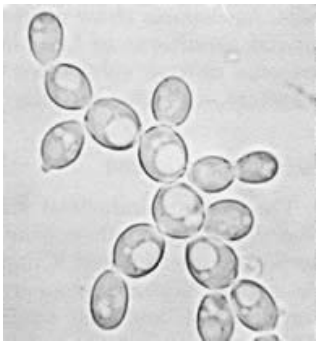


Figure 2.3 Chain of yeast cells (pseudomycelium) produced by budding (www, 2005)

During the process of buding, the nucleus divides, one daughter nucleus passing into the bud, the other remaining in the mother cell. Most known yeasts reproduce by budding such as *S. cerevisiae.*

The fission yeasts reproduce by transverse division. The parent cell elongates, the nucleus divides, and a transverse wall (septum) in laid down some where near the middle, separating the mother cell into two uninucleate daughter cells. This septum is formed by annular growth beginning at the wall and proceeding in ward. The new wall thickens before the daughter cells separation (Conti and Naylor, 1959).

b) Sexual reproduction

Sexual union in the yeasts takes place either between two somatic cells or between two ascospores which assume the function of copulating gametangia, unite and form a zygote cell. Eventually an ascus forms which contains ascospores, their number depending on the number of nuclear divisions which take place and on the subsequent development of the nuclei. Four or eight ascospores per ascus are the usual number, but other numbers may also be encountered. Figure 2.4 shows the

reproduction of yeast, proceeding by the formation of buds on the cell surface, but sexual reproduction can be induced under special condition. In the sexual cycle, a normal diploid cell divides by meiosis, and sporulation gives rise to asci, or spore cells, that usually contain four haploid ascospores. The ascospores are of two mating types; a and α. Each type can develop by budding into order haploid cells. The mating of an a haploid cell and an α haploid cell yields a normal α diploid cell. Haploid cells of the same sex also unite occasionally to form abnormal diploid cells (a/a or α/ α) that can reproduce only asexually, by budding in the usual way. The majority of industrial yeasts reproduce by budding (Glazer and Nikaido, 1995).

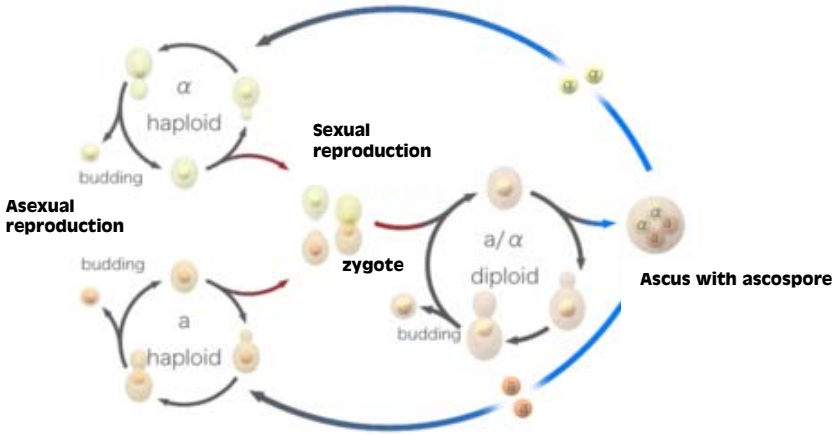


Figure 2.4 The reproduction of yeast by sexual and asexual reproduction (www, 2005)

Ascospores formed by yeasts are often globose or ovoid, as in *Debaryomyces, Saccharomyces, Schizosaccharomyces*, and *Saccharomycodes* (Figure 2.5). Other yeasts form different types of ascospores. Thus, in *Pichia* and some species of *Hansenula*, the ascospores are hat-shaped; in other species of *Hansenula* they may be hemispherical of shaped like the planet Saturn. Release of ascospores may occur

when the ascus wall deliquesces; this is the usual method of release in species with hat- or Saturn-shaped spores. In other species the germinating spores bud or form germ tubes, which results in bursting of the persistent ascus wall (Alexopolos *et al*., 1996). Miller (1989) pointed out that yeast ascospores are much more durable than somatic cells and have the ability to withstand even snail gut enzyme, a distinct advantage in their natural environment.

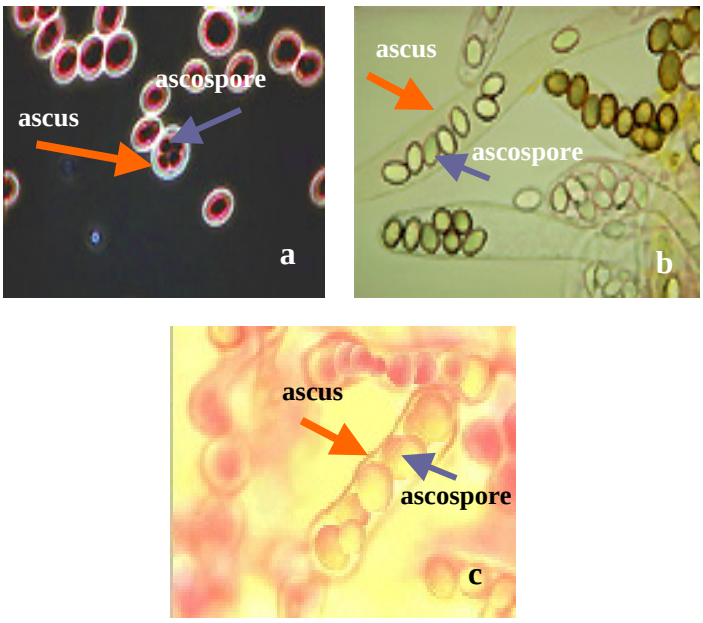


Figure 2.5 Yeast ascospores; a) an ascus with four ascospores of *S. cerevisiae*, b) cylindrical asci of *Ascobolus stercorarius*, with eight ascospores in each ascus and c) shows at least three asci with ascospores of *Sch. octosporus* (www, 2005)

However, morphological, physiological and biochemical tests have commonly been used for phenotypic characterization of yeast species. These methods are often unreliable, due to strain variability and, therefore, do not allow differentiation between yeast strains belonging to the same species. Genetic characterization using molecular

techniques provides more powerful means of strain identification and differentiation among strains (Recek *et al*., 2002).

For identification based on genomic analysis, ribosomes are an indispensable component of the protein synthesis apparatus, and their structures are strictly conserved, the DNA component of the small subunit ribosome has proved to be an important and useful molecular clock for quantifying evolutionary relationships between organisms. Generally, the rate of base substitutions, deletions or insertions in various regions of the rRNA gene is not uniform; some areas are highly conserved and unchanged through millions of years, some are highly variable and others are semiconserved (Nishimura and Mikata, 2000). Application of gene sequence analyses to yeast systematics has shown conflict between the placement of species on gene trees and their classification from phenotype. For example, the genus *Wingea* had been described because of the uniqueness of its lenticular ascospores, but phylogenetic analysis of rRNA sequences has placed it in the genus *Debaryomyces*, where species are generally characterized by roughened, spheroidal ascospores (Kurtzman and Robnett, 1994).

These is now a widespread pattern of disparity between phenotype and genotype as means for classifying yeasts, and these differences have been demonstrated from analyses of 18S ribosomal DNA (rDNA) (James *et al*., 1994; Cai *et al*., 1996; James *et al*., 1997), internal transcribed spacer (ITS) rDNA (James *et al*., 1996) and 26S rDNA (Kurtzman and Robnett, 1995 and 1998), leaving little doubt that phenotype is a poor predictor of genetic relationships among species (Kurtzman and Robnett, 2003).

2.3 Yeast killer toxin

Yeast killer toxins are protein compounds, which are active against members of the same species or closely related species, and the activities of these toxins are analogous to the activities of bacteriocins in bacterial species (Lowes *et al*., 2000).

The killer strain has immune to the effect of its own toxin (Magliani *et al*., 1997, Bartunek *et al*., 2001; Schmitt and Breinig, 2002; Ceccato-Antonini *et al*., 2004;). Killer, sensitive, and neutral phenotypes of *S. cerivesiae* were first described by Makower and Bevan (Marquina *et al*., 2002). When sensitive and killer cells grow in the same culture medium, a high proportion of the sensitive cells will died. The neutral cells do not kill sensitive cells, nor are killed by the killer ones (Ceccato-Antonini *et al*., 2004). At present, killer activity has been found in about 80 species representing almost 20 yeast genera (Wickner, 1985; Bruenn, 1986; Young and Yagiu, 1987) are shown in Table 2.6. There exist many classes of killer yeast strains differing particularly in the spectrum of their activity against sensitive strains, in their cross-reactivity, genetic determination of killer toxin and killer toxin immunity, and in features and molecular mechanisms of killer toxin action (Bartunek *et al*., 2001).

Table 2.6 Yeast species for which killer activity has been reported

Yeast species	**Reference**	**Yeast species**	**Reference**
Candida albicans	Rogers and Bevan (1978)	*Pichia acaciae*	Worsham and Bolen (1990)
C. dattila	Choi *et al*. (1990)	*P. amethionina*	Starmer *et al*. (1987)
C. glabrata	Sriprakash and Batum (1984)	*P. anomala*	Sawant *et al*. (1989)
		P. antillensis	Starmer *et al*. (1987)
C. guilliermondii	Polonelli *et al*. (1987)	*P. bimundalis*	Polonelli *et al*. (1987)
C. holmii	Nagornaya *et al*. (1989)	*P. cactophila*	Starmer *et al*. (1987)

Table 2.6 (Continued)

Yeast species	Reference	Yeast species	Reference
C. krusei	Lehmann *et al*. (1987a)	*P. canadensis*	Lehmann *et al*.(1987a)
C. maltosa	Polonelli *et al*. (1987)	*P. ciferrii*	Nomoto *et al*. (1984)
C. neodendra	Suzuki *et al*. (1989)	*P. fabianii*	Polonelli *et al*. (1987)
C. parapsilosis	Zekhnov *et al*. (1989)	*P. farinosa*	Suzuki and Nikkuni (1989)
C. pseudotropicalis	Polonelli *et al*. (1987)	*P. guilliermondii*	Zekhnov *et al*. (1989)
C. sonorensis	Starmer *et al*. (1987)	*P. holstii*	Polonelli *et al*. (1987)
C. valida	Yokomori *et al*. (1988)	*P. inositovora*	Hayman and Bolen (1990)
C. versatilis	Vaughan-Martini *et al*. (1988)	*P. jadinii*	Vaughan-Martini *et al*. (1988)
		P. kluyveri	Zorg *et al*. (1988)
Cryptococcus albidus	Young and Yagiu (1978)	*P. membranifaciens*	Golubev and Blagodaskaya (1993)
Cryp. laurentii	Vaughan-Martini *et al*. (1988)	*P. mexicana*	Starmer *et al*. (1987)
		P. minuta var. *nonfermentans*	Polonelli *et al*. (1987)
Cryp. podzolicu	Starmer et al. (1987)		
Cystofilobasidium bisporidii	Golubev and Kuznetsova (1989)	*P. ohmeri*	Zekhnov *et al*. (1989)
		P. opuntiae	Starmer *et al*. (1987)
Debaryomyces carsonii	Golubev (1991)	*P. petersonii*	Nomoto *et al*. (1984)
	Golubev (1990)	*P. pini*	Zekhnov *et al*. (1989)
D. polymorphus	Polonelli *et al*. (1987)	*P. quercuum*	Zekhnov *et al*. (1989)
D. vanrijiae	Vaughan-Martini *et al*. (1988)	*P. spartinae*	Polonelli *et al*. (1987)
		P. thermotolerans	Ganter and Starmer (1992)
Filobasidium capsuligenum	Zekhnov *et al*. (1989)	*Rhodotorula fujisanensis*	Golubev (1992a)
	Golubev and Kuznetsova (1991)	*R. glutinis*	Golubev (1989)
Hanseniaspora uvarum	Radler *et al*. (1990)	*R. mucilaginosa*	Golubev and Churkina (1990)
		R. pallida	Golubev (1992b)
Kloeckera apiculata	Rosini and Cantini (1987)	*Saccharomyces cerevisiae*	Bussey *et al*. (1990)
K. japonica	Starmer *et al*. (1987)		

Table 2.6 (Continued)

Yeast species	Reference	Yeast species	Reference
Kluyveromyces aestuarii	Vaughan-Martini and Rosini (1989)	*S. paradoxus*	Naumov (1985)
		S. unisporus	Nagornaya *et al*. (1989)
Kluy. dobzhanskii	Vaughan-Martini and Rosini (1989)	*Sporidiobolus johnsonii*	Golubev and Tsiomenko (1985)
Kluy. lactis	Stark *et al*. (1990)	*Spor. pararoseus*	Golubev *et al*. (1988)
Kluy. lodderae	Vaughan-Martini and Rosini (1989)	*Trichosporon capitatum*	Morace *et al*. (1983/84)
Kluy. marxianus	Lehmann *et al*. (1987b)	*Williopsis californica*	Vustin *et al*. (1988a)
Kluy. phaffii	Vaughan-Martini and Rosini (1989)	*W. pratensis*	Vustin *et al*. (1988b)
Kluy. wickerhamii	Vaughan-Martini and Rosini (1989)	*W. saturnus*	Ohta *et al*. (1984)
		W. beijerinckii	Vustin *et al*. (1988a)
Kluy. wikenii	Rosini and Cantini (1987)	*W. saturnus* var. *mrakii*	Yamamoto *et al*. (1988)
Metschnikowia pulcherrima	Vustin *et al*. (1990)	*W. saturnus* var. *sagentensis*	Lehmann *et al*. (1987b)
		W. saturnus var. *subsufficiens*	Vustin *et al*. (1988a)

The capability to produce killer toxin can confer an advantage over more sensitive competitive strains growing in a fermentative process (Soares and Sato, 2000). Recently, an interest in the development of bacteriocins as food preservatives and the use of the killer factors for industrial applications has increased (Santos *et al*., 2000). Investigations revealed that the occurrence of the killer phenotype in yeast is widespread in alcohol fermentations for beverage production such as in breweries,

saké, wine, plants and more recently in sugarcane producing plants (Soares and Sato, 2000).

The assay method established the optimal culture conditions for toxin production and activity (Marquina *et al*., 2002). One of the most important conditions for detecting killer activity is the pH of the test medium. Killer activity is expressed under acidic conditions, usually at a pH within the range of 3-6. As a rule, yeast killer toxins are most active at pH 4-5 (Wood and Bevan, 1968; Young and Yagiu, 1978; Middlebeek *et al*., 1979; Tolstorukov *et al*., 1989). With some exceptions (Ohta *et al*., 1984; Vustin *et al*., 1989), killer toxins are rapidly inactivated at increased temperatures, and 15-20 °C is the preferred temperature range for incubation during the assay for killer activity. In contrast to the toxin of *Kluyveromyces lactis* is active at the pH range of 4.4 to 5.8 and at the temperature up to 40 °C, but inactivated at 50 °C (Wilson and Whittaker, 1989).

Yeast killer toxins are more stable in agar medium than in liquid, and agitation causes their inactivation (Wood and Bevan, 1968; Wilson and Whittaker, 1989). The concentration of sensitive cells influences the sensitivity of the bioassay; in the case of an abundant lawn of the inhibition zone can be narrow and rapidly overgrown or not developed at all. In addition, the composition of the medium and buffer solution may contribute to the sensitivity of the assay (Panchal *et al*., 1985). As a rule, nutritionally rich organic media are more suitable than synthetic media. In most case, glucose-yeast extract-peptone agar or malt agar with sodium citrate-phosphate buffer are used. It is apparent that both the level and expression of killer toxin activity depends on a number of variables. In particular, the assay condition can be crucial for detecting

killer with low activity of those organisms that are weakly sensitive (Kurtzman and Fell, 1997).

Ribéreau-Gayon *et al*. (2000) described the action of a killer strain on a sensitive strain is easy to demonstrate in the laboratory on an agar culture medium at pH 4.2-4.7 at 20 °C. The sensitive strain is inoculated into the mass of agar before it solidifies; then the strain to be tested is inoculated in streaks on the solidified medium. If it is a killer strain, a clear zone in which the sensitive strain cannot grow encircles the inoculum streaks.

2.4 Ethanol fermentation

The fermentation of sugar to ethanol by yeast has an important among the different processes that are used in industry. The yeasts, which are of primary interest to industrial operations, are *S. cerevisiae*, *S. uvarum* (*carlsbergensis*), *Sch. pombe*, and *Kluyveromyces* species.

Yeasts metabolize glucose to ethanol by the glycolysis pathway. The overall net reaction (Figure 2.6) involves the production of 2 moles each of ethanol, CO_2, and ATP per mole of glucose fermented. Therefore, on a weight basis, each gram of glucose can theoretically give rise to 51 % alcohol. The yield attained in practical fermentations, however, does not exceed 90-95% of the theoretical value. This is due to the requirement for some nutrients to be utilized in the synthesis of new biomass and other cell maintenance-related reactions. Side reactions also occur in the fermentation (usually to glycerol) which many consume up to 4-5% of the total substrate. If these reactions could be eliminated, an additional 2.7% yield of ethanol from substrate would result (Roehr, 2001).

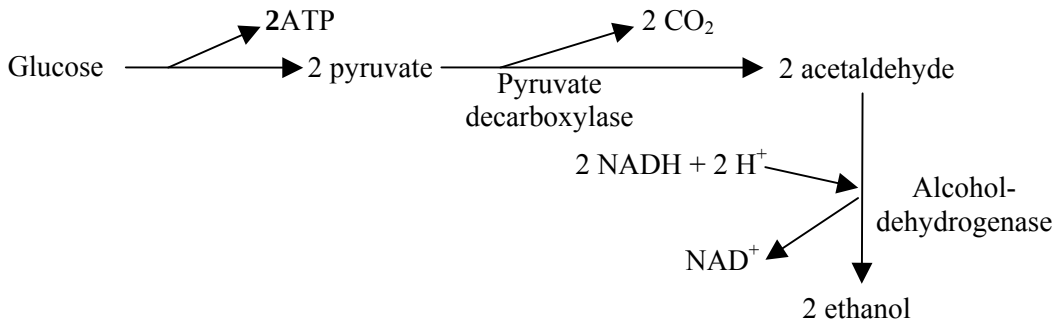


Figure 2.6 The ethanol fermentation pathway results in the formation of ethanol and carbon dioxide (Norr *et al.,* 2003)

The reducing power of NADH, produced by glycolysis, must be transferred to an electron acceptor to regenerate NAD^+. In ethanol fermentation, it is not pyruvate but rather acetaldehyde, its decarboxylation product, which serves as the terminal electron acceptor. With respect to glycolysis, ethanol fermentation contains two additional enzymatic reactions, the first of which (catalyzed by pyruvate decarboxylase), decarboxylates pyruvic acid which have thiamine pyrophosphate (TPP) as cofactor (Ribéreau-Gayon *et al*., 2000).

2.4.1 Thermotolerant yeasts and their ethanol production

Ethanol production by thermotolerant yeasts has been extensively studied, because thermotolerant yeasts are capable of growth and fermentation during the summer months in non-tropical countries as well as under tropical climates (Ueno *et al*., 2001). Cooling costs during the process of ethanol production are expensive; hence, by using thermotolerant yeasts cooling and distillation costs can be reduced (Sree *et al*., 1999; Ueno *et al*., 2002). Considerable difficulties are associated with fermentation in tropical areas owing to the lack of heat tolerance in conventional

industrial yeast strains. These difficulties include high ambient temperature, especially in the summer, coupled with an exothermic fermentation reaction, the compound effect of which leads to inhibition of yeast fermentation ability (Anderson *et al.,* 1986). In general, mesophilic yeasts with upper limit growth temperature between 28 °C and 38 °C. This is the reason why the operating temperature must be maintained between 30 and 35 °C in typical yeast fermentation reactors (Ueno *et al*., 2001). However, there are only a limited number of reports on the successful selection and isolation of yeasts capable of growth or fermentation at or above 40 °C (Pellegrini *et al.,* 1999; Sree *et al.,* 1999). As the temperature increases, productivity decreases sharply because of greater ethanol inhibition (Sree *et al.,* 1999). Moreover, Anderson *et al*. (1986) and Ueno *et al*. (2003) reported that thermotolerant yeast can produce > 6% ethanol within 24 hours at 40 °C.

2.4.1.1 *Kluyveromyces* spp.

The morphology of *Kluyveromyces* spp. was described by Kurtzman and Fell (1997), cells are ovoidal, ellipsoidal, cylindrical to elongate as shown in Figure 2.7. Pseudomycelium may be formed and true hyphae are not produced.

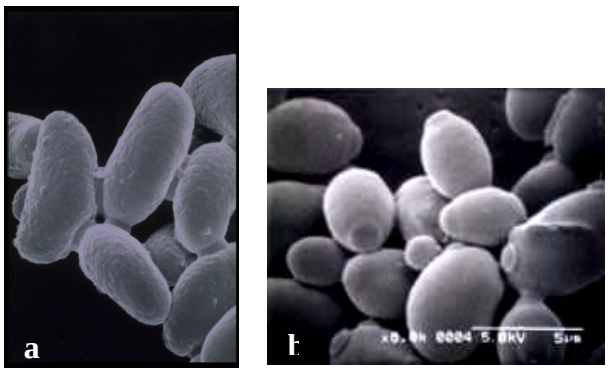


Figure 2.7 Yeast cell morphology; a) *Kluyveromyces marxianus* and b) *K. lactis*, (www, 2005)

As summerized in Table 2.2, it appears that *Kluyveromyces* strains were the most thermotolerant of the seven genera examined (Hacking *et al*., 1984). Banat *et al*. (1992) isolated thermotolerant strains of *Kluyveromyces*, *K. marxianus* capable of growth and fermentation at 52 °C were isolated from soil samples of the distillery environment in India. One of *K. marxianus* strains, IMB3, has been reported to be capable of ethanol production at 45 °C during growth on glucose, cellobiose (Barron *et al*., 1994), sucrose (Fleming *et al*., 1993) and lactose-containing media (Brady *et al*., 1994). Brady *et al*. (1995) found when *K. marxianus* IMB3 grown on 2% (w/v) glucose, the organism produced a maximum concentration of 8.5 g/L ethanol, which represented 83% of the maximum theoretical yield.

2.4.1.2 *Saccharomyces* spp.

As shown in Figure 2.8, *Saccharomyces* spp. Cells are globose, ellipsoidal or cylindroidal (Kurtzman and Fell, 1997).

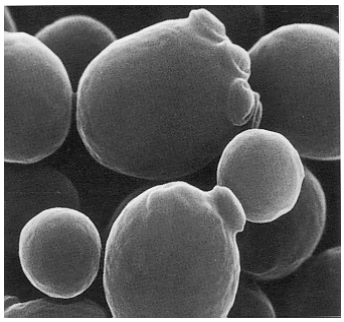


Figure 2.8 Yeast cell morphology; *Saccharomyces* sp., (www, 2002)

Torija *et al*., (2003) reported the influence of fermentation temperature of *S. cerevisiae* from 15 °C to 35 °C, the growth in yeast varied according to temperature. The usual growth curve, with a series of short-lag, exponential, stationary and decline

phases, was observed at 25 °C and 30 °C, whereas, at 35 °C, a high amount of yeast died. This high yeast mortality may have induced a slower final of fermentation, and can produce stuck fermentations with higher sugar contents. In fact, these results agree with previous reports that yeast viability decreases as the temperature increases (Ough, 1966; Nagodawithana *et al*., 1974; Casey *et al*., 1984). The effect of higher temperatures is a premature end of fermentation, which means that fermentation is incomplete and the ethanol concentration is low (Larue *et al*., 1980). Moreover, Lee *et al*., (1980) examined the fermentation kinetics of *S. uvarum (carlsbergensis)* in batch culture over the temperature range 25 °C to 43 °C. As indicated in Table 2.7, the ethanol yield was constant over this temperature range. The optimum temperature for growth was 33 °C while the maximum rate of ethanol production was constant at 37 °C to 43 °C. It was also shown that increasing concentrations of ethanol exerted greater degree of inhibition on the growth rate than on ethanol formation.

Table 2.7 Effect of temperature on the kinetic parameters of *S. uvarum (carlsbergensis)* (Slapack *et al*., 1987)

Parameters	Temperature (°C)					
	25	**30**	**33**	**37**	**40**	**43**
Maximum specific growth rate (hr^{-1})	0.19	0.23	0.26	0.23	0.21	0.16
Maximum ethanol production rate (g/g/hr)	0.94	1.15	1.33	1.44	1.44	1.43
Ethanol yield (g/glucose)	0.377	0.377	0.377	0.377	0.377	0.377

The percentage of the different *Saccharomyces* strains change considerably during fermentation. This may be related to their sensitivity to ethanol toxicity, some strains always disappeared when the concentration of ethanol in the medium was high.

Also, the ethanol tolerance of some yeast species depends on the temperature (Casey and Ingledew, 1986; D'Amore and Stewart, 1987; Gao and Fleet, 1988), and this could be the case of some *Saccharomyces* strains. This may explain why the presence of some strains decrease at some temperatures, but were able to finish the fermentation at other temperature (Torija *et al*., 2003).

2.4.1.3 *Candida* spp.

Candida spp. cells are globose, ellipsoidal, cylindroidal, or elongate, occasionally ogival, triangular of lunate (Kurtzman and Fell, 1997).

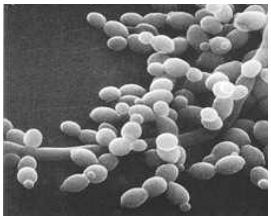


Figure 2.9 Yeast cell morphology; *Candida* sp., (www, 2001).

McCracken and Gong (1982) examined fermentation of glucose at 40 °C of eight *Candida* species isolated from sugar cane compost. Strains, HT1, HT4 and HT7 gave the highest ethanol yields (Table 2.8). Strain HT4 with a maximum temperature about 48 °C was examined carefully to see if it would be suitable for either simultaneous saccharification and fermentation (SSF) cellulose process or for one that would be used in combination with xylose isomerase to carry out isomerization and fermentation of xylose. At 45 °C, it yielded 4.3% (w/v) ethanol in 2 days from 10% (w/v) glucose. The initial fermentation rate was relatively constant over a temperature range of 30 °C to 50 °C. At 50 °C, about 2% (w/v) ethanol was produced from 4.5%

(w/v) glucose in 6 hours. It also fermented the glucose portion of neutralized ad pretreated sugar cane bagasse at 45 °C.

Table 2.8 Different species of *Candida* capable of fermentation at temperature ≥ 40 °C (McCracken and Gong 1982)

Strain	Temperature (°C)	Time (hours)	Substrate	Ethanol concentration (% w/v)
Candida acidothemophilum	40	14	10% pretreated rice straw	2.3
C. brassicae IFO 1664	40	24	cellulose	0.24
	45	48	cellulose	0.19
C. lusitaniae Y-5394	41	NA	10% cellobiose	4.57
C. obtusa ATCC 24009	40	NA	14%(w/v) glucose	1.76
C. pseudotropicalis YCa9	40	63	14.2%(w/v) glucose	6.87
	45	NA	14%(w/v) glucose	3.99
C. pseudotropicalis NCYC143	40	NA	14%(w/v) glucose	3.57
C. tropicalis NCYC405	40	NA	14%(w/v) glucose	3.43
C. utilis YCa25	40	62	14.1%(w/v) glucose	3.43
Candida sp. HT4	45	48	10%(w/v) glucose	4.32
	50	6	4.5%(w/v) glucose	2.00
Candida sp. HT1	45	48	10%(w/v) glucose	4.07
Candida sp. HT2	45	48	10%(w/v) glucose	0.39
Candida sp. HT3	45	48	10%(w/v) glucose	3.42
Candida sp. HT5	45	48	10%(w/v) glucose	1.98
Candida sp. HT6	45	48	10%(w/v) glucose	3.65
Candida sp. HT7	45	48	10%(w/v) glucose	4.09
Candida sp. HT8	45	48	10%(w/v) glucose	3.20

NA= not available

Candida sp. HT4 also fermented glucose at 50 °C; however, at this temperature the rate was much slow, probably as a result of cell death. The results also indicated that significant quantities of ethanol were produced from xylose by *Candida* sp. HT4 at 45 °C, when xylose isomerase was added to the medium (McCracken and Gong 1982).

2.4.2 Effect of ethanol on yeast fermentation

A limitation of ethanol fermentation is the capacity of yeast to tolerate ethanol concentration, because ethanol inhibits alcoholic fermentation, which limits the concentration of ethanol which can be produced by a given strain of yeast. The maximum concentration of ethanol which can be produced by yeast varies with species up to 20% by volume. The degree of inhibition is also related to other environmental factors, in particular high sugar concentration and high temperature which cause the inhibition of ethanol fermentation. Ethanol, which is produced during fermentation, is more inhibitory to cell growth than that from an exogenous source (Wayman and Rarekh, 1990).

Navarro (1980) studied the high intracellular ethanol concentrations were a consequence of resistance to diffusion through the membrane to the outside. At elevated temperature, the rate of ethanol production increased faster than the rate of excretion. Navarro and Durand (1978) also concluded that the effects of temperature on ethanol accumulation in *S. uvarum*. They found growth was arrested when a critical intracellular ethanol concentration had been reached, and this intracellular accumulation was greater at higher temperatures.

The toxic effect of ethanol has also been attributed to damaging the cell membrane or changing its properties. The extent of ethanol tolerance of certain yeasts is highly strain dependent and appears to be related to the unsaturated fatty acid and the fatty acyl composition of the plasma membrane (Wayman and Rarekh, 1990).

2.5 Effects of nutrients and pH on ethanol production by thermotolerant yeasts

2.5.1 Nutrients

Yeasts grow in simple media which contain fermentable carbohydrates to supply energy and carbon skeleton for biosynthesis, adequate nitrogen for protein synthesis, mineral salts and one or more growth factors. Sources of carbon included monosaccharides, disaccharides and trisaccharides (Priest and Campbell, 1996).

The metabolic activities of yeasts are greatly affected by the temperature at which they grow. Temperatures above the optimum lower the growth rate, oxygen solubility and also change the cellular composition of yeasts. It is known that under oxygen-limited conditions, yeasts require nutritional supplements for growth (Slapack *et al*., 1987 and Thomas *et al*., 2002). An increase in temperature does not inhibit substrate uptake nor does it significantly alter enzyme levels (Slapack *et al*., 1987).

Helena da Cruz *et al*. (2003) concluded that nitrogen and carbon are the main nutrients in fermentation medium and this implies that the mutual interaction of these nutrients may play an important role in the metabolism of yeasts. The supplementation of the growth media, containing maltose or glucose, with a more complex structural nitrogen source such as peptone induced higher biomass

accumulation and ethanol production. In *S. diastaticus*, Amore *et al*. (2002) reported by doubling the nutrient components in the medium, resulting in the production of 9.1% (w/v) ethanol. Increasing the fermentation temperature from 40 to 45 °C resulted in a decrease in the rate and extent of glucose utilization and ethanol production. Most yeast grows well on a variety of amino acids, purines, and pyrimidines as the sole source of nitrogen. They require trace amounts of biotin, thiamine, pyridoxine, calcium pantothenate and inositol for the maximum growth and fermentation rate (Wayman and Parekh, 1990). Amore *et al*. (2002) have also shows that role of magnesium in relieving the detrimental effect of high temperature may to some extent be related to the requirement of some of the glycolytic enzymes for this cation. In addition, increasing the cell density also resulted in an increase in ethanol production at the higher temperature.

2.5.2 pH

Hydrogen ion concentration has a significant influence on industrial fermentation due as much to its importance in controlling bacterial contamination as its effect on yeast growth, fermentation rates and by-product formation. The best ethanol yields are generally obtained at pH 4.5-4.7. At higher pH, more glycerol and organic acids are formed at the expense of ethanol (Wayman and Parekh, 1990).

Under fermentation conditions, the intracellular pH of *S. cerevisiae* is usually maintained between 5.5 and 5.75 when the external pH is 3.0 or between 5.9 and 6.75 when the external pH is varied between 6.0 and 10.0. The gap between the extracellular pH and the intracellular pH widens, greater stress is placed on the cells and more energy is expended to maintain the intracellular pH within the range that

permits growth and survival of the yeast. A greater proportion of glucose is converted to ethanol if the pH is adjusted to 4.5. This increased conversion is independent of the presence of nutrient supplements in the medium (Thomas *et al*., 2002). If the pH is adjusted to 7 or above, acetic acid is produced from acetaldehyde due to the increased activity of aldehyde dehydrogenase due to glycerol production which inhibits ethanol fermentation (Wang *et al*., 2001).

CHAPTER III

MATERIALS AND METHODS

3.1 Isolation and screening of thermotolerant yeasts

Thermotolerant yeasts were isolated from silage samples, at Suranaree University of Technology farm on March 2005. Ten grams of silage sample was soaked in 90 ml phosphate buffer and shaken vigorously. Each 100 µl of suspension was spread onto a plate containing a yeast extract-malt extract (YM) agar, which consisted of 3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose and 15 g agar in 1 liter water, initial pH 5.5 (Kreger-van Rij, 1984), and was incubated anaerobically at 40 °C for 3 days. Single colony formed was picked and the cells were observed under microscope.

3.2 Selection of thermotolerant yeast with ethanol production activity

According to the methods of Sree *et al*. (2000) and Ueno *et al*. (2001), the efficiency of ethanol production of thermotolerant yeast strain was tested. A yeast extract peptone dextrose (YPD) liquid medium, which contained 20 g glucose, 20 g peptone and 10 g yeast extract in 1 liter water, was used for ethanol production test. The medium was adjusted to pH 5.5 with 1 N HCl. One hundred µl cell suspensions of the isolated thermotolerant yeast strain taken from actively growing culture was inoculated into 7 ml of YPD liquid medium in a test tube containing a durham tube. Fermentation was recognized by the accumulation of CO_2 gas trapped in the inner

durham tube (Ueno *et al*., 2001; Ueno *et al*., 2002 and 2003). All culture was incubated at 40 °C for 3 days. The ethanol production was determined by using a gas chromatograph equipped with capillary PE-1 column (AutoSystem XL, Perkin Elmer, U.S.A.). The analysis of ethanol was operated on flame-ionization detector and an inlet system using the splitless injection technique, injector and detector temperature were 250 and 300 °C, respectively. Oven program was 37 °C for 5 min and increase from 37 to 245 °C at 10 °C/ min. Helium gas was used as carrier gas, adjusted to 14 psi. The ethanol production was primarily identified by comparing the retention time of the gas chromatographic peak with ethanol standard.

3.3 Optimization of temperature for yeast growth

Following Sree *et al*. (1999), 50 ml of YPD medium was distributed into 125 ml screw cap Erlenmeyer flask were inoculated with thermotolerant yeast from actively growing culture. All cultures were incubated at 30 °C, 37 °C, 40 °C and 45 °C for 72 h. The initial optical density of each flask was read off on spectrophotometer (Ultraspec 2000 UV/Visible Spectrophotometer, Pharmacia Biotech, England) at 660 nm against the medium as blank. The increase in optical density in a flask was recorded as evidence of growth.

3.4 Detection of yeast killer toxin

The assay of isolated thermotolerant yeast killer toxin was performed by means of streak-plate agar diffusion assay (Comitini *et al*., 2004). Approximately 10^5 cells/ml of the sensitive yeast strain *S. cerevisiae* EC 1118, *Escherichia coli* ATCC 25922 and *Bacillus subtilis* ATCC 6633 were suspended in each 20 ml malt

agar buffered at pH 4.4 (0.1 M citrate-phosphate buffer). Killer yeast strain *S. cerevisiae* K1-V1116 and isolated thermotolerant yeast were streaked on the agar surface, incubated at 20 °C for 72 h. Killer activity of isolated thermotolerant yeast was evident as a clear zone of inhibition to sensitive strain surrounding the streak similarly killer strain.

3.5 Detection of ethanol tolerance of thermotolerant yeast

The medium for the detection of ethanol tolerance of thermotolerant yeast was modified from Osho (2005). YPD liquid medium was used for detecting thermotolerant yeasts for ethanol tolerance. The medium was sterilized at 121 °C for 15 min in an autoclave and cooled. One ml of various concentrations of absolute ethanol was varied from 5 to 25% (v/v), and then added to different flask of the same medium to constitute varying percentages of ethanol differing by 5% (v/v) from one flask to the others. Forty ml portion of the medium was distributed into 125 ml flask, and then inoculated with selected thermotolerant yeasts. The initial optical density of each flask was read off on spectrophotometer (Ultraspec 2000 UV/Visible Spectrophotometer, Pharmacia Biotech, England) at 660 nm against the medium as blank. All cultures were incubated at 40 °C for 5 days. The increase in optical density in a flask was recorded as evidence of growth. The concentration of alcohol at which the growth of yeasts was just inhibited was assessed as the ethanol tolerance of yeasts.

3.6 Identification of the selected thermotolerant yeast

3.6.1 Morphological characterization

According to the method of Kreger-van Rij (1984) and Kurtzman and Fell (1997), the morphology of the vegetative cells was grown in liquid and on solid media.

3.6.1.1 Growth on solid medium

The morphology of cells of thermotolerant yeast and their appearance on solid medium, on YPD agar was examined, after incubating at 40 °C for 3 days. The following features of the appearance of cultures were recorded; texture, color and surface of colonies. Their ascospore and pseudomycelium formation were determined.

3.6.1.1.1 Ascospore formation

Thermotolerant yeast was examined for ascospore formation applied from Kurtzman *et al*. (2005). The culture was initially incubated for 2 days at 37 °C to facilitate growth, and then further incubated at 25 °C to induce ascospore formation. The culture was examined for ascospores at approximately weakly intervals for 3 weeks. Ascospore formation was detected by staining the heat-fixed preparation (Kreger-van Rij 1984) carbol-fuchsine and steamed gently for about 5 min. Slide was decolorized with 95% ethanol containing 1% concentrated hydrochloric acid. The slide was rinsed in water and counter stained with 1% methylene blue; the mature ascospores stain red and vegetative cells blue.

3.6.1.1.2 Pseudomycelium formation

Following to Kreger-van Rij (1984), the formation of pseudomycelium was investigated by slide culture technique. A Petri dish was containing a U-shaped glass-rod supporting glass slide, was sterilized by dry heat at 180 °C for 2 h. YPD agar was melted and poured into a second Petri dish. The glass slide was quickly removed from the glass rod with a flame-sterilized, and was dipped into the agar after which it was replaced on the glass rod support.

After solidification of the agar on the slide, the thermotolerant yeast was inoculated very lightly in two lines along slide and a sterile coverslip was placed over part of the lines. Some sterile water was poured into the Petri dish to prevent the agar from drying out. The culture was incubated at 25 °C for 5 days. For observation, the slide was taken out of Petri dish and the agar was wiped off the back of the slide. The edges of the streak under and around the coverslip were examined microscopically.

3.6.1.2 Growth in liquid medium

The morphology of cells was cultured in YPD liquid medium. Cells from a young actively growing culture were inoculated into test tube containing 7 ml of medium, incubated at 40 °C for 3 days. The culture was examined the growth of thermotolerant yeast visually on the surface of YPD liquid medium and the shape of cells by compound microscope (Alphaphot-2, Nikon, Japan) and scanning electron microscope (JSM-6400, Scaning Microscrope, JEOL, Japan).

3.6.2 Biochemical characterization

API Candida strip (bioMérieux®sa, France) was used to investigate the biochemical reaction. This strip consists of 10 tubes containing dehydrated substrates, which enable the performance of 12 identification tests (sugar acidification of enzymatic reactions). The reactions produced during incubation were revealed by spontaneous color changes. After 24 h of incubation at 37 °C, the reactions were read visually according to the reading table and identification was obtained by using the identification software.

3.6.3 Molecular characterization

3.6.3.1 Genomic DNA extraction

DNA of thermotolerant yeast was extracted by following steps; yeast cell were collected form YPD broth by centrifugation (Centrifuge 5415C, Eppendorf, Germany) at 5,000×g for 5 min. After centrifugation, two cycles of the pellet was resuspended in 500 µl of TEN buffer (TE buffer and 0.1 M NaCl) and centrifugation at 5,000×g for 5 min, supernatant was discarded. Then 200 µl of 20% (v/v) sucrose in TEN buffer, 100 µl of 10% (w/v) SDS and 20 µl (2.0 mg/ml) lysozyme were added into the sample before incubated at 37 °C for 1 hour. The mixture, after the addition of 75 µl 5 M NaCl and two times of 500 µl phenol: chloroform: isoamyl alcohol (25: 24: 1) was centrifuged at 14,000×g for 10 min. The upper phase was transferred to the new tube. The nucleic acid in aqueous phase was precipitated with and equal volume of cold isopropanol and 50 µl of 3 M sodium acetate, intermittent vortexing, then incubated at -20 °C for 1 hour. The nucleic acid was separated by centrifugation at 14,000×g for 10

min, washed with 70% (v/v) ethanol, dried and dissolved in TE buffer containing 1/10 RNase, then stored at 4 °C for further analysis.

3.6.3.2 PCR amplification

The genotypic identification of thermotolerant yeast was conducted by reading of the nucleotide sequence of DNA encoding 18S rRNA (rDNA). The rDNA was amplified using universal fungal primers (Ueno *et al*., 2001; Ueno *et al*., 2002; Ueno *et al*., 2003), forward primer, NS1 (5'-GTAGTCATATGCTTGTCTC-3') and reverse primer, NS2 (5'-GGCTGCTGGCAC CAGACTTGC-3') (White *et al*., 1990).

The PCR reaction was done in a 50 µl reaction mixture using 2.5 U of *Taq* polymerase (Promega, U.S.A.), the buffer supplied by Promega, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 500 nM (each) primer. The reaction was run in a Thermal cycler (GeneAmp®PCR System 2400, Perkin Elmer, U.S.A.) by heating at 95 °C for 5 min, and then amplification was performed with 35 cycles at 95 °C for 30 second, 53 °C for 30 second and 72 °C for 120 second, followed by elongation at 72 °C for 10 min. The amplification result was detected on 1% agarose gel electrophoresis staining with 10 mg/ml of ethidium bromide. Before being sequenced, the amplified fragment was purified with a Wizard® SV Gel and PCR Clean-Up System (Promega, USA) from agarose gel. Nucleotide sequences were analyzed by Macrogen (Macrogen, Inc., Korea). All the sequences were compared for their similarity with reference yeast strains by a BLAST search.

3.6.3.3 Sequence analysis

The sequence data was compared to gene libraries (GenBank) with BLAST and FASTA programs. Multiple sequence alignments of translated gene sequence were carried out with the program CLUSTALW (version 1.83). Genetic distances were estimated by using the Maximum parsimony with bootstrap method employed by PHYLIP (Felstein 1993). The tree was displayed with the TREEVIEW program (Page 1996).

3.7 Optimization of some ethanol production conditions

3.7.1 Nitrogen source

Table 3.1 shows the composition of M9 minimal medium (Sambrook and Russell, 2001), the basal medium use for determined the suitable nitrogen source. Five sources of nitrogen; sodium nitrate ($NaNO_3$), ammonium chloride (NH_4Cl), ammonium sulphate [$(NH_4)_2SO_4$], diammonium hydrogen phosphate [$(NH_4)_2HPO_4$] and ammonium dihydrogen phosphate ($NH_4H_2PO_4$) were substituted nitrogen source in 5X M9 salts and various the concentrations of each nitrogen source in 5X M9 salts was tried from 0 to 25 g/l, differing by 5 g/l from one flask to the others.

Fifty ml of the medium was distributed into 125 ml screw cap Erlenmeyer flask and adjusted to pH 5.5 with 1 N HCl. For inoculum preparation, the thermotolerant yeast was grown aerobically in YPD medium. After incubated at 40 $^{\circ}$C for 24 h, 10% inoculum size was centrifuged at 14,000×g for 5 min and washed the pellet with sterile distilled water before inoculated in the medium. The initial optical density of each flask was read off on spectrophotometer (Ultraspec 2000 UV/Visible

Spectophotometer, Pharmacia Bitech, England) at 660 nm. The YPD medium was used as blank. All cultures were incubated at 40 °C for 3 days.

Measurement the growth of yeast by spectrophotometry at 660 nm (Norrell and Messley, 1997; Ueno *et al*., 2001) and ethanol production (Section 3.2) for every 6 h in first day, every 12 h in second day and every 24 h for a day. Selected the concentration and source of nitrogen for the thermotolerant yeast was produced highest ethanol.

Table 3.1 The composition of M9 minimal medium (Sambrook and Russell, 2001)

Composition per liter	
5X M9 salts	200 ml
1M $MgSO_4$	2 ml
20% solution of carbon source	20 ml
1M $CaCl_2$	0.1 ml
5X M9 salts (composition per liter)	
$Na_2HPO_4.7H_2O$	64 g
KH_2PO_4	15 g
NaCl	2.5 g
NH_4Cl	5 g

3.7.2 Glucose concentration

The basal medium and methodology for examined the optimum glucose concentration was described in section 3.6.1 except the addition of different glucose concentration, and nitrogen source in 5X M9 salts and incubation time were different.

Glucose concentrations were varied from 0 to 25% (v/v), differing by 5% (v/v) from one flask to the others. 10 g/l $(NH_4)_2SO_4$ were used as a source of nitrogen. All cultures were incubated at 40 °C for 2 weeks. Similar to section 3.6.1, the highest ethanol production was selected from various glucose concentration for further analysis.

3.7.3 Initial pH

The basal medium and methodology were followed section 3.6.2. 5% (v/v) glucose solution and 10 g/L $(NH_4)_2SO_4$ in 5X M9 salts were used to determined the optimization of pH for ethanol production. The pH was adjusted in the range between 2.0 to 8.0 with 1 N HCl or 1N NaOH according to Thomas *et al*. (2002). The optimum conditions for ethanol production were applied to scale up in 2 L fermenter.

3.8 Ethanol production in 2 L fermenter

For the preparation of inoculum, yeast cells from a freshly prepared slant were transferred aseptically into a 250 ml Erlenmeyer flask containing 150 ml of YPD medium. The flask was incubated aerobically at 40 °C for 24 h. The suitable glucose concentration, nitrogen source and pH (section 3.6) were used for ethanol production in fermenter, the pH maintained by addition of 2 N HCl and 2 N NaOH. Batch experiments were performed in a 2 L fermenter (micro DCU-300, B. Braun Biotech international, Germany) with a working volume of 1.5 Lat 40 °C for 72 h. The first two batch experiments were flushed with air to 0.5 vvm for 24 h and other two batches were flushed continuously with air to 0.5 vvm for 72 h. The stirring rate was fixed at 200 rpm until the fermentation end. During the whole fermentation process,

samples were periodically withdrawn for analysis of ethanol production and growth estimation as described in section 3.6.1.

Titrable acidity (TA) was investigated by titration with 0.1 N NaOH at pH 8.2 (Ough and Amerine, 1986), and the TA concentration calculated as follows:

$$\text{Titrable acidity (g/L tartaric acid)} = \frac{(\text{ml base})\,(\text{N base})\,(0.075)\,(1000)}{\text{ml sample}}$$

where: ml sample = sample volume (ml)

ml base = volume of sodium hydroxide used for titration (ml)

N base = normality of sodium hydroxide solution

Determination of some organic acids was analyzed by high performance liquid chromatographic (HPLC). The thermo separation product (tsp) HPLC system composed of autoinjecter AS 3000 and UV 6000 LP detector was set at 210 nm. The Phenomenex® Rezex ROA organic acid column (300 x 7.8 nm) was operated at 55 °C. The 0.01 N H_2SO_4 was used as mobile phase at the flow rate of 0.5 ml /min (www, 1999).

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Isolation of thermotolerant yeasts

A total of 15 isolates of yeast-like colonies were isolated from silage samples at SUT farm, hot spring water and waste water samples of starch and sugar industries. One isolate was isolated from silage samples, 2 isolates were isolate from starch industry, 3 isolates were isolated from starch industry and 9 isolates were isolated from hot spring water.

4.2 Selection of thermotolerant yeasts with ethanol production activity

4.2.1 Screening of yeast morphology under microscope

All isolates of yeast-like colonies were observed under compound microscope. The number of yeast was only one strain from silage samples at 40 $^{\circ}$C. The thermotolerant yeast strain S1 was screened from these colonies according to test for their ethanol production at 40 $^{\circ}$C. Cell morphology was morphologically observed under a microscope as shown in Figure 4.1.

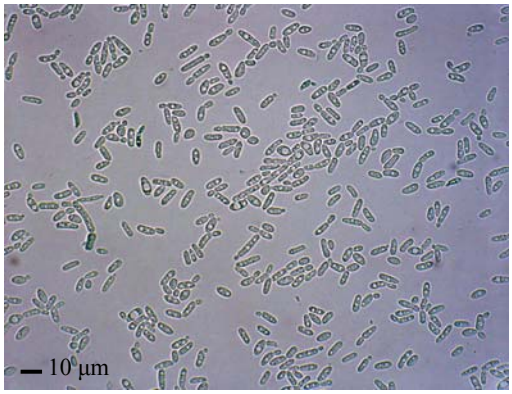


Figure 4.1 Cell morphology of vegetative cells of the thermotolerant yeast strain S1 cultivated in YPD broth at 40 °C for 3 days

4.2.2 Study of optimum growth temperature of selected thermotolerant yeast

The effect of temperature on the growth of yeast was study under the range of 30-45 °C, and the result was showed in Figure 4.2. The optimum temperature which gave the highest cell dry weight was at 37 °C. At temperature higher than 40 °C, the growth of yeast was decreased. In this study, ethanol fermentation was occurred at temperature higher than optimum temperature because yeast currently used for industrial fermentation are rapidly inactivated at 33-35°C (Laluce *et al*., 1987). Significant cooling costs would be eliminated, especially during the summer or in tropical countries, with fermenting temperatures of 40 °C and above. Additional energy requirements involving heating and subsequent cooling, often required in the preparation of many substrates for fermentation, could be drastically reduced in processes operated at these temperatures. Fermentation at temperatures higher than the optimum requires the selection of unconventional yeast starters capable of eluding the inhibitory effect of ethanol, which is increasing with temperature, and consequently of

attaining maximum yield levels. Only a few screening surveys have been carried out for the ability of yeasts to grow and ferment at or above 40 °C (D'Amore *et al*., 1989, Anderson *et al*., 1986 and Hacking *et al*., 1984).

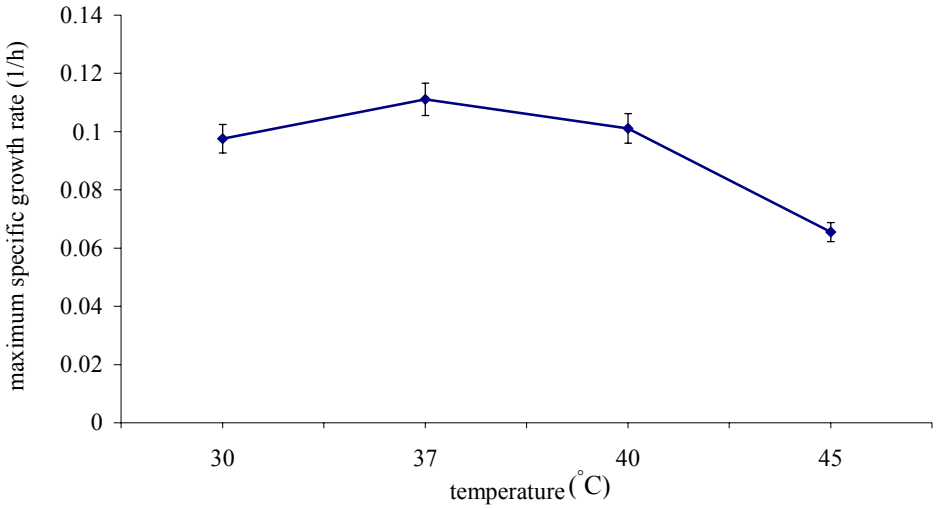


Figure 4.2 The optimum growth temperature of the thermotolerant yeast strain S1 cultivated in YPD broth at 40 °C for 3 days

4.3 Identification of the thermotolerant yeast strain S1

4.3.1 Morphological and physiological characteristics

Figure 4.3 shows the features of the appearance of cultures when cells grown in YPD broth and on YPD agar. After 3 days of incubation at 40 °C, heavy, dry climbing pellicles were formed on the surface of YPD medium. The growth was butyrous and light cream color on YPD agar.

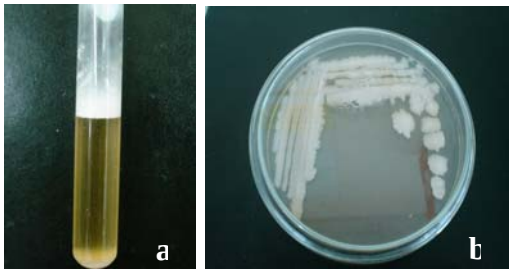


Figure 4.3 The growth of yeast cells in YPD medium; a), and on YPD agar; b) after incubating at 40 °C for 3 days

The cell morphology of the thermotolerant yeast strain S1 under compound microscope (Figure 4.4) and scanning electron microscope (Figure 4.5) are ovoidal to elongate, (2.7-4.2) x (5.6-10.1) µm, single or in pairs, budding cells are present and pseudomycelia are developed.

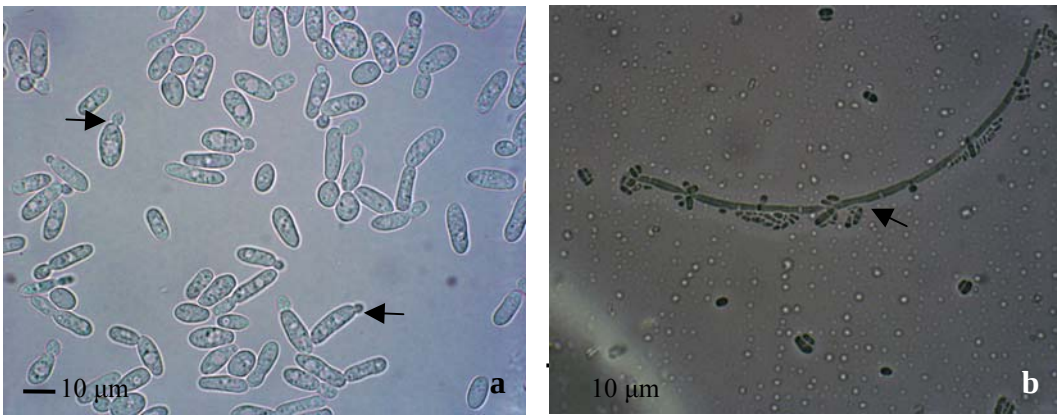


Figure 4.4 Morphological characteristics of the thermotolerant yeast strain S1 under compound microscope; a) budding cells (1000x) and b) pseudomycelium formation (400x) as shown by arrows

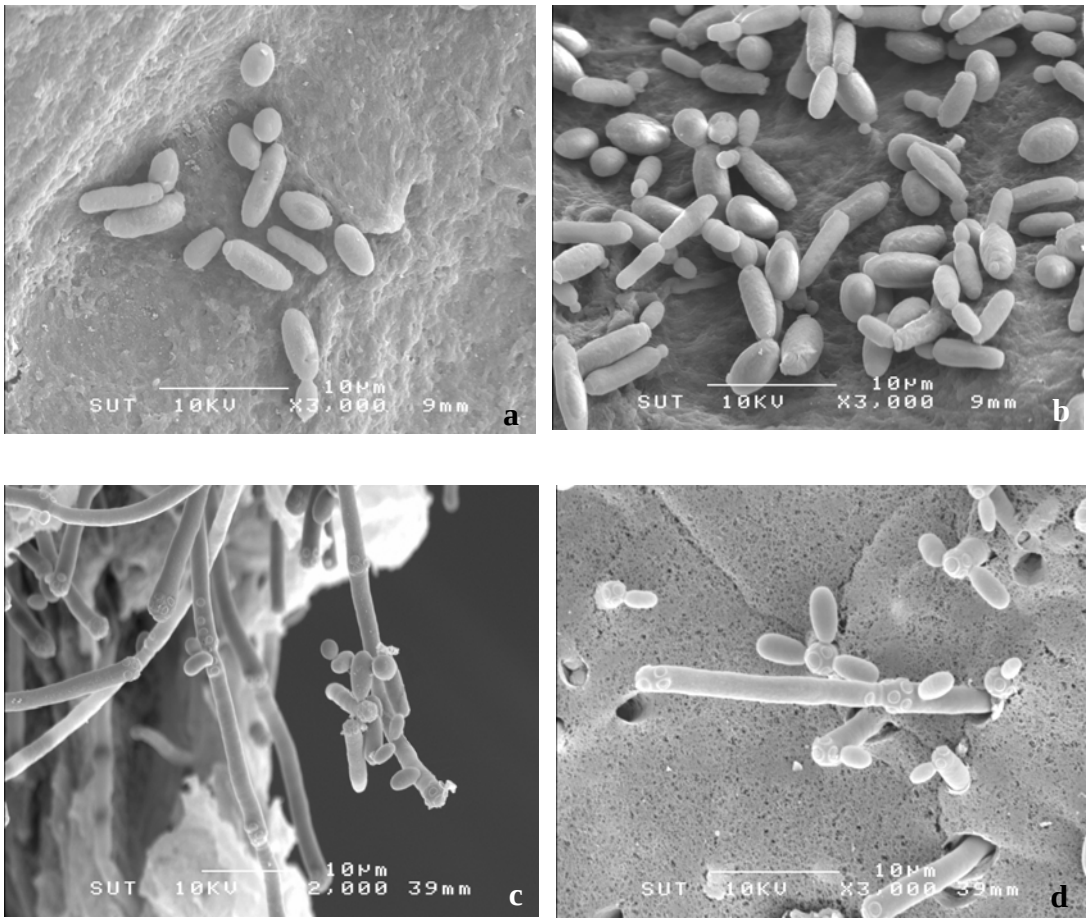


Figure 4.5 SEM micrographs of the thermotolerant yeast strain S1; a) vegetative cell in YPD broth at 40 °C for 24 h, b) budding cell in YPD broth at 40 °C for 24 h and c, d) pseudomycelium formation in YPD agar at 40 °C for 7 days (Scale bars = 10 μm)

Following to the method of Kreger-van Rij (1984) and Kurtzman and Fell (1997), ascospore formation is use for indication of the ascomycetous yeasts. As shown in Figure 4.6, ascospore formed on YPD agar after 3 weeks at 25 °C. Asci are persistent and form 1-2 spheroidal ascospores.

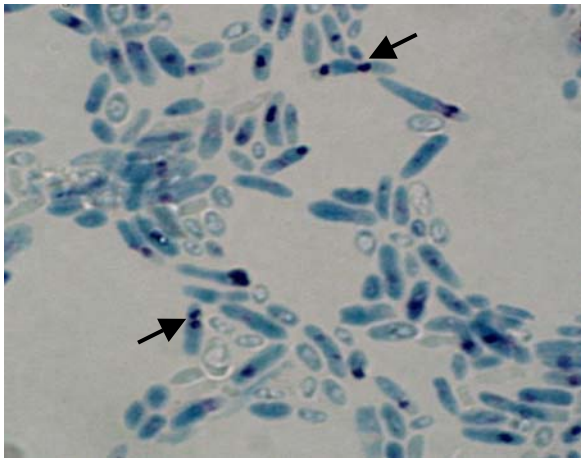


Figure 4.6 Ascospores (arrows) of the thermotolerant yeast strain S1

The thermotolerant yeast strain S1 was identified based on biochemical characterization by using API Candida strip (bioMérieux®sa, France). The result which was compared the similarity percentages of sugar acidification and/or enzymatic reactions patterns with reference strain of the APILAB Plus software (version 2.0) was demonstrated in Table 4.1.

From the result of API Candida strip, only glucose showed the positive, which was the characteristic of *Candida krusei*. Additionally, *Candida krusei* is considered to represent the anamorphic form of *Issatchenkia orientalis* because the type strains show significant (93-100%) DNA base sequence complementarity (Kurtzman *et al.*, 1980). It was isolated from a wide variety of habitats including humans and animals (Kurtzman and Fell, 1997).

Table 4.1 Biochemical characteristics of the selected thermotolerant yeast isolated form silage

Characteristic	Reference strain (*Candida krusei*)	The thermotolerant yeast strain S1
Sugar acidification		
1. Glucose	+	+
2. Galactose	-	-
3. Saccharose	-	-
4. Trehalose	-	-
5. Raffinose	-	-
Enzymatic reactions		
6. beta-Maltosidase	-	-
7. alpha-Amylase	-	-
8. beta-Xylosidase	-	-
9. beta-Glucuronidase	-	-
10. Urease	-	-
11. N-Acetyl-beta-glucosaminidase	-	-
12. beta-Galactosidase	-	-

Symbol: +, positive; -, negative

4.3.2 Genetic analysis

The thermotolerant yeast strain S1 was genetically characterized using 18S rDNA analysis. Genomic DNA of this yeast was extracted and used as DNA template for PCR amplification. The PCR reaction was performed by NS1 and NS2 primers.

The single amplified DNA fragment of approximate size 600 bp was generated (Figure 4.7).

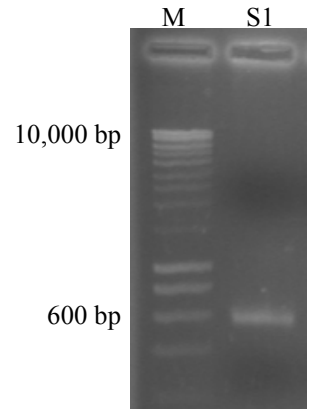


Figure 4.7 Agarose gel electrophoresis of PCR-amplified fragment of 18S rDNA of the thermotolerant yeast strain S1

4.3.3 18S rDNA sequence analysis

The partial sequence of 18S rDNA of the thermotolerant yeast strain S1 (Figure 4.8) was obtained from the sequence analysis, and compared with other 18S rDNA sequences of yeasts from GenBank database. When examined by BLAST similarity analysis, the 18S rDNA sequence from S1 was produced closely related with *Issatchenkia sp*. The result showed that the S1 strain has 99.5% similarity of 18S ribosomal RNA gene sequence with *Issatchenkia* sp. (Figure 4.8-4.10).

```
  1 TTTCCTCAGT CCGTTTAGTA TTTACATTGT ACTCATTCCA ATTACAAGAC
 51 CAAAGGCCCT GTATCGTTAT ATATTGTCAC TACCTCCCTG TGTCAGGATT
101 GGGTAATTTG CGCGCCTGCT GCCTTCCTTG GATGTGGTAG CCGTTTCTCA
151 GGCTCCCTCT CCGGAATCGA ACCCTTATTC CCCGTTACCC GCGAAAACCA
201 TGGTGGGACA CTATTATATA TCACTAGCTG ATGATGACAA ATTATATCTT
251 TTTATGCACC ATCTGCCGTC CCCCGGTCCT GTTATTTCAC AAATTATCAT
301 TAATTACCTT TATACCGGTC CACCCCATAA TCATTCTTTC ACTCCATAAA
351 TGCCCCCCTT CTAGAGGTCA GATAACGCGC CACTCCTCTA TAATTTACAC
401 TCTCTATTGC CCATCTATAC TAGCTAGCGT ACCACTTACT GAAACCCCCC
451 CCCAATTTCA CTCTTCCATC CCTCTTTTTC CTCCCCATAC ATTCAACACA
501 TACCTTATAC ATTGTTATAG TTATACCTCT ATTCCCCATC TTTTCTCTTT
551 CAACCAACAA CCTCCAAAAC ATCTTTTTAT CCCCTTTTAT AATTCATCAT
601 ACTCCCCCCC TTCACATCCC CACACTACAC AAAAGCCTAA TTCTTTAATT
651 TTACCCCTTC CACATTTATC ACCACCACCA ACCTCCCCTT ATAAAAACCA
701 ATAAAATAAT ACCCCCACTT ATTATATCCT CACCACACCC TAATTTCCAC
751 CCCCCCTTTC ACCTTAATAC TATATAACCT CTACTACGCC CTTTATCTC
```

Figure 4.8 Partial 18S rDNA sequence of the thermotolerant yeast strain S1

```
Query  18   GTATTTACATTGTACTCATTCCAATTACAAGACCAAAGGCCCTGTATCGTTATATATTGT  77
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  365  GTATTTACATTGTACTCATTCCAATTACAAGACCAAAGGCCCTGTATCGTTATATATTGT  306

Query  78   CACTACCTCCCTGTGTCAGGATTGGGTAATTTGCGCGCCTGCTGCCTTCCTTGGATGTGG  137
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  305  CACTACCTCCCTGTGTCAGGATTGGGTAATTTGCGCGCCTGCTGCCTTCCTTGGATGTGG  246

Query  138  TAGCCGTTTCTCAGGCTCCCTCTCCGGAATCGAACCCTTATTCCCCGTTACCCGCGAAAA  197
            |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  245  TAGCCGTTTCTCAGGCTCCCTCTCCGGAATCGAACCCTTATTCCCCGTTACCCGTGAAAA  186

Query  198  CCATGGT  204
            |||||||
Sbjct  185  CCATGGT  179
```

Figure 4.9 18S rDNA of S1 which was performed in the GenBank data library by using Basic Local Alignment Search Tool program (BLAST, http://www.ncbi.nlm.nih.gov/BLAST)

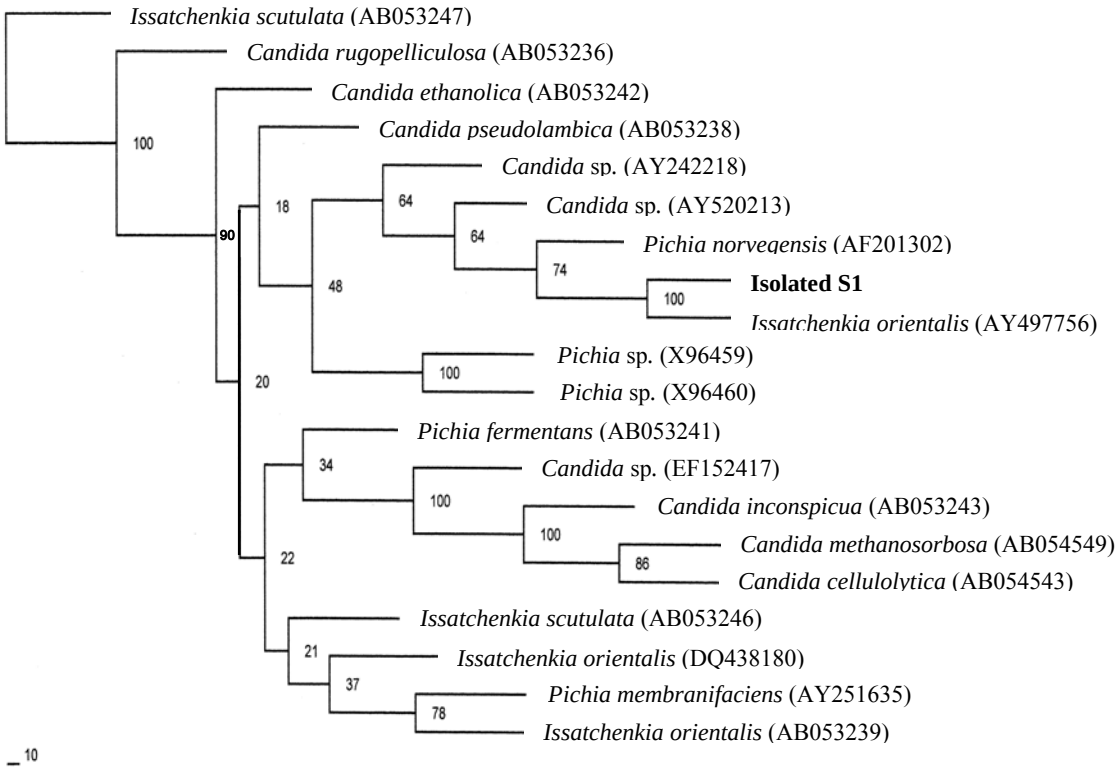


Figure 4.10 Phylogenetic relationship of the thermotolerant yeast strain S1. Branch lengths are proportional to the numbers of nucleotide and amino acid changes, and the numerals given on the branches are the frequencies with which a given branch appeared in 100 bootstrap replications. Reference sequences were retrieved from GenBank under the accession numbers in parentheses.

4.4 Some characteristics of the thermotolerant yeast strain S1

4.4.1 Evaluation of the killer activity

The thermotolerant yeast strain S1 was tested for killing activity when compared with a killer strain, *S. cerevisiae* K1-V1116. The thermotolerant yeast strain S1 was not able to kill any of *S. cerevisiae* EC 1118, *Escherichia coli* ATCC 25922 and *Bacillus subtilis* ATCC 6633 target strains, but *S. cerevisiae* K1-V1116 was active against the whole set of target strains. As shown in Table 2.6, killer activity has not been reported from *Issatchenkia* sp.

4.4.2 Ethanol tolerance of the thermotolerant yeast strain S1

Figure 4.11 showed the maximum specific growth rate in increase the ethanol concentration from 5 to 25% (v/v) compared with control (0% (v/v) ethanol), which not contain any ethanol in YPD medium. The thermotolerant yeast strain S1 was able to grow in ≥ 5% (v/v) ethanol concentration. Except in control, growth rate was highest in 5% (v/v) and remained lowest in 25% (v/v). Only slight differences were observed in the growth rates with increasing ethanol concentration from 5 to 15% (v/v) and 20 to 25% (v/v). Ekunsanmi and Odunfa (1990) assert that the ethanol tolerance is an advantage when a yeast is being considered for industrial use especially where ethanol is being produced. Jimenez and Benitez (1986) and Du Preez *et al*. (1987) pointed out that ethanol tolerance is particularly important since ethanol tolerance can hardly be avoided during fermentation although substrate inhibition can be avoided through stepwise addition of substrate. However, the ethanol tolerances of some yeast species depend on the temperature (Casey and Ingledew, 1986; D'Amore and Stewart, 1987; Gao and Fleet, 1988). The resistance of a strain to specific temperature and ethanol concentration may be related to the natural conditions of their origination isolation area (Torija *et al*., 2003).

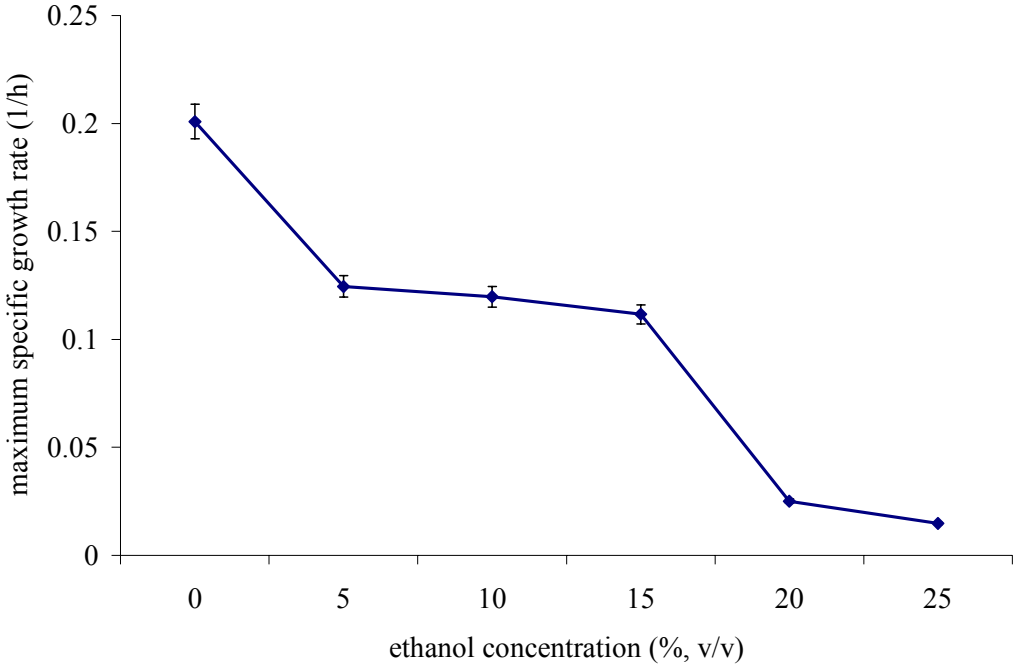


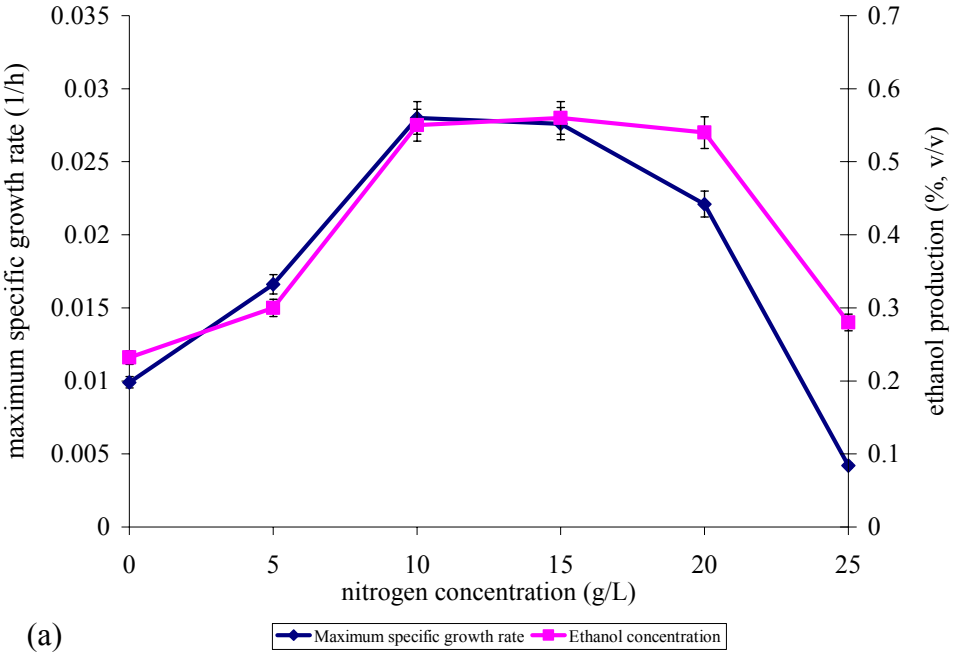
Figure 4.11 The Maximum specific growth rate of the thermotolerant yeast strain S1 in increasing ethanol concentrations (%, v/v) in YPD medium

4.5 Optimization of some ethanol production conditions

4.5.1 Effects of nitrogen source

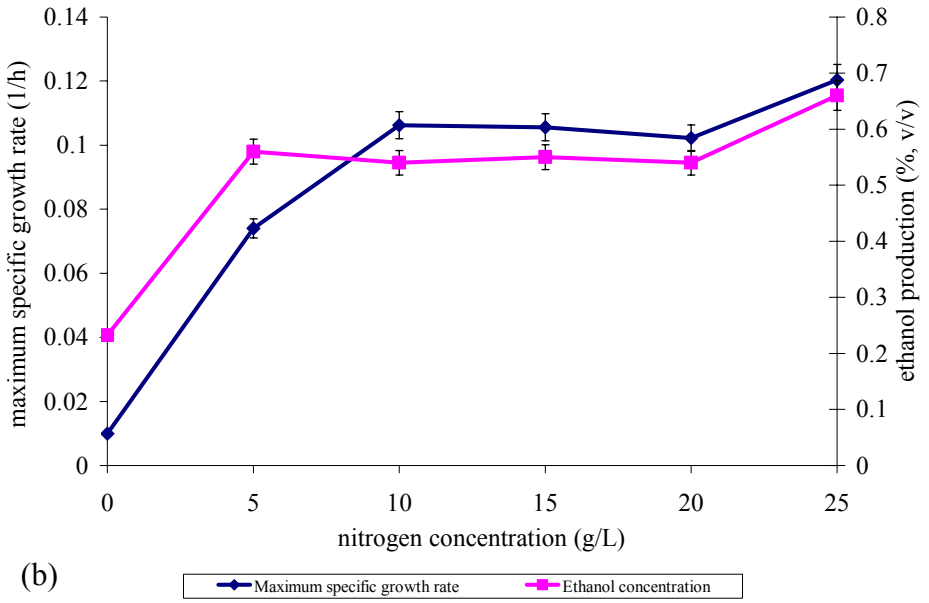
Nitrogen is necessary for the growth and multiplication of yeasts and it also influences the ethanol productivity (Bafrncová *et al*., 1999). In this study, the effect of various inorganic nitrogen sources ($NaNO_3$, NH_4Cl, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ and $NH_4H_2PO_4$) and their concentrations were examined (Figure 4.12). To observe easily the effects of different concentrations and sources of nitrogen on ethanol production,

the relatively low concentration of glucose (1%, v/v) in M9 minimal medium was used. The results showed that $NH_4H_2PO_4$ was the best nitrogen source for growth, which slightly decreased from NH_4Cl, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ and $NaNO_3$, respectively. However, the ethanol production was not related to the growth of the yeast when the highest ethanol was produced in M9 minimal medium, which 10 g/L $(NH_4)_2SO_4$ was used as nitrogen source (Figure 4.13).

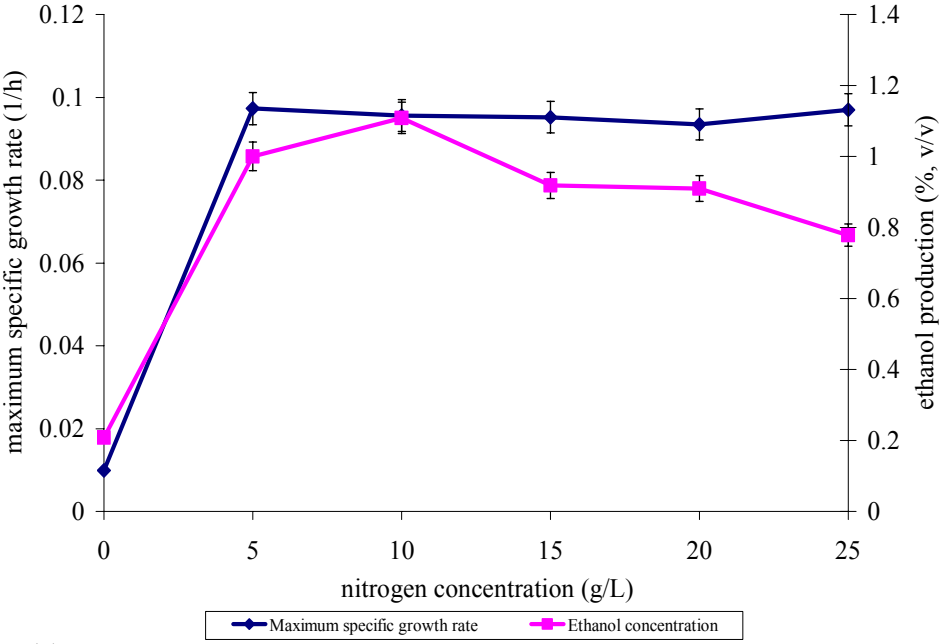


(a)

Figure 4.12 Effect of various sources and concentration of nitrogen for ethanol production by the thermotolerant yeast strain S1 at 40 °C for 3 days in M9 minimal medium, 1% (v/v) glucose, 10% inoculum size, pH 5.5; a) $NaNO_3$, b) NH_4Cl, c) $(NH_4)_2SO_4$, d) $(NH_4)_2HPO_4$ and e) $NH_4H_2PO_4$

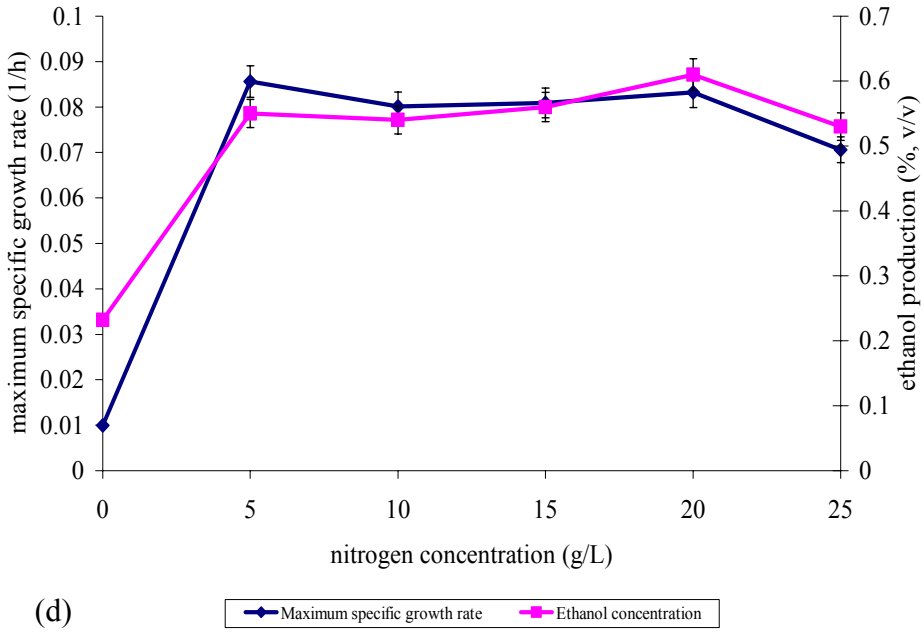


(b)

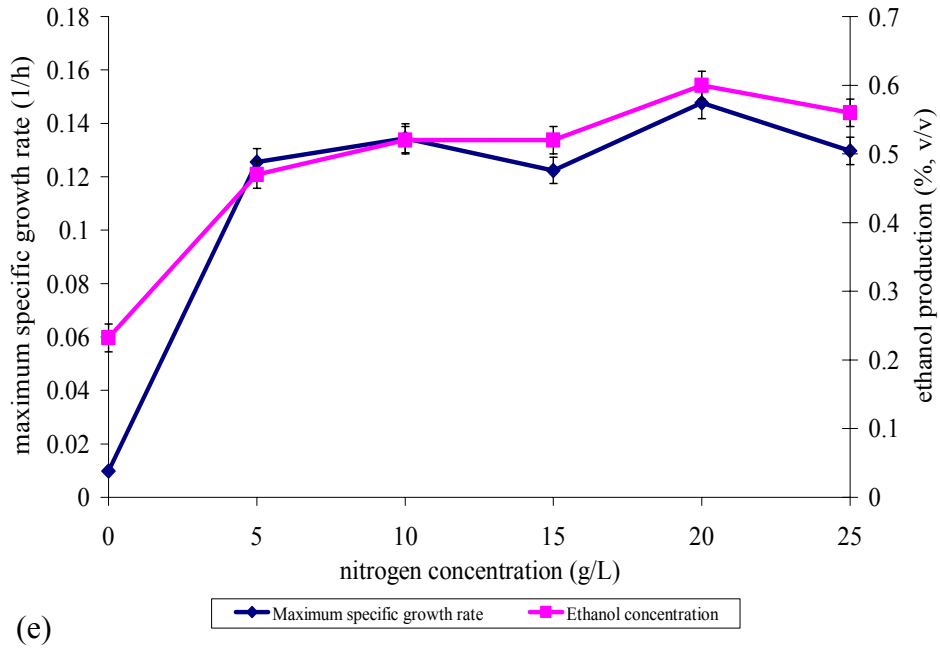


(c)

Figure 4.12 (Continued)



(d)



(e)

Figure 4.12 (Continued)

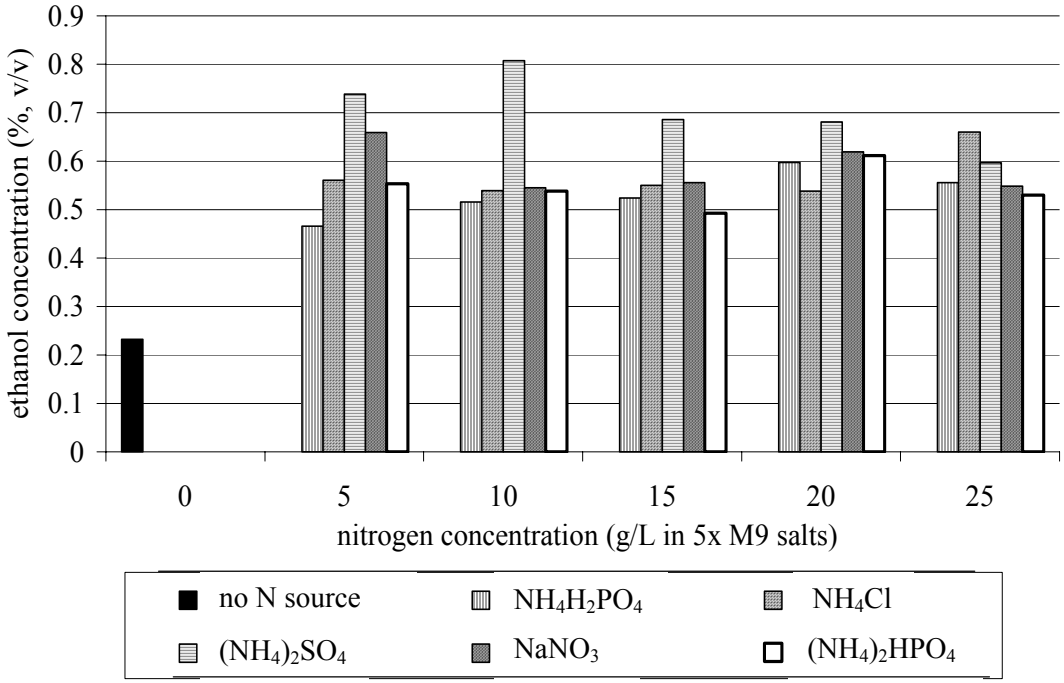


Figure 4.13 Ethanol production in various concentrations and sources of nitrogen in M9 minimal medium, 1% (v/v) glucose, pH 5.5 at 40 °C for 72 h

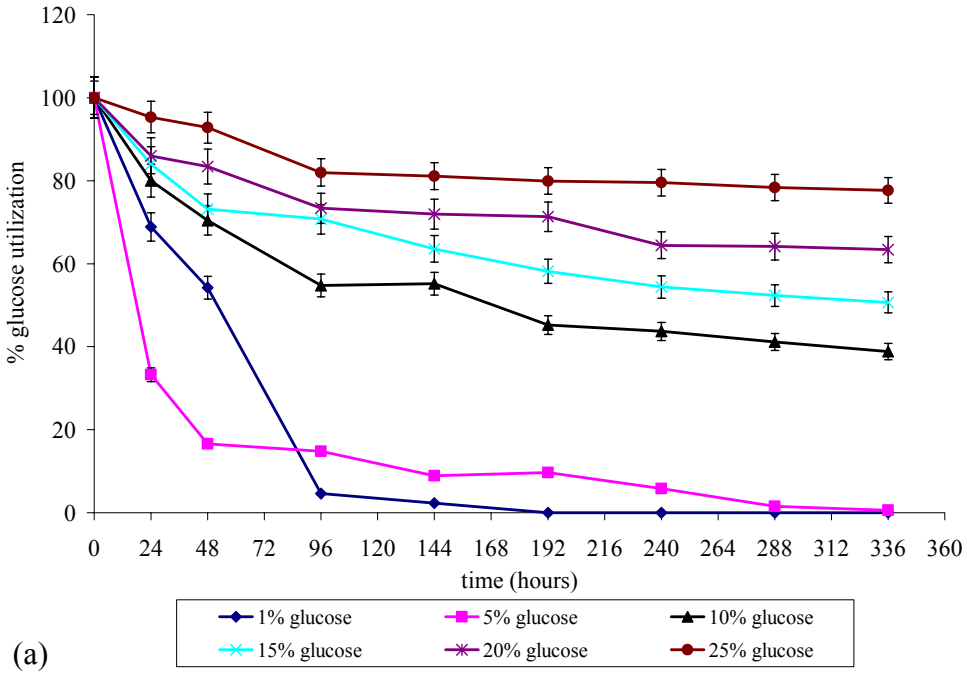
Walker (1998) reported that ammonium sulphate is a commonly used as nitrogen source for yeast growth media since it also provides a source of assimilable sulphur. Some yeast can also grow on nitrate as a source of nitrogen and if able to do so, may also utilize low, sub-toxic concentration of nitrate. Moreover, in the media at initial pH values below 6.0, nitrous acid is formed, which is known to be toxic to the yeast (Rose, 1987). Therefore the addition of $NaNO_3$ was found to reduce the production activity in this experiment, which is similar to Isono and Hoshino (2000). Actually, the source of nitrogen for yeasts is usually provided by organic compounds,

some natural and semi-natural media are based on peptone, yeast extract, wort, etc. Ammonium salts (sulphate, phosphate, and nitrate) are also utilized by the cells. Ammonium salts of organic acid are better utilized than salts of inorganic acids, since their decomposition gives rise to weak acids that can serve as and additional carbon source. Utilization of inorganic salts gives rise to strong inorganic acid that change the pH and have an inhibitory effect on cells (Kocková-Kratochvílová, 1990).

4.5.2 Effect of glucose on ethanol production

Since the previous experiment implied that 10 g/L $(NH_4)_2SO_4$ had the efficiency to ethanol production. The optimization of glucose concentration was observed by various the concentration of glucose from 0 to 25% (v/v) in M9 minimal medium, pH 5.5 and 10 g/L $(NH_4)_2SO_4$ as a nitrogen source. The effect of glucose concentration on ethanol production and growth of yeast is presented in Figure 4.14. It could be seen that ethanol production was slowly increased with the period of incubation. The ethanol production in the fermentation broth was increased with the 5% (v/v) glucose but decreased beyond that. The final glucose utilization in the fermentation broth was found to be used up at the glucose concentration equal to or below 5% (v/v), but above the glucose concentration of 5% (v/v), the final glucose utilization became quite appreciable. The growth of yeast and its maximum ethanol concentration show in Figure 4.15. The maximum specific growth rate and maximum ethanol concentration were increasing with an increase of glucose concentration for 5% (v/v). A reduction of ethanol production and growth of yeast were decreased when glucose concentration was greater than 5% (v/v).

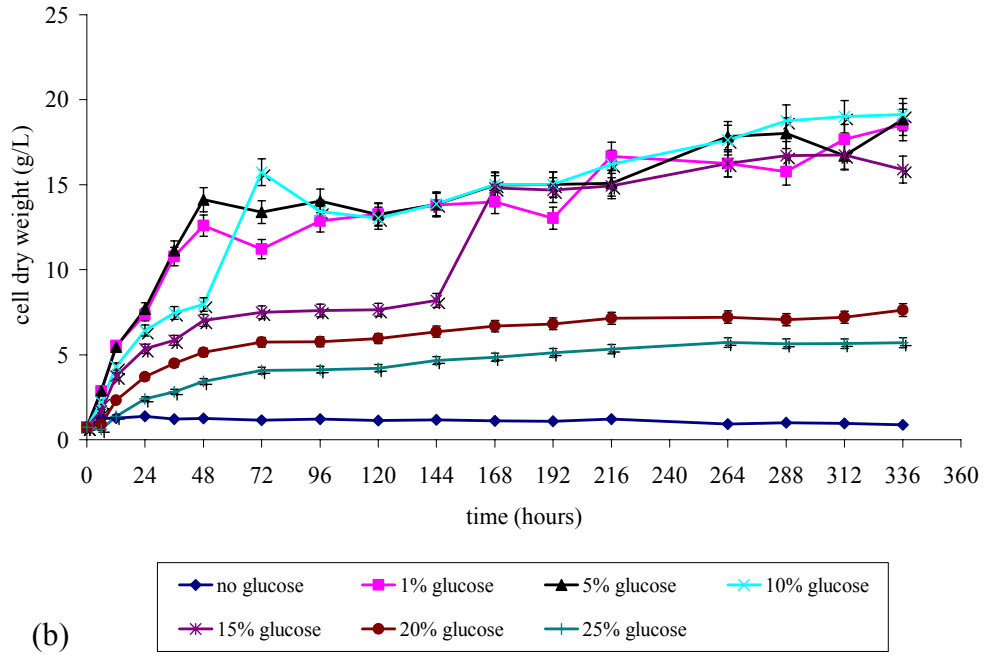
It is apparent that high glucose concentration can be efficiently converted into ethanol by the thermotolerant yeast strain S1. The results also indicate to a certain extent the inhibitory effects of high glucose concentration on the ethanol production. Glucose inhibition is related principally to osmotic effects (Bajpai and Margaritis, 1987). As pointed out by Ghose and Tyagi (1979), a high concentration of glucose in the medium inhibits the growth of yeast cells and the production of ethanol, and the inhibitory effect is attributed to high osmotic pressure. This inhibitory effect is one of the major problems for ethanol production.



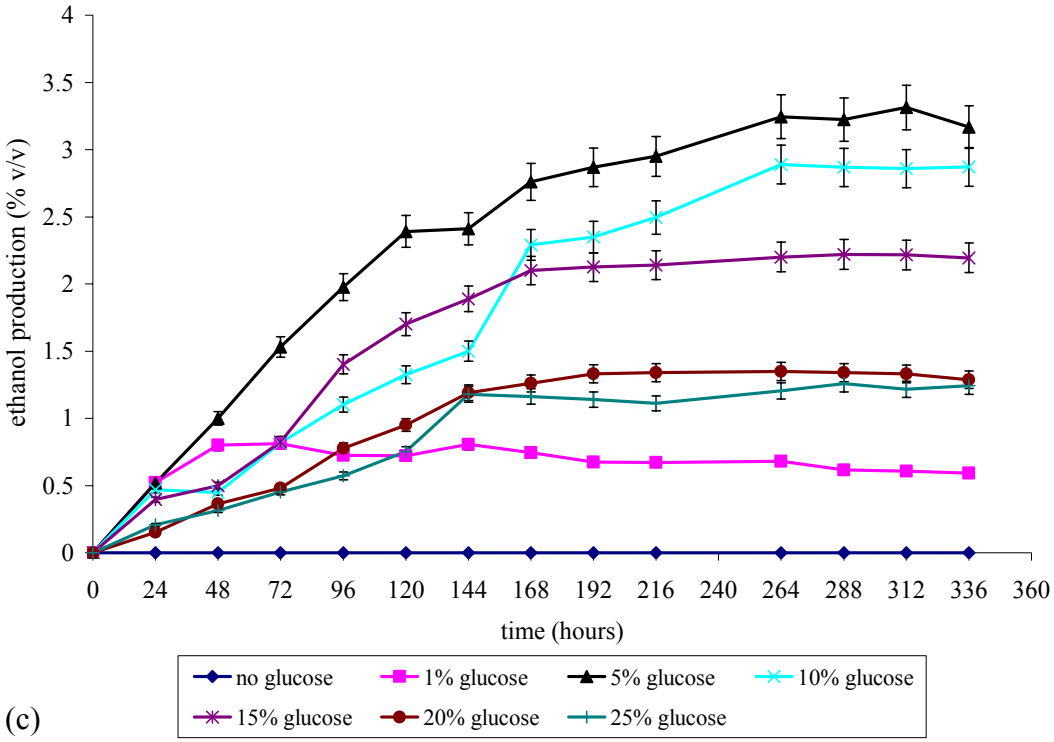
(a)

Figure 4.14 Percentage of glucose utilization, growth and ethanol production by the thermotolerant yeast strain S1 in M9 minimal medium containing various glucose concentrations and 10 g/L $(NH_4)_2SO_4$ as a nitrogen source, 5% inoculum size, pH 5.5 at 40 $^{\circ}$C for 336 h; a) glucose

concentration, b) growth of the thermotolerant yeast strain S1, and c) ethanol production



(b)



(c)

Figure 4.14 (Continued)

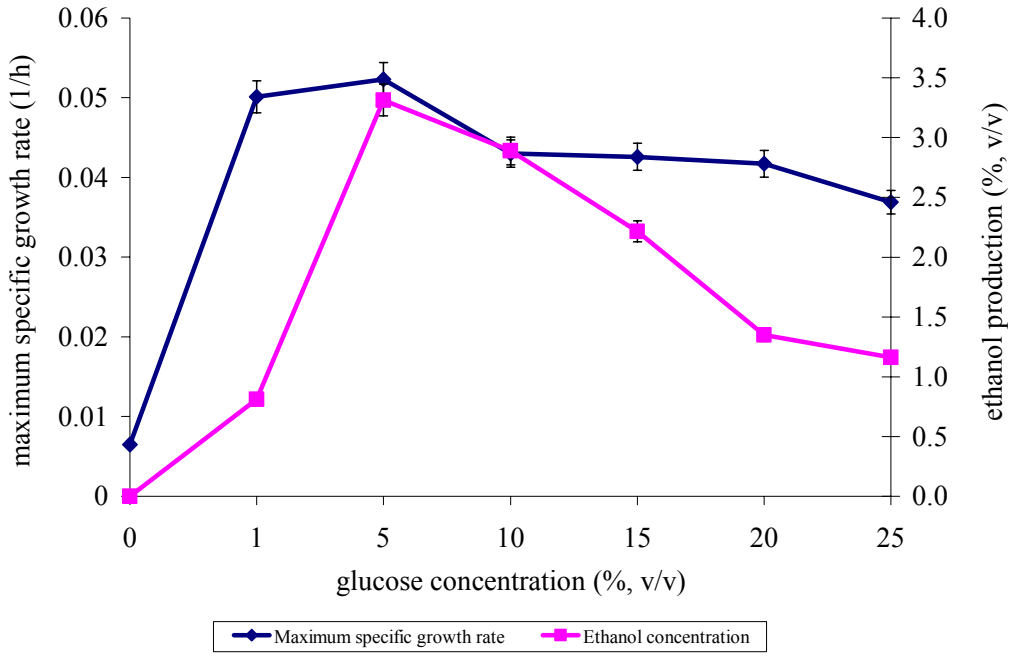


Figure 4.15 Effect of glucose concentration on growth and ethanol production in M9 minimal medium when use $(NH_4)_2SO_4$ as a nitrogen source

4.5.3 Effect of pH on ethanol production

As results from the previous study, the ethanol production was examined in M9 minimal medium which 10 g/L $(NH_4)_2SO_4$ was used as a nitrogen source and 5% (v/v) glucose as a carbon source. The growth rate of yeast was increased along with the pH level increase to 2.5. The pH of the medium had no more different effect on the growth rate at pH 2.5 to 6.8, but beyond that the growth rates were decreased. However, medium pH had an important impact on the final ethanol production in the

medium. A reduction in the final ethanol produced was observed as the pH of the medium below 4.0 and above 6.5. At medium pH 4.0 to 6.5, the final ethanol productions were slightly decreased. However, the maximum final ethanol production was observed in the medium pH equal to 4.0 (Figure 4.16).

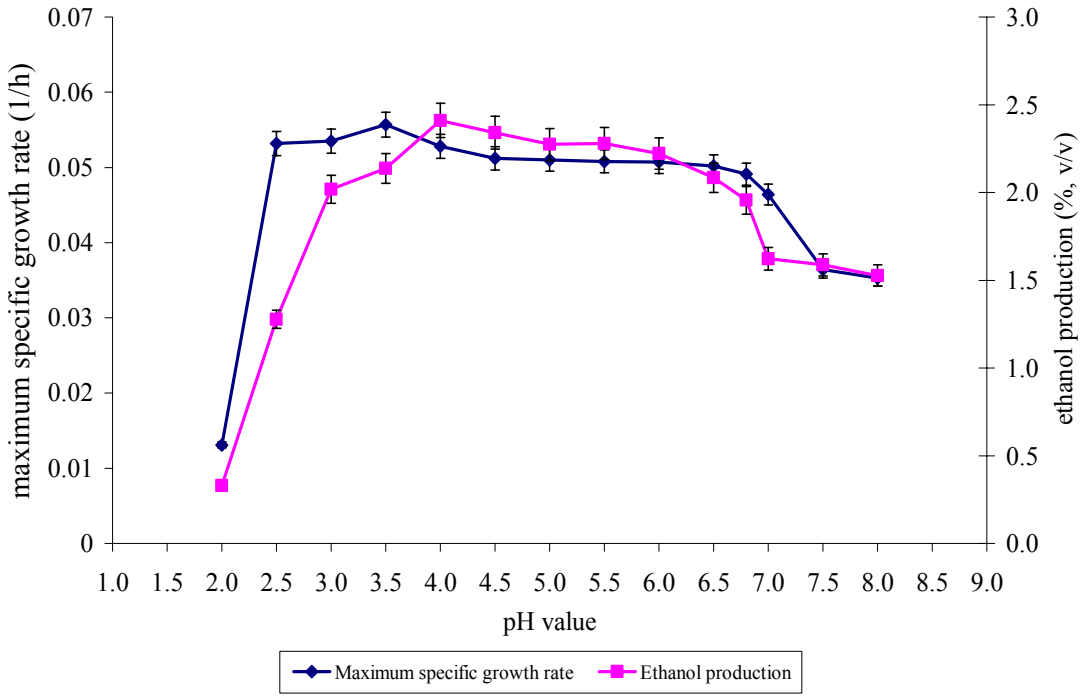


Figure 4.16 Maximum specific growth rate and ethanol production of the thermotolerant yeast strain S1 at 40 °C in M9 minimal medium with 5% (v/v) glucose as a carbon source, 10 g/L $(NH_4)_2SO_4$ as a nitrogen source and 5% inoculum size at various pHs

In general, yeast is an acidophilic organism and, as such, grows better under acidic conditions. The optimal pH range for yeast growth can vary from pH 4.0 to 6.0, depending on temperature, the presence of oxygen, and the strain of yeast. There is a paucity of literature on the physiological basis for this preferred pH range, but it most

likely is due to the optimum pH value for the activity of plasma membrane-bound proteins, including enzymes and transport proteins (Narendranath and Power, 2005). During growth, it is important for the yeast to maintain a constant intracellular pH. There are many enzymes functioning within the yeast cell during growth and its metabolism. Each enzyme works best at its optimal pH, which is acidic because of the acidophilic nature of the yeast itself. When the extracellular pH deviates from the optimal level, the yeast cell needs to invest energy to either pump in or pump out hydrogen ions in order to maintain the optimal intracellular pH (Narendranath *et al*., 2001 and Thomas *et al*., 2002). If the extracellular pH deviates too much from the optimal range, it may become too difficult for the cell to maintain constant intracellular pH, and the enzymes may not function normally. If the enzymes are deactivated, the yeast cell will not be able to grow and make ethanol efficiently (Narendranath and Power, 2005). That is the most likely explanation for the observed reduction in ethanol production when the initial medium pH was lowered from 4.0 to 2.0 and higher than 6.5 (Figure 4.16).

As compared with sources and concentrations of nitrogen, the maximum specific growth rate (μ_{max}) of optimization of glucose and pH were low (Figure 4.12, 4.15 and 4.16). In this case, inoculum size was directly effect on the growth rate. Particularly, $(NH_4)_2SO_4$, the first experiment was used with 10% inoculum size, then μ_{max} near 0.01 to 0.1 h^{-1}. The two latter experiments, the inoculum size was lower than 10%, thus μ_{max} was low (0.005 to 0.05 h^{-1}). In contrat, ethanol production in various glucose concentrations and pH were high resulting from different glucose concentration served as substrate in production of ethanol.

4.6 Ethanol production in 2 L fermenter

Two liters of batch fermentations were run for the purpose of determining the ethanol production by the thermotolerant yeast strain S1. A 2 L fermenter was charged with 1.5 L of M9 minimal medium, at glucose concentration of 5% (v/v) and 10 g/L $(NH_4)_2SO_4$ as carbon and nitrogen source, respectively. The fermentation was run aseptically at 40 °C and pH 4.0. Both temperature and pH were controlled.

The glucose consumption, ethanol and cell production and titritrable acidity (TA) during the different aeration time processes are shown in Figure 4.17. Figure 4.17a, it can be observed that the fermentation occurred in two phases: a first phase was characterized by a concomitant growth, ethanol production and glucose consumption and a second phase where growth and glucose consumption were dropped but cells kept producing ethanol. In contrast, Figure 4.17b, a first phase similar to Figure 4.17a, but a second phase where growth and glucose consumption stopped and ethanol production was decreased. Indeed, after depletion of glucose and accumulation of ethanol, the metabolism in alcoholic fermentation changes. The fermentation product, ethanol, becomes a substrate and is degraded if oxygen is present. This change in metabolism is known as the diauxic shift (Pronk *et al*., 1996). However, glucose was lowered when the air flushed continuously for 72 h. The titrable acidity values were not different.

Wayman and Parekh (1990) reported that ethanol fermentation is not a wholly anaerobic process. Dissolved oxygen concentration is an important variable for industrial alcohol production. In many instances, active starter cultures are grown under aerobic or semiaerobic conditions to improve yeast yields and growth rates. Even if sufficient nutrients such as sugar, nitrogen and vitamins are present, it is the

dissolved oxygen in the solution which limits the population of yeast in the fermentation medium. Once the dissolved oxygen is depleted during fermentation, the yeast population growth slows and eventually ceases. Also during this time the metabolism of yeast switches from aerobic respiration to anaerobic alcoholic fermentation, and the synthesis of alcohol occurs until the sugar is depleted or until a limiting ethanol concentration is obtained.

Kinetic parameters in shake flask level and in 2 L fermenter were shown in Table 4.2. The growth rate of yeast in shake flask level was lower than in fermenter, this may results from inoculum size and aeration. In fermenter, when started aeration for 24 h, μ_{max} was low but ethanol yield was high when compared with aeration along with fermentation. It seems that the aeration of fermentation might effect to ethanol production. However, the time of highest of ethanol production in both experiments was 18 to 21 h (Figure 4.17). Addition of glucose in 18^{th} h may gave high ethanol production since glucose was rapidly decreased.

Values obtain for ethanol yield (% of theoretical) are comparable with data compiled by Hughes *et al*., (1984) for a strain of *K. marxianus* grown in glucose medium (glucose, yeast extract, NaCl, $CaCl_2$, KH_2PO_4, $FeCl_3$, $MgSO_4.7H_2O$ and NH_4Cl) at 40 °C in shake flask. These workers recorded ethanol yield (% theoretical yield) of 92.2%. Anderson *et al*., (1986) showed the isolation and characterization of naturally occurring strains of *K. marxianus* var. *marxianus* which ferment carbohydrate to ethanol at efficiencies between 85 and 90% at temperature up to 47 °C as Ueno *et al*., (2001) reported *Torulaspora debrueckii* show the high ethanol yield in 5% glucose solution (without nutrient and metallic salts) of 72.1% compared with 57.0% for the brewer's yeast at 25 °C.

Table 4.2 Kinetic parameters for ethanol production of the thermotolerant yeast strain S1 in shake flask and fermenter (different aeration time) with 5% (v/v) of glucose and 10 g/L $(NH_4)_2SO_4$ in M9 minimal medium, pH 4.0 at 40 °C

Kinetic Parameter	Shake flask	Aeration time (h)	
		24	72
Maximum specific growth rate, μ_{max} (h^{-1})	0.0528	0.1484	0.1634
Maximum ethanol yield (g/g)	0.3804	0.3879	0.3223
Maximum ethanol yield (% theoretical)	74.58	76.06	63.20
Maximum ethanol production rate (g/g/h)	0.00113	0.0216	0.0153
Maximum volumetric production (g/L/h)	0.057	0.872	0.725
Volumetric production (g/L/h)	0.057	0.168	0.0296
Fermentation time (h)	336	72	72

As indicated in Figure 2.6, carbon dioxide and ethanol are produced from glucose in equimolar amounts. Additional reactions also take place in the fermenter, leaking to small amounts of such minor by-products as glycerol, acetic acid, lactic acid, succinic acid, acetaldehyde and furfural (Glazer and Nikaido, 1995). Of these, some organic acids were determined when compare with standards: lactic acid, citric acid, acetic acid, succinic acid, tartaric acid, malic acid and fumaric acid by using HPLC technique. The major unknown peak (amount 40x of acetic acid) was found at the retention time of 13.5 minutes (Figure 4.18-4.20). From this result, unknown by-product might be involved in some metabolisms of this yeast during fermentation.

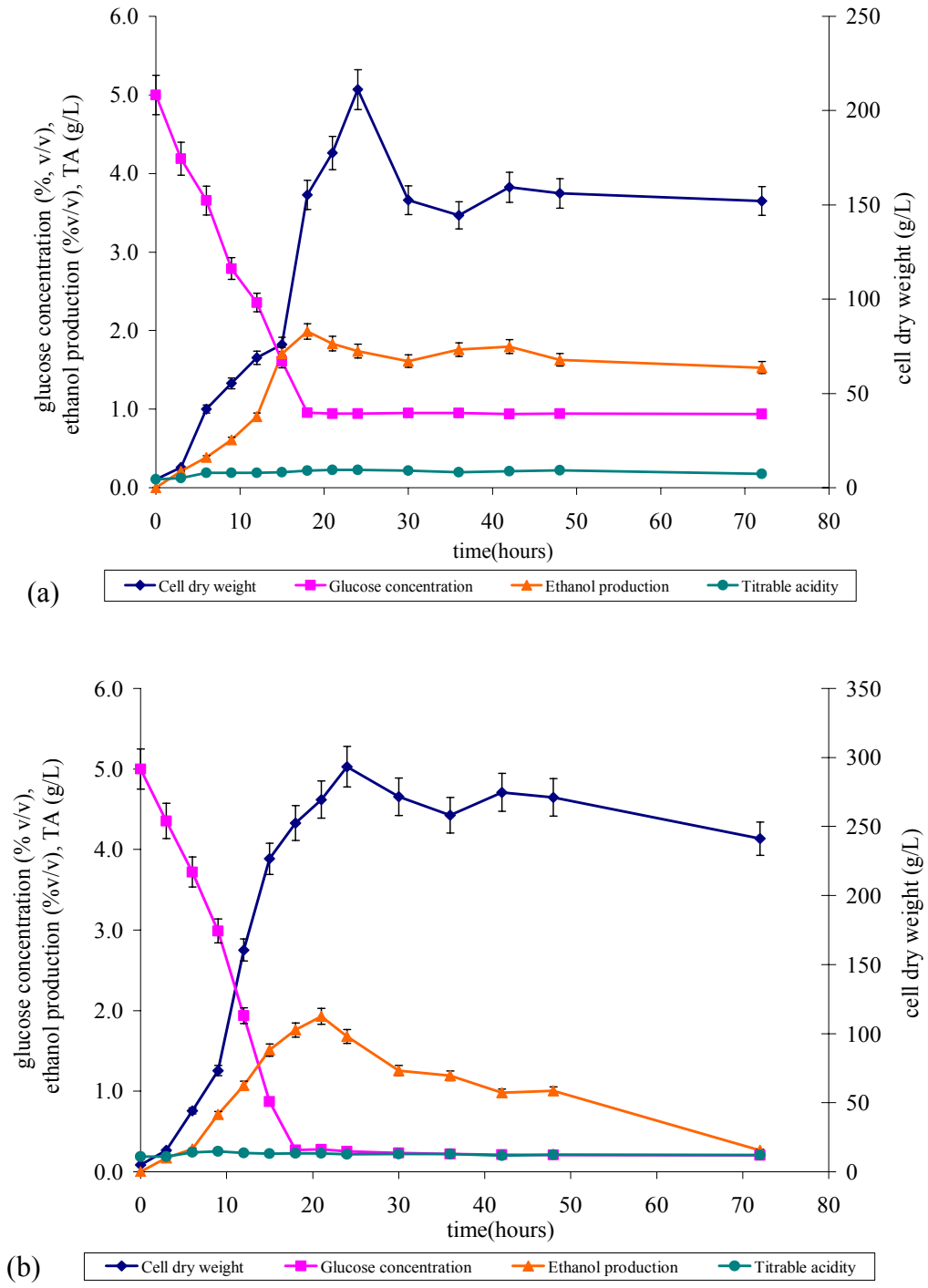


Figure 4.17 Changes in the glucose concentration, ethanol production, growth of yeast and titritrable acidity in batch fermentations with 0.5 vvm of

aeration, 200 rpm and 10% inoculum size at 40 °C; a) aeration with in 24 h, and b) aeration for 72 h

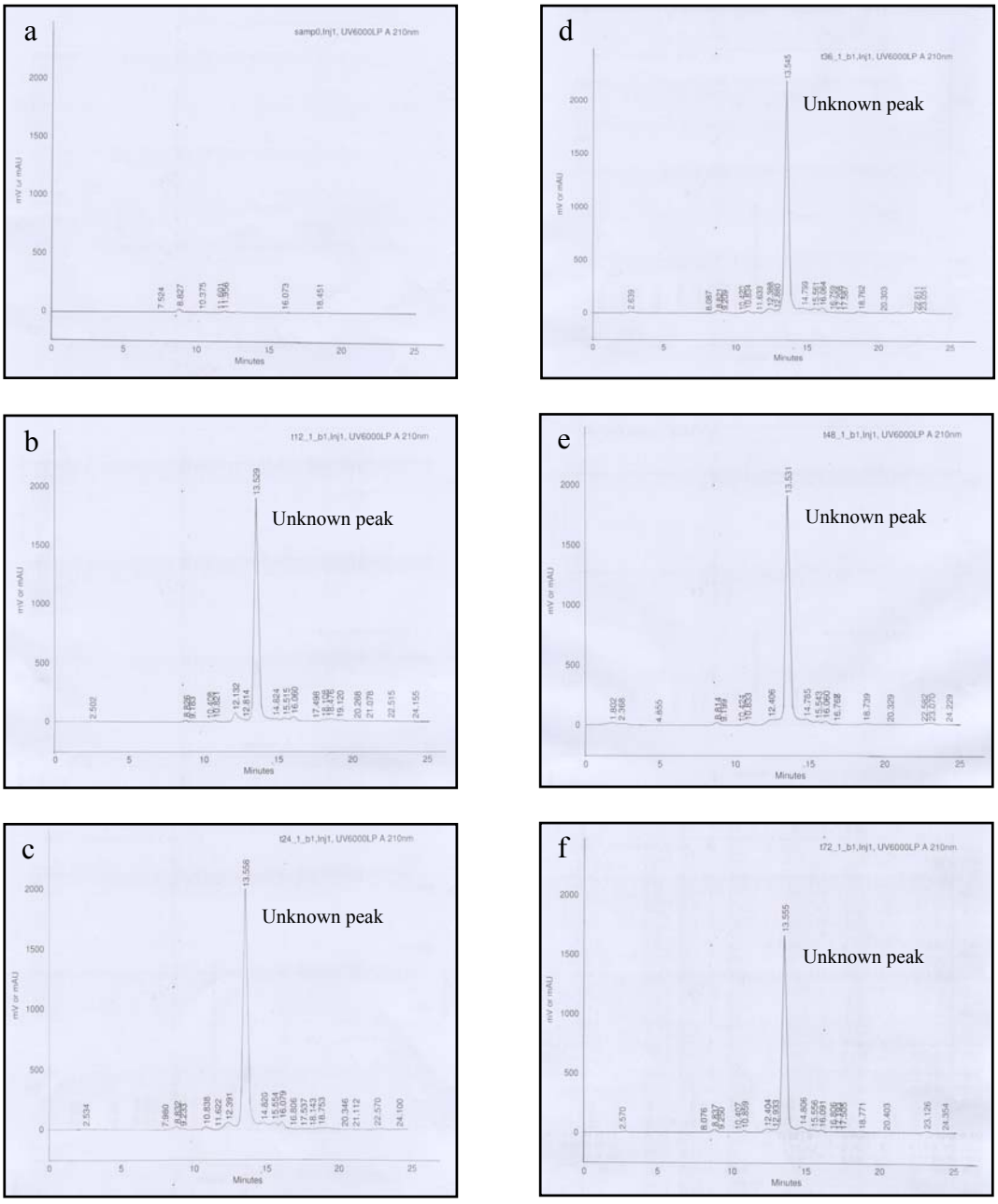


Figure 4.18 Unknown peak detection by HPLC at the retension time of 13.5 minutes when batch fermentation was performed by the thermotolerant yeast strain S1 in M9 minimal medium, 5% (v/v) glucose, 10 g/L $(NH_4)_2SO_4$ as carbon and nitrogen source, respectively, pH 4.0 at 40 °C for 72 h with

started aeration for 24 h; a) start of fermentation, b) 12 h, c) 24 h, d) 36 h, e) 48 h, and f) 72 h

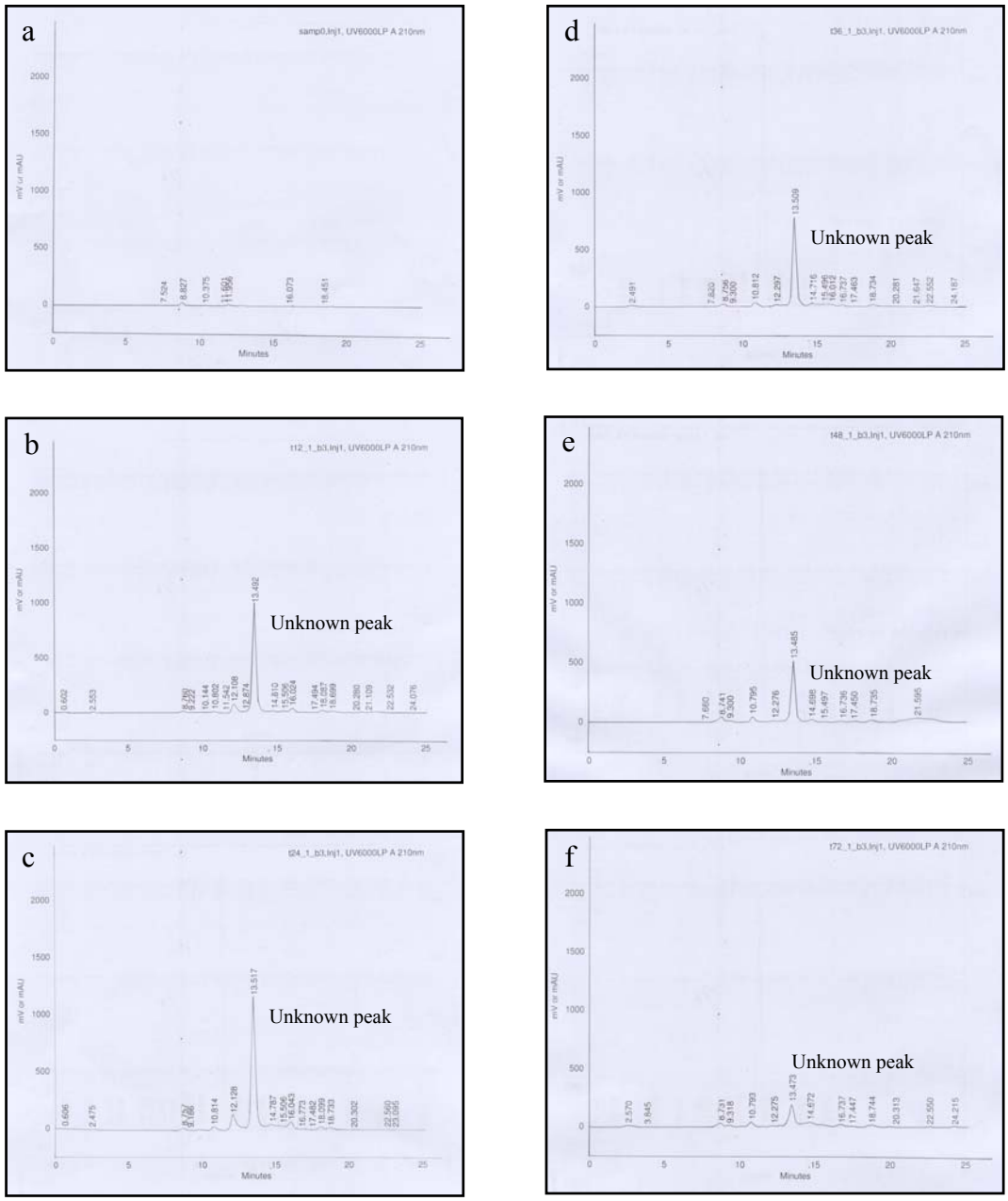


Figure 4.19 Unknown peak detection by HPLC at the retension time of 13.5 minutes when batch fermentation was performed by the thermotolerant yeast strain S1 in M9 minimal medium, 5% (v/v) glucose, 10 g/L $(NH_4)_2SO_4$ as carbon and nitrogen source, respectively, pH 4.0 at 40 °C for 72 h with

aeration a long with fermentation; a) start of fermentation, b) 12 h, c) 24 h, d) 36 h, e) 48 h, and f) 72 h

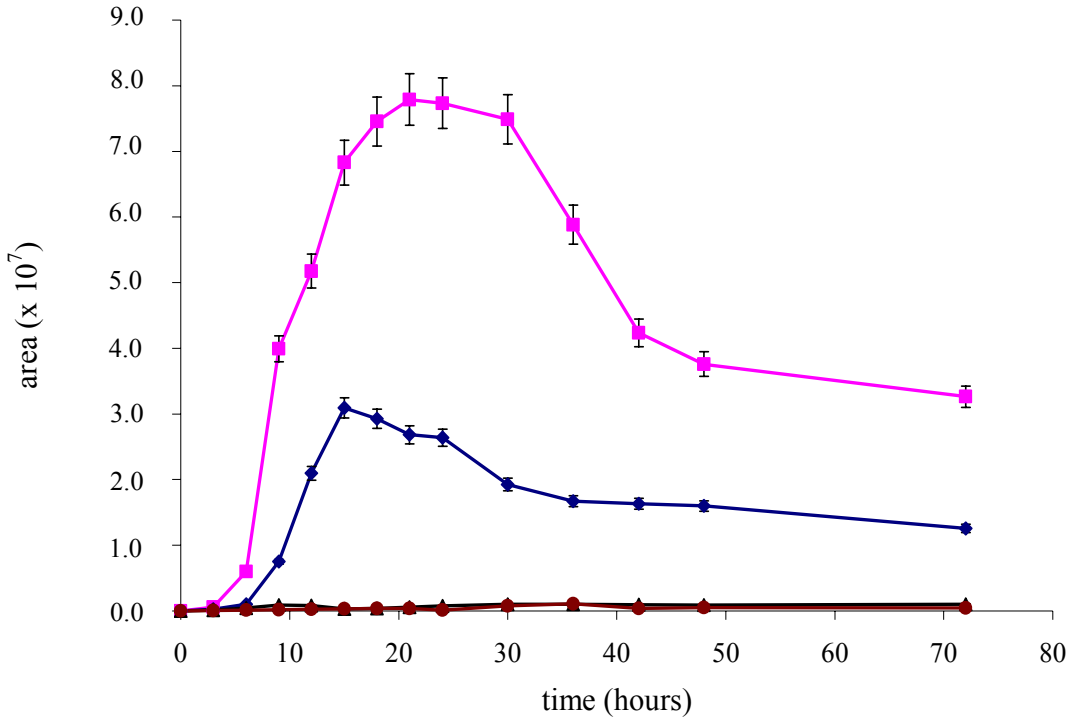


Figure 4.20 Detection of unknown compound and acetic acid by HPLC during fermentation; aeration started for 24 h (-■-), aeration along with fermentation (-◆-), acetic acid when aeration 24 h (-▲-), acetic acid when aeration along with fermentation (-●-)

CHAPTER V

CONCLUSION

The thermotolerant yeast was isolated from silage produced at Suranee University of Technology farm. The cell morphology of the yeast cells under microscope is ovoidal to elongate, single or in pairs. Budding cells are present and pseudomycelia are developed. Based on biochemical characterisics from API Candida strip, the thermotolerant yeast strain S1 was identified as *Candida krusei*. Moreover, from the results of the comparision of PCR-amplified 18S rDNA sequence the thermotolerant yeast strain S1 was closely related to *Issatchenkia* sp. The S1 strain did not produce killer toxin when tested with *S. cerevisiae* EC 1118, *E. coli* ATCC 25922 and *B. subtilis* ATCC 6633, and it was able to tolerate ethanol in YPD medium containing ethanol up to 25% (v/v), the growth was highest in 5% (v/v) ethanol.

The ethanol production at elevated temperature, 40 $^{\circ}$C, was preliminary tested in YPD medium, pH 5.5. In shake flask experiment, to observe the effect of nitrogen source, carbon source and pH for ethanol production, M9 minimal medium was used. The thermotolerant yeast strain S1 was able to produced ethanol up to 74.58% theoretical from 5% (v/v) glucose in M9 minimal medium pH 4.0 when ammonium sulfate as a nitrogen source. The optimum condition for ethanol production in shake flask experiment was observed in 2 liters fermenter. The yeast gave the highest cell dry weight and produced ethanol within 24 hours. After end of aerobic fermentation,

the ethanol production was decreased within 72 hours. In contrast to aeration for 24 hours, ethanol production was stable with 76.06% theoretical yield. Additionally, the production of some organic acid using HPLC technique was investigated. Amount 40x of unknown compound when compare with acetic acid was detected at the retension time of 13.5 minutes couple with the growth of yeast and its ethanol production. Therefore, ethanol production by thermotolerant yeast strain S1 in M9 minimal medium should be considered because this yeast was produced high amount unknown compound, that might be involved in some metabolisms of this yeast during ethanol fermentation.

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