ETHANOL PRODUCTION BY THERMOTOLERANT *ISSATCHENKIA* SP. S1

Tapanawat Muaenjang

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การผลิตเอทานอลโดยเชื้อยีสต์ทนร้อน *Issatchenkia* sp. S1

นายฐปณวัชร์ หมื่นแจ้ง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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มหาวิทยาลัยเทคโนโลยีสุรนารี
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ETHANOL PRODUCTION BY THERMOTOLERANT *ISSATCHENKIA* SP. S1

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

Thesis Examining Committee

(Assoc. Prof. Dr. Neung Teaumroong)
Chairperson

(Asst. Prof. Dr. Chokchai Wanapu)
Member (Thesis Advisor)

(Asst. Prof. Dr. Apichat Boontawan)
Member

(Prof. Dr. Pairote Sattayatham)
Vice Rector for Academic Affairs

(Asst. Prof. Dr. Suwayd Ningsanond)
Dean of Institute of Agricultural Technology

ฐปณวัชร์ หมื่นแจ้ง : การผลิตเอทานอลโดยเชื้อยีสต์ทนร้อน *Issatchenkia* sp. S1 (ETHANOL PRODUCTION BY THERMOTOLERANT *ISSATCHENKIA* SP. S1) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.โชคชัย วนภู, 108 หน้า.

การศึกษายีสต์ทนร้อนเพื่อการผลิตเอทานอลเป็นไปอย่างแพร่หลาย เนื่องจากยีสต์ทนร้อนสามารถเจริญและทำให้เกิดกระบวนการหมักได้ดีในประเทศเขตร้อน วัตถุประสงค์ของการศึกษาครั้งนี้คือหาสภาวะที่เหมาะสมต่อการเจริญและการผลิตเอทานอลของยีสต์ *Issatchenkia* sp. S1 ในอาหาร enrich medium การใช้แหล่งคาร์บอนโดยยีสต์ *Issatchenkia* sp. S1 ซึ่งทำการเปรียบเทียบชนิดของแหล่งคาร์บอนในอาหารเลี้ยงเชื้อ YM พบว่า *Issatchenkia* sp. S1 เจริญได้น้อยในอาหารเลี้ยงเชื้อที่ใช้ซูโครส แลคโตส กลีเซอรอล แมนนิทอล มอลโตส แป้งมันสำปะหลังและแป้งมันฝรั่งเป็นแหล่งคาร์บอน แต่เมื่อใช้น้ำตาลกลูโคสหรือฟรุกโตสเป็นแหล่งคาร์บอนในอาหารเลี้ยงเชื้อ YM พบว่ายีสต์สามารถเจริญได้ดี และใช้แหล่งคาร์บอนทั้งสองชนิดเพื่อหาความเข้มข้นที่เหมาะสมต่อการเจริญและการผลิตเอทานอลสำหรับการทดลองใน flask จากทดลองการเลี้ยงยีสต์ *Issatchenkia* sp. S1 ใน flask พบว่าการผลิตเอทานอลสูงสุดเมื่อทำการการเลี้ยงยีสต์โดยใช้ความเข้มข้นของกลูโคสที่ 100 กรัมต่อลิตรในอาหารเลี้ยงเชื้อ YM ที่ทำการเติม 2 กรัมต่อลิตร แอมโมเนียมซัลเฟต ทำการเขย่าที่ความเร็วรอบ 200 รอบต่อนาที และบ่มเชื้อที่อุณหภูมิ 40 องศาเซลเซียส (μ = 0.205 ± 0.008 ต่อชั่วโมง Q_p = 2.328 ± 0.040 กรัมต่อลิตรต่อชั่วโมง, Y_{ps} = 0.511 ± 0.009 กรัมต่อกรัม และความเข้มข้นของเอทานอลสูงสุด = 55.877 ± 0.962 กรัมต่อลิตร) จากนั้นศึกษาการผลิตเอทานอลในถังหมักขนาด 2 ลิตร ซึ่งทำการศึกษาอัตราการให้อากาศและความเร็วในการกวน พบว่า การกวนที่ความเร็ว 500 รอบต่อนาทีและไม่มีการให้อากาศ โดยใช้อาหารเลี้ยงเชื้อ YM ที่มีน้ำตาลกลูโคส 100 กรัมต่อลิตรและทำการเติม 2 กรัมต่อลิตร แอมโมเนียมซัลเฟต ที่ 40 องศาเซลเซียส ให้ผลสูงสุดของ μ (0.370 ± 0.009 ต่อชั่วโมง) Q_p (1.886 ± 0.056 กรัมต่อลิตรต่อชั่วโมง) Y_{ps} (0.516 ± 0.026 กรัมต่อกรัม) และความเข้มข้นของเอทานอลสูงสุดที่ 49.991 ± 1.495 กรัมต่อลิตร จากนั้นได้ทำการผลิตเอทานอลในถังหมักขนาด 10 ลิตร ซึ่งพบว่าผลิตได้น้อยกว่าในถังขนาด 2 ลิตร (μ = 0.355 ± 0.012 ต่อชั่วโมง Q_p = 1.335 ± 0.104 กรัมต่อลิตรต่อชั่วโมง Y_{ps} = 0.431 ± 0.005 กรัมต่อกรัม และความเข้มข้นของเอทานอลสูงสุด = 42.434 ± 1.699 กรัมต่อลิตร) นอกจากนี้ ได้ทำการพัฒนาการผลิตเอทานอลในถังหมักขนาด 10 ลิตร โดยใช้ระบบแบบ fed-batch โดยทำการเติมน้ำตาลกลูโคสเพื่อเพิ่มการผลิตเอทานอล พบว่าการเติมน้ำเชื่อมที่มีน้ำตาลกลูโคส 350 กรัม ปริมาตร 1 ลิตร ที่เวลา 12 ชั่วโมงและ 400 กรัม ปริมาตร 1 ลิตร ที่เวลา 24 ชั่วโมงหลังจากเริ่มต้นการหมัก ให้ผลสูงสุดของการผลิตเอทานอล (Q_p = 1.716 ± 0.150 กรัมต่อลิตรต่อชั่วโมง Y_{ps} = 0.506 ± 0.011 กรัมต่อกรัม และความเข้มข้นของเอทานอลสูงสุด = 77.810 ± 1.879 กรัมต่อลิตร) นอกจากนี้ ได้ทำการวิเคราะห์

หาการผลิตกรดอินทรีย์ในถังหมัก 10 ลิตร พบว่าเป็นกรดออกซาโลอะซิติก (oxaloacetic acid) ซึ่งจะผลิตในช่วงที่มีการเจริญของยีสต์และลดลงเมื่อน้ำตาลกลูโคสถูกใช้จนหมด ความเข้มข้นสูงสุดของกรดออกซาโลอะซิติกคือ 3.035 ± 0.252 กรัมต่อลิตรที่ 30 ชั่วโมงหลังจากเริ่มต้นการหมัก

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2551 ลายมือชื่ออาจารย์ที่ปรึกษา_____

TAPANAWAT MUAENJANG : ETHANOL PRODUCTION BY THERMOTOLERANT *ISSATCHENKIA* SP. S1. THESIS ADVISOR : ASST. PROF. CHOKCHAI WANAPU, Ph.D., 110 PP.

ETHANOL PRODUCTION/ THERMOTOLERANT *ISSATCHENKIA* SP.

Ethanol production by thermotolerant yeast has been extensively studied, because thermotolerant yeasts are capable of growth and fermentation during the summer periods in non-tropical countries as well as under tropical climates. The aim of this study was to optimized conditions for growth and ethanol production of thermotolerant *Issatchenkia* sp. S1 in enrich medium. The utilization of carbon sources by *Issatchenkia* sp. S1 was varied with sort of carbons supplemented in YM medium. *Issatchenkia* sp. S1 showed weakly grown in YM medium with sucrose, lactose, glycerol, manitol, maltose, cassava starch or potato starch as carbon source. When either glucose or fructose was used as carbon source in YM medium, the better growth of *Issatchenkia* sp. S1 was performed. Glucose and fructose were used for determining the optimal concentration for growth and ethanol production in flask experiments. The cultivation of *Issatchenkia* sp. S1 in flask experiment found that when cultured *Issatchenkia* sp. S1 in YM medium supplemented with 100 g/L of glucose and 2 g/L $(NH_4)_2SO_4$ under shaking condition at 200 rpm and incubation at 40 $^{\circ}$C showed the highest ethanol production (μ = 0.205 ± 0.008 h^{-1}, Q_p = 2.328 ± 0.040 g/L/h, Y_{ps} = 0.511 ± 0.009 g/g and maximum ethanol concentration = 55.877 ± 0.962 g/L). In 2 L fermenter experiment, aeration rate and agitation speed were varied. The agitation speed at 500 rpm with no aeration in YM medium with 100 g/L

and 2 g/L $(NH_4)_2SO_4$ at 40 °C showed the highest of μ (0.370 ± 0.009 h^{-1}), Q_p (1.886 ± 0.056 g/L/h), Y_{ps} (0.516 ± 0.026 g/g) and ethanol concentration (49.991 ± 1.495 g/L). The ethanol production by *Issatchenkia* sp. S1 was scale up to 10 L fermenter. The results in 10 L batch culture were lower than that of 2 L fermenter (μ = 0.355 ± 0.012 h^{-1}, Q_p = 1.335 ± 0.104 g/L/h, Y_{ps} = 0.431 ± 0.005 g/g and maximum ethanol concentration = 42.434 ± 1.699 g/L). Furthermore, the ethanol production by *Issatchenkia* sp. S1 in 10 L fermenter was improved by fed-batch operation by adding glucose for increasing the production of ethanol. A liter of both glucose syrup at 350 g and 400 g were added at 12 h and 24 h after fermentation showed the highest ethanol production (Q_p = 1.716 ± 0.150 g/L/h, Y_{ps} = 0.506 ± 0.011 g/g and maximum ethanol concentration = 77.810 ± 1.879 g/L). Additionally, the production of some organic acids detected by using HPLC technique was investigated in 10 L batch fermentation. Oxaloacetic acid (OAA) is major organic acid produced during log phase and reduced when glucose depleted. The highest concentration of OAA was 3.035 ± 0.252 g/L at 30 h after fermentation.

School of Biotechnology Student's Signature_____

Academic Year 2008 Advisor's Signature_____

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LIST OF ABBREVIATIONS

ADP	=	adenosine diphosphate
ATP	=	adenosine triphosphate
$^{\circ}C$	=	degree Celsius
CO_2	=	carbon dioxide
DCM	=	dry cell mass (g/L)
DNA	=	deoxyribonucleic acid
GC	=	gas chromatography
g	=	gram
g/g	=	gram of substrate per gram of ethanol
g/L	=	gram per liter
mg/L	=	milligram per liter
h	=	hour
h^{-1}	=	reciprocal hours
HCl	=	hydrochoric acid
HPLC	=	high performance liquid chromatography
L	=	liter
mL	=	milliliter
mL/min	=	milliliter per minute
µL	=	microliter
mm	=	millimeter

LIST OF ABBREVIATIONS (Continued)

nm	=	nanometer
µm	=	micrometer
mM	=	millimolar
M	=	molarity
nM	=	nanomolar
N	=	normality
NA	=	not available
NADH	=	nicotinamide adenine dinucleotide
$NADP^+$	=	nicotinamide adenine dinucleotide phosphate
NaOH	=	sodium hydroxide
$(NH_4)_2SO_4$	=	diammonium sulfate
OAA	=	oxaloacetic acid or oxalacetic acid
OD	=	optical density
PC	=	pyruvate carboxylase
PCR	=	polymerase chain reaction
PPC	=	phosphoenolpyruvate carboxylase
psi	=	pound per square inch
Q_p	=	volumetric ethanol production (g/L/h)
rpm	=	round per minute
rDNA	=	ribosomal deoxyribonucleic acid
rRNA	=	ribosomal ribonucleic acid
SEM	=	scanning electron microscope

LIST OF ABBREVIATIONS (Continued)

SSF	=	simultaneous saccharification and fermentation
TCA	=	tricarboxylic acid cycle
T_{max}	=	maximum temperature
T_m	=	melting temperature
T_{min}	=	minimum temperature
T_{opt}	=	optimum temperature
tsp	=	thermo separation product
tRNA	=	transfer ribonucleic acid
v/v	=	volume by volume
vvm	=	volumes of air per volume of liquid per minute
w/v	=	weight by volume
YPD	=	yeast extract peptone dextrose
YM	=	yeast extract-malt extract
Y_{ps}	=	yield of product per substrate (g/g)
μ	=	specific growth rate (h^{-1})
+	=	positive
-	=	negative

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 glycolysis under anaerobic condition. The overall reaction can be summarized as follow:

$$C_6H_{12}O_6 + 2\ ADP + 2\ Pi \xrightarrow{Yeast} 2\ C_2H_5OH + 2\ CO_2 + 2\ ATP$$

Glucose Ethanol

At the beginning of the 20th century, several kinds of raw materials were exploited for ethanol production, such as molasses or agricultural product, 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 are sugar, starch and lignocellulose. The first raw material group, sugar refers to sugarcane as well as molasses and sugar-beet. The second group, starch from such crops as cassava, cereals and potatoes. The last group, lignocelluloses covered 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 optimum temperature ranges between 25 °C and 30 °C at pH values of 3.5-6.0. The efficiency of ethanol fermentation is dropping off rapidly at higher temperature. In many tropical countries, summer temperatures frequently reach ≥ 35 °C and in the typical ethanol fermentation processes carried out at ambient temperatures with no cooling system an increase of up to 11 °C can be experienced due to exothermic metabolic reactions (Burrows, 1970).

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 chance of contamination is minimized.

1.2 Research objectives

The purposes of this study are as follows:

1.2.1 to optimize conditions for growth and ethanol production of *Issatchenkia* sp. S1 in enriched medium.

1.2.2 to determine growth kinetic parameters for *Issatchenkia* sp. S1.

1.3 Research hypothesis

The thermotolerant yeast could promote high yield of ethanol at the optimum conditions. Moreover, the thermotolerant yeast has efficiency for ethanol production.

1.4 Scope and limitation of the study

The optimum conditions and kinetic parameters were determined in laboratory scale using enrich medium in batch and fed-batch culture operations.

1.5 Expected results

The thermotolerant *Issatchenkia* sp. S1 show high efficient of ethanol production in enrich medium when operate at optimum conditions in both batch and fed-batch reactors.

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).

With regard to 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.

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 used 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*
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. Microorganisms 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 psychophilic yeast, temperature limits of growth, 2 to 20 $^{\circ}$C; mesophilic yeast, temperature limits of growth, 5 to 35 $^{\circ}$C; thermophilic yeast, temperature limits of growth, 28 to 45 $^{\circ}$C; and thermotolerant yeast, temperature limits of growth, 8 to 42 $^{\circ}$C. The term is usually used to describe yeast, which grows at temperature slightly above the T_{max} of most types of yeast 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 $\geq$ 37 $^{\circ}$C. Some literatures refer to thermophilic yeasts, where the organisms can only grow up to 37 $^{\circ}$C, while

yeasts, which grow at temperatures exceeding 45 °C, are referred 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 mesophilic yeasts had a T_{min} of 5 °C to 10 °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 types of yeast, 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 (Hacking *et al*., 1984).

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 Thermotolerant *Issatchenkia* sp. S1

Thermotolerant *Issatchenkia* sp. S1 was isolated from silage samples at Suranaree University of Technology (SUT) farm by Sripiromrak (2006). This strain was identified based on biochemical characterization by using API Candida strip (bioMérieux®sa, France). The characteristic of *Candida krusei* was used as referent strain (Table 2.3). Additionally, *C. 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). The partial sequence of 18S rDNA of this strain was obtained from the sequence analysis, and compared with other 18S rDNA sequences of yeasts from GenBank database. The 18S rDNA sequence was produced closely related with *Issatchenkia* sp. (Sripiromrak, 2006) (Figure 2.1).

The cell morphology of *Issatchenkia* sp. S1 under compound microscope (Figure 2.2) and scanning electron microscope (Figure 2.3) 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. *Issatchenkia* sp. S1 is ascomycetous yeasts. They could form ascospore on YPD agar after 3 weeks at 25 $^{\circ}$C (Figure 2.4).

The optimal temperature of *Issatchenkia* sp. S1 studied by Sripiromrak (2006) was 37 $^{\circ}$C. Moreover, it could grow up to 45 $^{\circ}$C in YPD broth at pH 5.5. The optimal pH of *Issatchenkia* sp. S1 was 4.0 in M9 medium with 50 g/L glucose and 10 g/L ammonium sulfate.

Table 2.3 Biochemical characteristics of the selected thermotolerant yeast isolated form silage (Sripiromrak, 2006).

Characteristic	Reference strain (*Candida krusei*)	*Issatchenkia* sp. S1
Sugar acidification		
Glucose	+	+
Galactose	-	-
Saccharose	-	-
Trehalose	-	-
Raffinose	-	-
Enzymatic reactions		
beta-Maltosidase	-	-
alpha-Amylase	-	-
beta-Xylosidase	-	-
beta-Glucuronidase	-	-
Urease	-	-
N-Acetyl-beta-glucosaminidase	-	-
beta-Galactosidase	-	-

Symbol: +, positive; -, negative

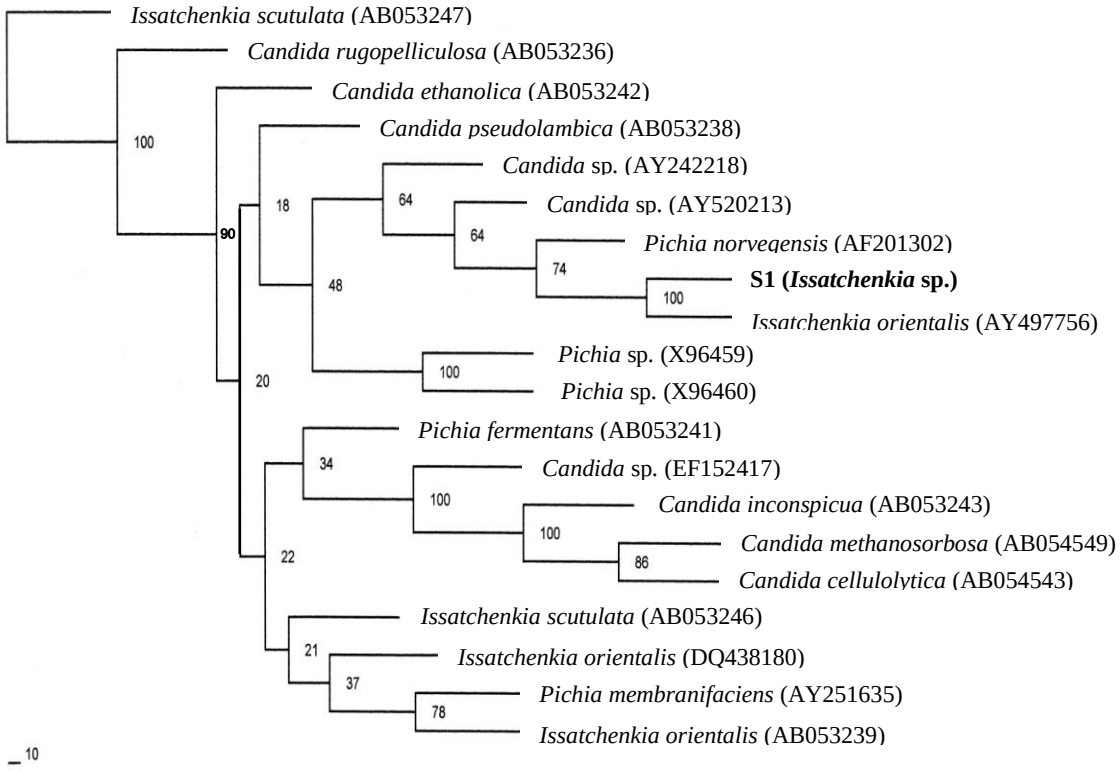
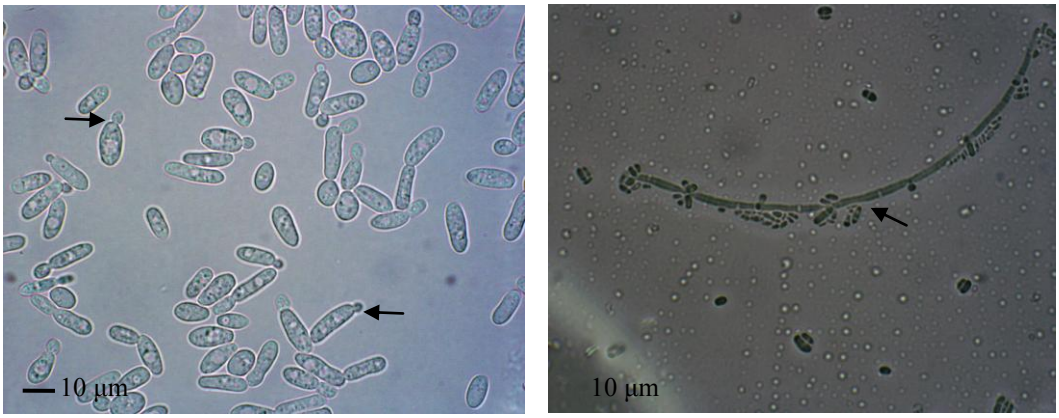
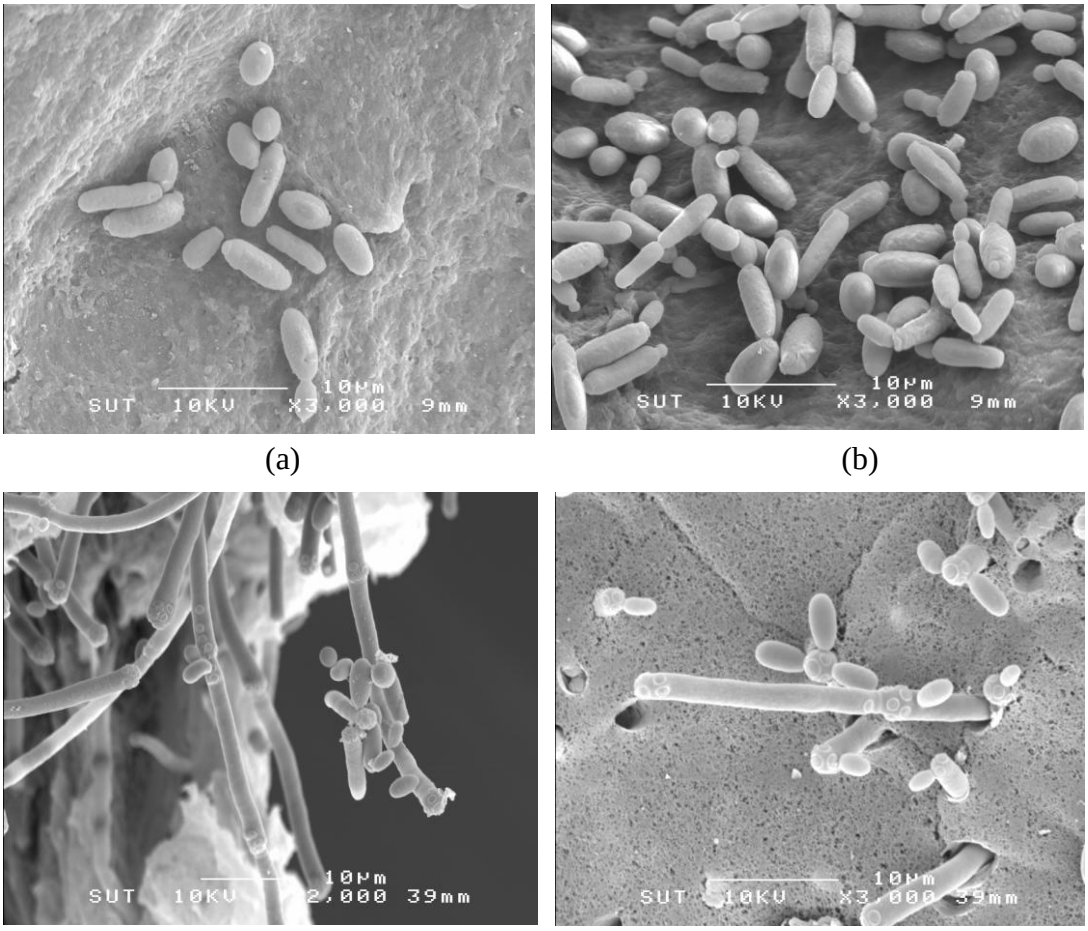


Figure 2.1 Phylogenetic relationship of *Issatchenkia* sp. strain S1. Branch lengths are proportional to the numbers of nucleotide and amino acid changes, and the numerals given on the branches are the frequencies with which a given branch appeared in 100 bootstrap replications. Reference sequences were retrieved from GenBank under the accession numbers in parentheses (Sripiromrak, 2006).



(a) (b)

Figure 2.2 Morphological characteristics of *Issatchenkia* sp. S1 under compound microscope; (a) budding cells (1000x) and (b) pseudomycelium formation (400x) as shown by arrows (Sripiromrak, 2006).



(a) (b)

(c) (d)

Figure 2.3 SEM micrographs of *Issatchenkia* sp. 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) and (d) pseudomycelium formation in YPD agar at 40 °C for 7 days at magnification for 2000 and 3000 time, respectively; Scale bars = 10 μm (Sripiromrak, 2006).

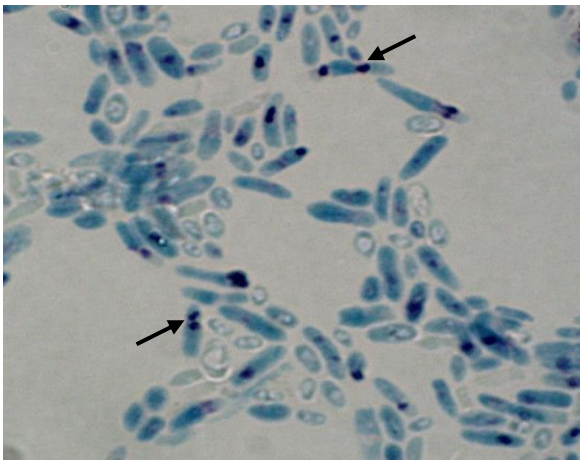


Figure 2.4 Ascospores (arrows) of *Issatchenkia* sp. S1 formed on YPD agar after 3 weeks at 25 °C (Sripiromrak, 2006).

2.2 Ethanol fermentation

In industry ethanol production, yeasts are important microorganism for fermentation of sugar to ethanol. The yeasts, which are of primary interest to industrial operations, are *S. cerevisiae*, *S. uvarum* (*carlsbergensis*), *Schizosacchromyces pombe*, and *Kluyveromyces* species.

Yeasts metabolize glucose to ethanol by the glycolysis pathway. The overall net reaction (Figure 2.5) involves the production of 2 moles each of ethanol, CO_2, and ATP per mole of glucose. Therefore, on a weight basis, each gram of glucose can theoretically give rise to 51% alcohol. The yield attained in practical fermentations does not exceed 90-95% of the theoretical value of mesophilic condition. 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 fermentation reactions could be eliminated, the ethanol production about 2.7% was obtained (Roehr, 2001).

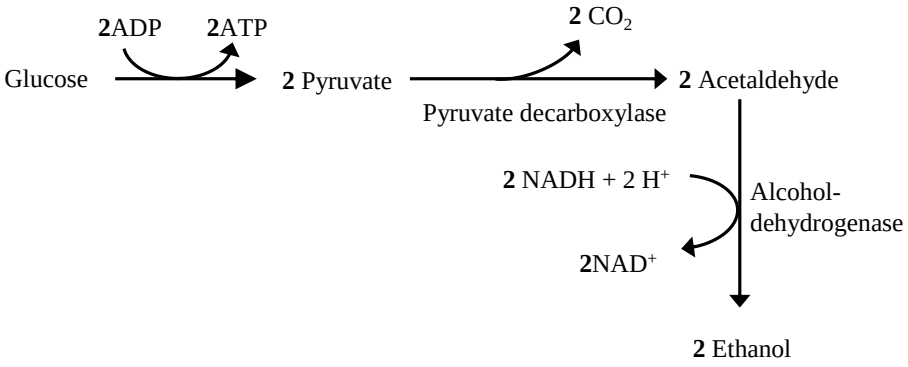


Figure 2.5 The ethanol fermentation pathway results in the formation of ethanol and carbon dioxide (Norr *et al.*, 2003).

The reducing power of NADH, produced in glycolysis pathway, 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. The ethanol fermentation contains two additional enzymatic reactions; the first of which (catalyzed by pyruvate decarboxylase), decarboxylates pyruvic acid which have thiamine pyrophosphate as cofactor; the second step reduces acetaldehyde into ethanol by NADH, this reaction is catalyzed by the alcohol dehydrogenase whose activate site contains a Zn^{2+} ion (Ribéreau-Gayon *et al*., 2000).

2.2.1 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 because of 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 $^{\circ}$C and 38 $^{\circ}$C. This is the reason why the operating temperature must be maintained between 30 and 35 $^{\circ}$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 $^{\circ}$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 $^{\circ}$C.

2.2.1.1 *Kluyveromyces* spp.

The morphology of *Kluyveromyces* spp. was described by Kurtzman and Fell (1998). The cells are ovoidal, ellipsoidal, cylindrical to elongate. Pseudomycelium may be formed and true hyphae are not produced.

As summarized 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, which 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 that *K. marxianus* IMB3 produced a maximum concentration of 8.5 g/L ethanol growing on 2% (w/v) glucose, which represented 83 % of the maximum theoretical yield.

2.2.1.2 *Saccharomyces* spp.

Cells are globose, ellipsoidal or cylindroidal (Kurtzman and Fell, 1997) shown in Figure 2.6. 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 (Table 2.4). 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 2.5, 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.

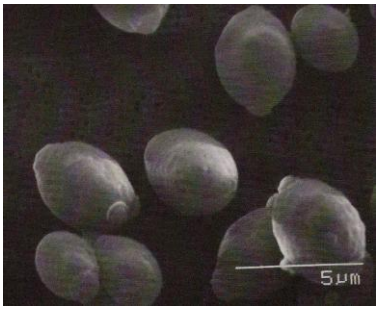


Figure 2.6 The cell morphology of *Saccharomyces* sp. (Fugeland and Edwards, 2007)**.**

Table 2.4 Effect of temperature on the kinetic parameters of *S. cerevisiae* (Torija *et al*., 2003).

Parameters	Temperature (°C)				
	15	**20**	**25**	**30**	**35**
Maximum ethanol production rate (g/L/h)	0.39	0.87	2.20	2.63	2.90
Ethanol yield (g ethanol/ g glucose)	0.475	0.472	0.457	0.456	0.404

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).

2.2.1.3 *Candida* spp.

Candida spp. cells are globose, ellipsoidal, cylindroidal, or elongate, occasionally ogival, triangular of lunate (Kurtzman and Fell, 1997) as shown in Figure 2.7. 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 2.6). Strain HT4 with a maximum temperature about 48 °C was 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.

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).

Table 2.5 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 (h^{-1})	0.19	0.23	0.26	0.23	0.21	0.16
Maximum ethanol production rate (g/L/h)	0.94	1.15	1.33	1.44	1.44	1.43
Ethanol yield (g ethanol/ g glucose)	0.377	0.377	0.377	0.377	0.377	0.377

Table 2.6 Different species of *Candida* capable of fermentation at temperature $\geq 40\ ^{\circ}C$ (McCracken and Gong, 1982).

Strain	Temperature ($^{\circ}C$)	Time (h)	Substrate	Ethanol concentration (g/L)
Candida acidothemophilum	40	14	10% pretreated rice straw	23
C. brassicae IFO 1664	40	24	cellulose	02.4
	45	48	cellulose	01.9
C. lusitaniae Y-5394	41	NA	100 g/L cellobiose	45.7
C. obtuse ATCC 24009	40	NA	140 g/L glucose	17.6
C. pseudotropicalis Yca9	40	63	142 g/L glucose	68.7
	45	NA	140 g/L glucose	39.9
C. pseudotropicalis NCYC143	40	NA	140 g/L glucose	35.7
C. tropicalis NCYC405	40	NA	140 g/L glucose	34.3
C. utilis YCa25	40	62	141 g/L glucose	34.3
Candida sp. HT4	45	48	100 g/L glucose	43.2
	50	6	45 g/L glucose	20.0
Candida sp. HT1	45	48	100 g/L glucose	40.7
Candida sp. HT2	45	48	100 g/L glucose	3.9
Candida sp. HT3	45	48	100 g/L glucose	34.2
Candida sp. HT5	45	48	100 g/L glucose	19.8
Candida sp. HT6	45	48	100 g/L glucose	36.5
Candida sp. HT7	45	48	100 g/L glucose	40.9
Candida sp. HT8	45	48	100 g/L glucose	32.0

NA= not available

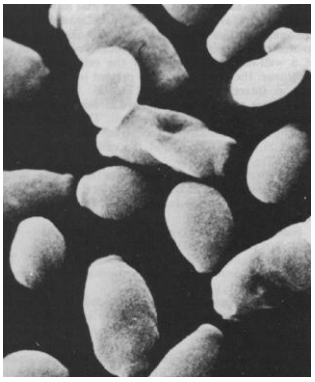


Figure 2.7 The cell morphology of *C. albicans* (De Nollin and Borgers, 1975)

2.2.1.4 *Issatchenkia* spp.

The genus *Issatchenkia* was described by Kudriavzev (1960) for yeasts, which were isolated from fruit juices and berries. Asexual reproduction is by multilateral budding on a narrow base. Cells are spheroidal, ellipsoidal or elongate, and pseudohyphae are present. Asci are unconjugated when formed from dipoid cells or may be conjugated if formed by pairing of complementary mating types. Asci are persistent and form 1-4 roughened spheroidal ascospores. Glucose is fermented. Nitrate is not assimilated (Kurtzman and Fell, 1998).

The literature of *Issatchenkia* sp. studied in ethanol production was not found. Only from Sripiromrak (2006) studied, the thermotolerant *Issatchenkia* sp. S1 was able to produced ethanol up to 74.58% of theoretical yield in M9 minimal medium with 5% (v/v) glucose and using ammonium sulfate as a nitrogen source at pH 4.0 at 40°C.

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

2.3.1 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. Amore *et al*. (2002) reported by doubling the nutrient components in the medium of *S. diastaticus*, resulting in the production of 9.1% (w/v) ethanol. Increasing the fermentation temperature from 40 to 45 $^{\circ}$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 shown 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.

2.3.2 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, and production glycerol inhibits ethanol fermentation (Wang *et al*., 2001).

2.4 By-products of ethanol fermentation

2.4.1 Glycerol

Glycerol is a sugar alcohol produced from dihydroxyacetone phosphate (Figure 2.8). The regeneration of NAD^+ in the first step of the glycerol-pyruric pathway is important during oxygen limitation, when oxidation of NADH in the mitochondrial membrane is inhibited (van Dijken and Scheffers, 1986). In addition to its importance in redox metabolism, glycerol also plays a role in the cellular protection against osmotic stress. During growth under high osmotic pressure, glycerol is produced and retained inside yeast cells where it functions as a compatible solute (Albertyn *et al*., 1994; Nevoigt and Stahl, 1997). Glycerol accumulates during growth in both high sugar and salt environments (Blomberg and Adler, 1992).

Ciani and Ferraro (1996) report glycerol production by *C. stellata* practical application in wine making was 15.10 g/L. And *S. cerevisiae* were found to form amounts of glycerol varying between 4.2 to 10.4 g/L (Radler and Schütz, 1982).

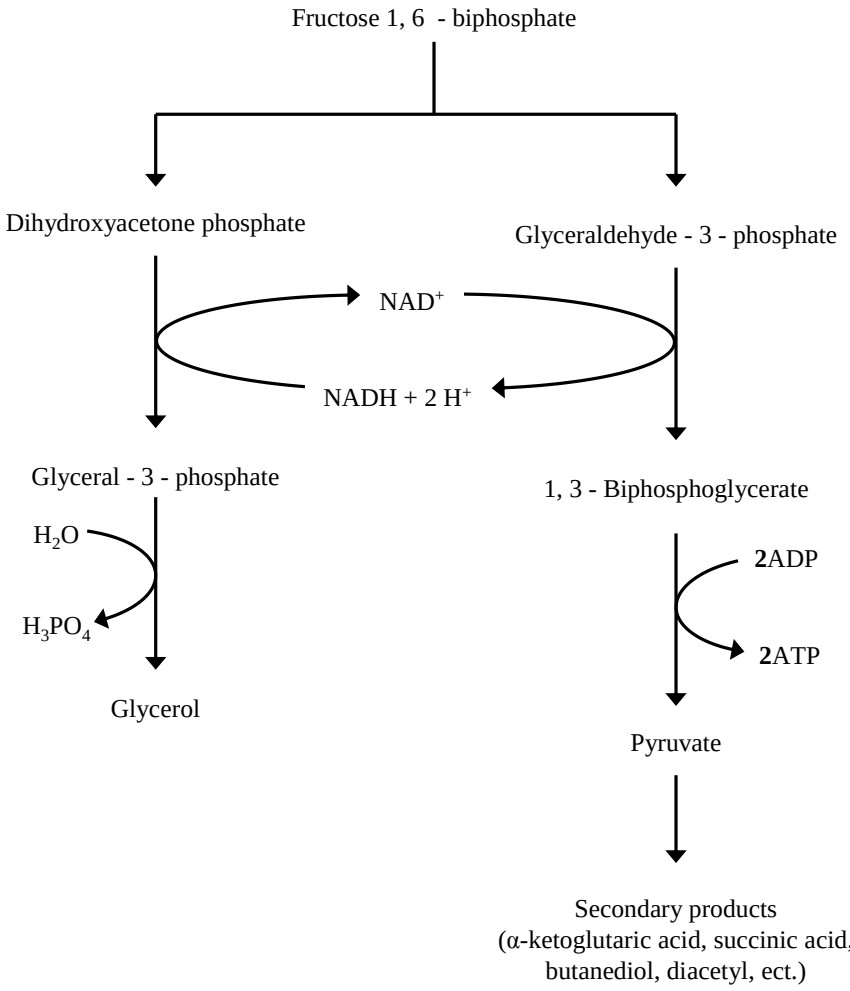


Figure 2.8 Glycero-pyruvic fermentation pathway (Ribéreau-Gayon *et al.*, 2000).

2.4.2 Organic acids

2.4.2.1 Acetic acid

Acetate are produced from the metabolic intermediate acetaldehyde by oxidation of acetaldehyde by the enzyme aldehyde dehydrogenase (Figure 2.9), reduced one molecule of NAD^+ or $NADP^+$ (Navarro-Aviño *et al.*, 1999). The

enzymatic reaction from acetaldehyde to ethanol is catalyzed by alcohol dehydrogenase, regenerating one molecule of NAD^+.

Gerós *et al*. (2000) reported that acetic acid produced by *Dekkera anomala* IGC 5153 grown in mineral medium with 120 g/L glucose was 7 g/L.

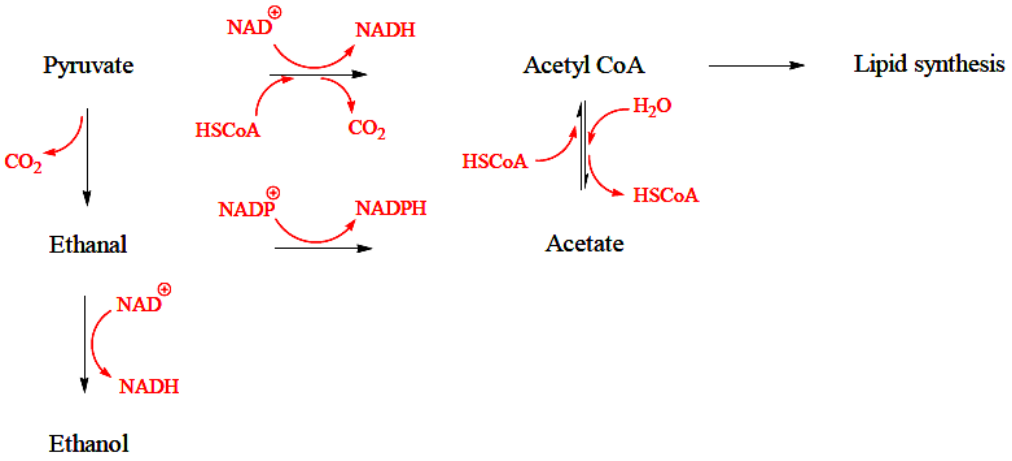


Figure 2.9 Acetic acid formation pathways in yeast (Ribéreau-Gayon *et al.*, 2000).

2.4.2.2 Lactic acid

Lactic acid is another secondary product of fermentation. It is derived from pyruvic acid, directly reduced by the enzyme lactate dehydrogenase (Figure 2.10). Lactic acid production by *Saccharomyces cerevisiae* expressing a *Rhizopus oryzae* lactate dehydrogenase gene was about 38 g/L (Skory, 2003). Lactic acid-alcoholic fermentation resulted in wine containing 5 to 8 g/L of lactic acid (Dequin *et al*., 1999).

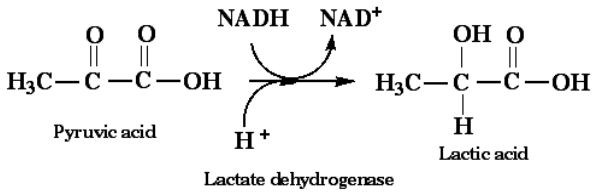


Figure 2.10 Lactate formation (Devlin, 2006).

2.4.2.3 Malic acid

Fermentative production of malic acid has been most successfully demonstrated with *Aspergillus flavus*, achieving 63% of the maximum theoretical yield of malic acid on glucose at high production rates and titers (Battat *et al*., 1991). Malic acid concentration obtained with *S. cerevisiae* is 12 g/L, which was achieved by overexpression of the cytosolic isoenzyme of malate dehydrogenase (Mdh2p gene) (Pines *et al*., 1997). The highest reported malic acid concentration obtained with *S. cerevisiae* thus far is 59 g/L which was three genetic modifications: (i) overexpression of the native pyruvate carboxylase encoded by PYC2 gene, (ii) high-level expression of an allele of the MDH3 gene, of which the encoded malate dehydrogenase was retargeted to the cytosol by deletion of the C-terminal peroxisomal targeting sequence, and (iii) functional expression of the *Schizosaccharomyces pombe* malate transporter gene SpMAE1 (Zelle *et al*., 2008). Another yeast, a natural isolate of *Zygosaccharomyces rouxii*, was shown to produce up to 75 g/L of malic acid in a complex medium containing 300 g/L glucose (Taing and Taing, 2007).

2.4.2.4 Citric acid

Citric acid is an intermediate of tricarboxylic acid cycle (TCA) and holds a key position in the metabolism of each microbial cell. However, under certain conditions of fermentation, fungi, bacteria, and yeasts produce citric acid in the excessive amounts. Production of citric acid by yeasts such as *Yarrowia lipolytica* and other *Candida* species are capable using various carbon sources and substrate (Tisnadjaja *et al*., 1996; Anastassiadis *et al*., 2001; Kamzolova et al., 2003; Anastassiadis and Rehm, 2005). Citric acid production by rapeseed-oil-grown yeast *Y. lipolytica* 187/1 amounted to 135 g/L (Kamzolova *et al*., 2005). And 167 g/L citric acid were produced continuously with the fill and drain technique at 4.85 days, at 80% air saturation using *C. oleophila* ATCC 20177 growth in basal medium containing 250 g/L glucose (Anastassiadis, 2006).

2.4.2.5. Succinic acid

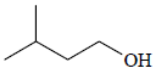
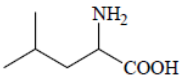
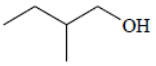
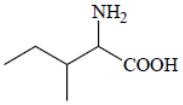
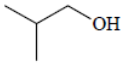
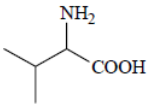
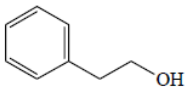
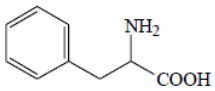
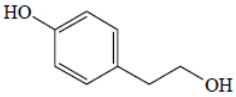
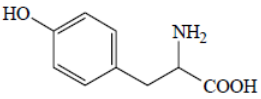
Taing and Taing (2007) reported succinic acid produced by *Zygosaccharomyces rouxii* V19 grown in YPG medium (yeast extract, 0.5%, peptone, 1.0%, glucose, 10%) was 7.7 g/L. Moreover, succinic acid was produced as secondary product in alcoholic fermentation led by *S. cerevisiae* about 0.70 – 0.89 g/L (Torija *et al*., 2003).

2.4.3 Higher alcohols and esters

The higher alcohols in a fermented beverage are the small aliphatic alcohols other than ethanol. They are formed by decarboxylation and reduction of the α-keto acid arising from deamination of the amino acid. After deamination of the amino acid

to obtain ammonium the yeast needs to deal with the α-keto acids and it can only excrete them after decarboxylation and reduction (Figure 2.11).

Table 2.7 The principle alcohol found in wine and their amino acid precursors (Ribéreau-Gayon *et al*., 2000)

Higher alcohol	Concentration in wine(mg/L)	Amino acid precursor
 3-methylbutan-1-ol or isoamyl alcohol	80-300	 leucine
 2-methylbutan-2-ol or active amyl alcohol	30-100	 isoleucine
 2-methylpropan-1-ol or isobutyl alcohol	50-150	 valine
 phenylethanol	10-100	 phenylalanine
 tyrosol	20-50	 tyrosine

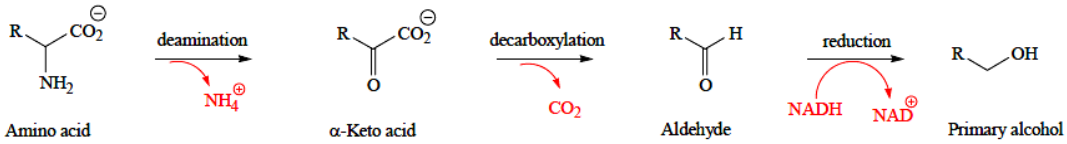


Figure 2.11 Formation of higher alcohol from amino acid (Ehrlich reaction) (Ribéreau-Gayon *et al*., 2000).

CHAPTER III

MATERIALS AND METHODS

3.1 Microorganism

Thermotolerant *Issatchenkia* sp. S1 isolated by Sripiromrak (2006) was used throughout this study. It was stored as frozen working stocks at -20 °C. The frozen working yeast was regenerated in 15 mL tube contained 10 mL YM broth (contained 10 g/L glucose, 5 g/L peptone, 3 g/L yeast extract and 3 g/L malt extract) for activating cell activity. And then tube was incubated at 40 °C for a day. A loop of thermotolerant yeast was cultured on YM agar plates and incubated at 40 °C for 2 days.

3.2 Inoculum preparation

Inoculum of *Issatchenkia* sp. S1 was prepared by transferring a loopfull of cells from the YM agar plate to 150 mL of inoculum YM broth. The medium was prepared in 250 mL Erlenmeyer flasks, at 40 °C and 200 rpm. The growth of inoculum was observed by a spectrophotometer (Ultraspec 2000 UV/Visible Spectrophotometer, Pharmacia Biotech, England) at 660 nm (section 3.3.1).

3.3 Analytical methods

3.3.1 Cell mass analysis

Issatchenkia sp. S1 cell growth and cell mass analysis were measured with a spectrophotometer at 660 nm against the medium as blank and gravimetrically, respectively. The gravimetric cell mass concentration measurement began by using a manifold filtration unit. Ten milliliters of *Issatchenkia* sp. S1 suspensions were filtered on the preweighed cellulose acetate membrane filters (13 mm, 0.45 μm, Whatman, England) placed under vacuum and washed once with deionized water, and then dried at 80 °C until constant weight was achieved (at least 24 h). Cell mass concentrations were calculated as dry cell mass (DCM, g/L).

3.3.2 Glucose and fructose analysis

Determination of glucose and fructose were analyzed by high performance liquid chromatographic (HPLC). The Thermo Separation Product (tsp) HPLC system composed of autosampler AS 3000 and RI-1530 detector (Jasco, Japan) was used. The Phenomenex® Rezex RPM-Monosaccharide column (300 x 7.8 nm) was operated at 75 °C. The deionized water was used as mobile phase at the flow rate of 0.6 mL/min.

3.3.3 Organic acid analysis

Determination of some organic acids was analyzed by high performance liquid chromatographic (HPLC). The 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 0.005 N H_2SO_4 was used as mobile phase at the flow rate of 0.5 mL/min.

3.3.4 Ethanol analysis

The ethanol production was determined using a gas chromatograph (GC) 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 increased 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.

3.4 Carbon sources utilization

To determine utilization of carbon sources; glucose, fructose, sucrose, lactose, glycerol, manitol, maltose, cassava starch and potato starch were selected. Each carbon source was replaced as glucose at 10 g/L in YM medium (pH 6.0) contained 3.0 g/L yeast extract, 3.0 g/L malt extract, 5.0 g/L peptone. Five hundred μL of *Issatchenkia* sp. S1 cell suspensions from actively growing culture after 8 h of cultivation time were inoculated into 15 mL test tube containing Durham tube and 9.5 mL of YM broth. Fermentation was recognized by the accumulation of CO_2 gas trapped in the inner Durham tube (Ueno *et al*., 2001). All cultures were incubated at 40 °C for 2 days. The turbidity and ferment gas were observed.

3.5 Optimization of glucose and fructose concentrations for ethanol production in static condition

For optimized concentration of glucose and fructose for ethanol production, glucose or fructose at 0, 50, 100, 150, 200 and 250 g/L were added as carbon source in YM medium. *Issatchenkia* sp. S1 cell suspensions from actively growing culture at 8 h after cultivation (7.5 mL) were inoculated into 142.5 mL of YM broth in 250 mL Erlenmeyer flask. The initial optical density of each flask was measured by spectrophotometer at 660 nm against the medium as blank. The increasing of optical density in a flask was recorded as evidence of growth. During fermentation, samples were periodically withdrawn for analysis of growth, ethanol production and glucose concentration by GC and HPLC, respectively.

3.6 Effect of shaking on ethanol production

From the previously experiment, glucose at 50 g/L and fructose at 100 g/L in YM broth were used in this experiment. *Issatchenkia* sp. S1 cell suspension from actively growing culture after 8 h of cultivation time (7.5 mL) was inoculated into 142.5 mL of glucose or fructose YM broth in 250 mL Erlenmeyer flask. In non static condition, *Issatchenkia* sp. S1 was studied with shaking at 200 rpm by refrigerated incubator shaker (innova^{TM} 4330, New Brunswick Scientific, N.J., USA) compared with static condition at the same temperature of 40 $^{\circ}$C.

3.7 Optimization of glucose and fructose concentrations for ethanol production in shaking condition

For optimized concentration of glucose and fructose for ethanol production, glucose or fructose were used as carbon source in YM medium at 0, 50, 100, and 150 g/L. *Issatchenkia* sp. S1 cell suspensions from actively growing culture (7.5 mL) were inoculated into 142.5 mL of YM broth in 250 mL Erlenmeyer flask with shaking at 200 rpm. The initial optical density of each flask was observed on spectrophotometer at 660 nm against the medium as blank. The increasing of optical density in a flask was recorded as evidence of growth. During fermentation, samples were periodically withdrawn for analysis of growth, ethanol production and glucose concentration.

3.8 Order of glucose and fructose consumption

For determine order of glucose and fructose consumption, YM medium with 25 g/L glucose and 25 g/L fructose were used as carbon sources in 250 mL Erlenmeyer flask with shaking at 200 rpm and incubated at 40 $^{\circ}$C. During fermentation, samples were periodically withdrawn for analysis of growth, ethanol production, and glucose and fructose concentration.

3.9 Effect of nitrogen source on ethanol production

This experiment was designed as in Table 3.1 for studied the effect of peptone, yeast extract, malt extract and ammonium sulfate ($(NH_4)_2SO_4$) on growth and ethanol

production. Each trial was added 100 g/L of glucose as carbon source. All flasks were incubated at 40 °C for 3 days with shaking speed at 200 rpm.

3.10 Batch Culture in 2 L fermenter for ethanol production

For the preparation of inoculum, *Issatchenkia* sp. S1 cells from a freshly prepared slant were transferred aseptically into a 250 mL Erlenmeyer flask containing 150 mL of YM medium. The inoculum of *Issatchenkia* sp. S1 was incubated at 40 °C for 8 h with non static condition (shaking speed at 200 rpm). The suitable glucose concentration, nitrogen source (section 3.8) were used for ethanol production in fermenter, the pH was controlled at 4.0 (± 0.05) maintained by adding of 1 N HCl or 1 N NaOH. Batch experiments were performed in a 2 L fermenter (micro DCU-300 and Biostat® B, B. Braun Biotech international, Germany) with a working volume of 1.0 L. Inoculum size was used at 5% v/v and operated at 40 °C for 48 h.

3.10.1 Effect of aeration

Six batch experiments were operated for determining the effect of aeration rate on ethanol production by continuously flushed with aeration rate at 0, 0.2 and 1.0 vvm, and operated at 100 rpm of agitation speed. During the whole fermentation process, samples were periodically withdrawn for analysis of growth, ethanol production and glucose concentration.

Table 3.1 Various concentrations of nitrogen sources for determination of effect on growth and ethanol production of *Issatchenkia* sp. S1.

Trial	Peptone (g/L)	Yeast extract (g/L)	Malt extract (g/L)	$(NH_4)_2SO_4$ (g/L)
1	0	0	0	0
2	0	0	0	2.0
3	0	0	1.5	2.0
4	0	0	3.0	2.0
5	0	1.5	0	2.0
6	0	1.5	1.5	2.0
7	0	3.0	0	2.0
8	0	3.0	3.0	2.0
9	2.5	0	0	2.0
10	2.5	0	1.5	2.0
11	2.5	1.5	0	2.0
12	2.5	1.5	1.5	0
13	2.5	1.5	1.5	2.0
14	5.0	0	0	2.0
15	5.0	0	3.0	2.0
16	5.0	3.0	0	2.0
17	5.0	3.0	3.0	0
18	5.0	3.0	3.0	2.0

3.10.2 Effect of agitation speed

Eight batch experiments were operated for determining the effect agitation rate ethanol production by operated agitation speed at 100, 200, 300, 400 and 500 rpm with no aeration. During the whole fermentation process, samples were periodically withdrawn for analysis of growth, ethanol production and glucose concentration.

3.11 Batch culture in 10 L fermenter for ethanol production

The suitable glucose concentration, nitrogen source (section 3.8), aeration rate and agitation speed (section3.9) were used for study the ethanol production in fermenter, the pH was controlled at 4.0 (± 0.05) maintained by addition of 1 N HCl or 1 N NaOH. Two batch experiments were performed in a 10 L fermenter (micro DCU-300, B. Braun Biotech international, Germany) with a working volume of 6.0 L at 40 °C for 60 h. During fermentation, samples were periodically withdrawn for analysis of ethanol production, growth and glucose concentration.

3.12 Fed-batch culture in 10 L fermenter for ethanol production

The suitable glucose concentration, nitrogen source (section 3.8), aeration rate and agitation rate (section3.9) were used for ethanol production in fermenter, the pH was controlled at 4.0 (± 0.05) maintained by addition of 1 N HCl or 1 N NaOH. Two batch experiments were performed in a 10 L fermenter with a working volume start at 6.0 L at 40 °C. Glucose syrup was added at 400 g (1 L) or 900 g (1 L of 400 g and 500 g/L) during fermentation process at 200 mL/min of addition rate for increasing the glucose concentration in fermenter. During fermentation, samples were periodically withdrawn for analysis of growth, ethanol production and glucose concentration.

3.13 Statistical analysis

The different of kinetic parameters of *Issatchenkia* sp. S1 were accessed by one-way ANOVA (SPSS® software for WINDOW™, Version 15.0; SPSS, Chicago.IL). Significance of differences was established at $P \leq 0.05$.

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Growth determination of *Issatchenkia* sp. S1 for inoculum preparation

The cultivation time of inoculum preparation was determined. Cell growth and glucose utilization are shown in Figure 4.1. Since, the inoculum had the highest activity at 8 to 9 hours of cultivation time. Therefore, 8 hours after incubation, *Issatchenkia* sp. S1 in YM broth was used as inoculum in all experiments.

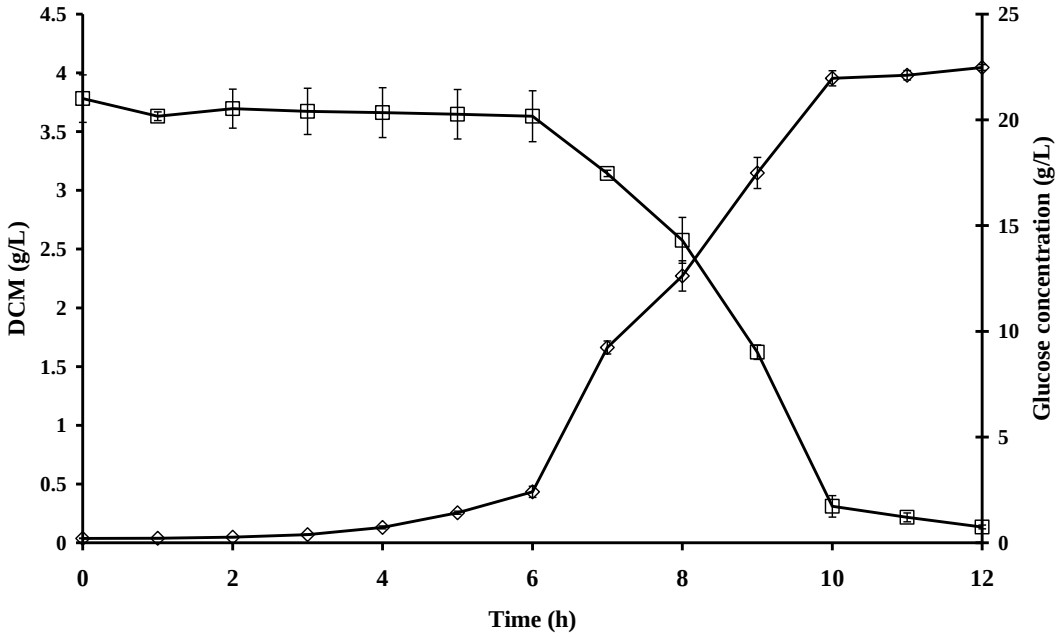


Figure 4.1 Cell growth and glucose utilization of *Issatchenkia* sp. S1 in 20 g/L of glucose in YM medium used as actively S1 yeast inoculum, incubation temperature at 40 °C with shaking speed at 200 rpm; ⊟ glucose concentration (g/L) and ◇ DMC (g/L).

4.2 Determination of carbon sources utilization

Issatchenkia sp. S1 was cultured in YM medium supplemented 9 carbon sources at 40 °C for 2 days. The results are shown in Table 4.1. *Issatchenkia* sp. S1 cultured in YM medium with glucose or fructose as carbon source gave strong utilization of glucose and fructose and produced large amount of fermented gas trapped with Durham tube. It also produced some organic acids because of decreasing of pH from 6.0 at initial cultivation to 4.5 at after 2 day. However, the YM medium supplemented with sucrose, lactose, glycerol, manitol, maltose, cassava starch and potato starch were shown very weak utilization. The pH was not changed

and no fermented gas in Durham tube. However, *Issatchenkia* sp. S1 had some growth although it could not used carbon sources, because it used other component from yeast extract, malt extract or peptone as carbon source.

Table 4.1 *Issatchenkia* sp. S1 cultured in YM medium supplemented with various carbon sources in tube with Durham tube at 40 °C for 2 days.

C sources	Turbidity	Fermented gas	Final pH
Glycerol	+	-	6.0
Glucose	+++	+	4.5
Fructose	+++	+	4.5
Mannitol	+	-	6.0
Maltose	+	-	6.0
Lactose	+	-	6.0
Sucrose	+	-	6.0
Potato starch	+	-	6.0
Cassava starch	+	-	6.0

+, positive; - , negative

4.3 Effect of glucose and fructose concentrations on ethanol production in static condition

Since the previous experiment shown that glucose and fructose are appropriate carbon sources in terms of growth and fermentation. This experiment aimed at study the effect of both carbon sources on ethanol production. The glucose and fructose concentrations were observed by various the concentrations of glucose and from 0 to 250 g/L in YM medium, initial pH 6.0 at 40 °C.

The effects of glucose concentration on growth, sugar utilization and ethanol production were determined under static conditions. The growth of *Issatchenkia* sp. S1 was shown in Figure 4.2a. The specific growth rate of *Issatchenkia* sp. S1 in YM

broth with glucose showed the highest when cultured with 50 and 100 g/L of glucose and decreased with higher glucose concentration (Table 4.2). The utilization of glucose cultured in YM medium with 50 g/L was completely utilized after 48 h of cultivation time. However, the consumption of glucose higher than 50 g/L (100–250 g/L) shown lower consumption rate (Figure 4.2b). Moreover, the ethanol production was slowly increased with the period of incubation. The ethanol production in the fermentation broth gave the highest concentration when fermented in the 50 g/L glucose and decreased when increased glucose concentration (Figure 4.2c). The maximum ethanol concentration was increased with an increasing of glucose concentration at 50 g/L. A reduction of ethanol production and growth of yeast were decreased when glucose concentration was greater than 50 g/L.

Results of *Issatchenkia* sp. S1 in YM medium with fructose were shown in Figure 4.3. The specific growth rate of *Issatchenkia* sp. S1 in YM medium with fructose was highest when cultured in 50 and 100 g/L of fructose and decreased with higher fructose concentration (Table 4.2). However, fructose was not completely utilized by *Issatchenkia* sp. S1. After the fermentation stopped, the residual fructose in the fermentation broth was detected about 8 g/L and 18 g/L in 50 g/L and 100 g/L fructose fermentation broth, respectively (Figure 4.3b). The consumption was decreased when fructose concentration was higher than 100 g/L. The ethanol production in the fermentation broth was increased with the 100 g/L glucose but decreased when increased concentration of fructose also. Ethanol production and growth of yeast were decreased when fructose concentration was greater than 100 g/L.

It is apparent that high glucose or fructose concentration can be converted into ethanol by *Issatchenkia* sp. S1. The results also indicated that the inhibitory effects of high glucose or fructose concentration have effect on the ethanol production. Glucose or fructose 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.

The reasons for the difference between the glucose fermentation rate and the fructose fermentation rate are unclear, but one of the first steps in hexose metabolism is generally thought to be involved. Sugar transport across the plasma membrane is the primary step in hexose metabolism. Another potential source of the difference is hexose phosphorylation, as glucose and fructose are both phosphorylated by the hexokinases Hxk1 and Hxk2 but with different efficiencies and the glucokinase Glk1 phosphorylates glucose but not fructose (Entian, 1997). The potential contributions of hexose transport and phosphorylation to the rates of glucose and fructose utilization are not known. Furthermore, other mechanisms may also be involved in limiting fructose utilization (Guillaume *et al*., 2007).

(a)

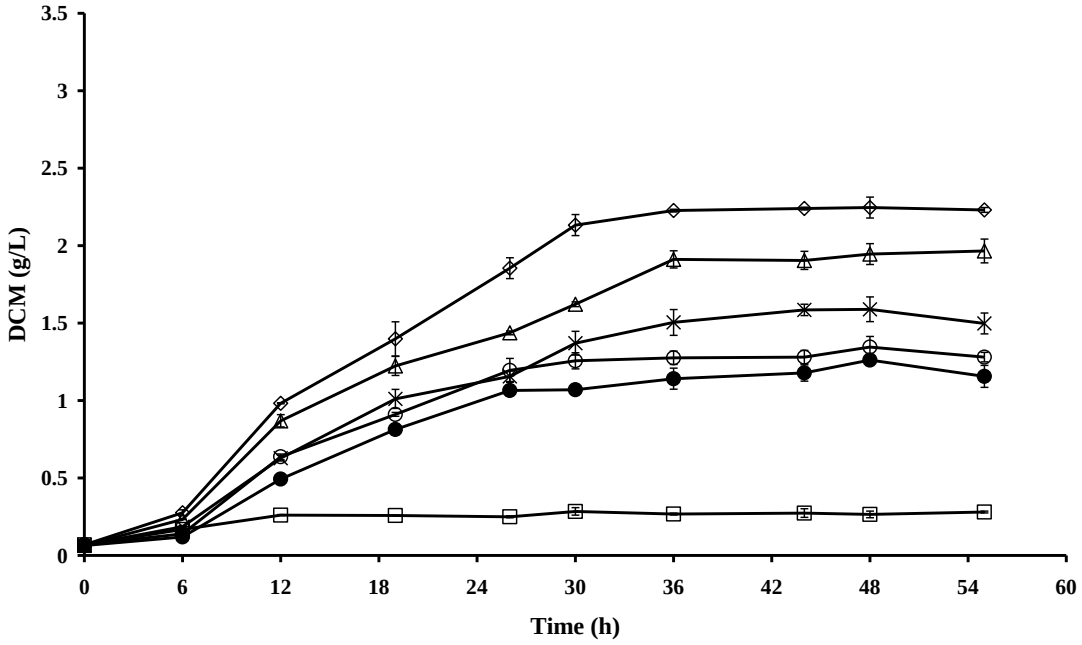
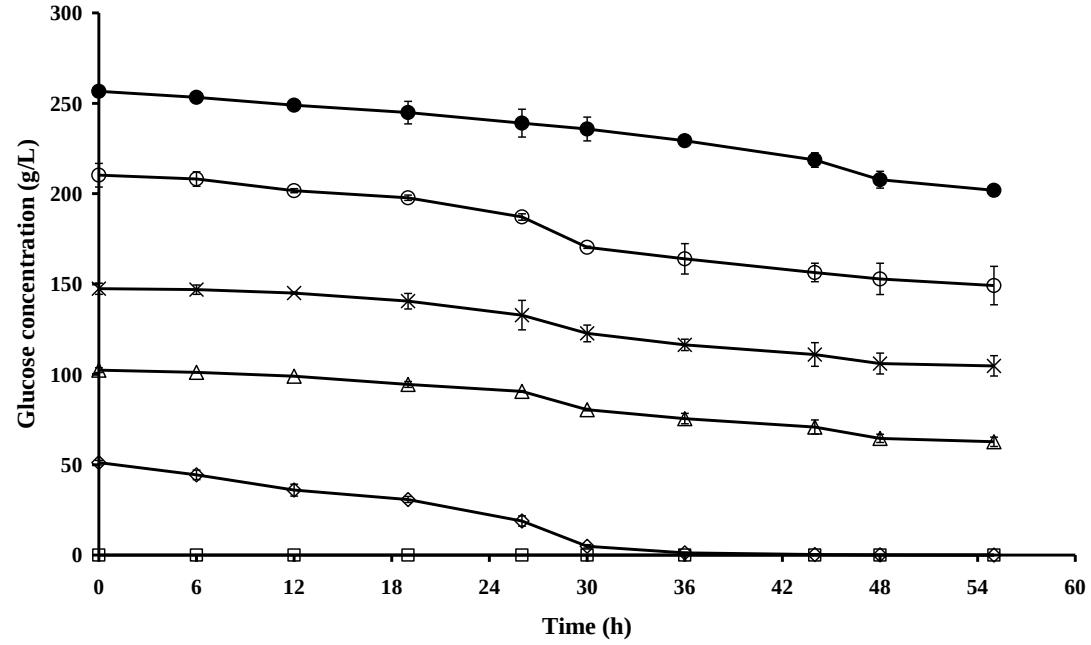


Figure 4.2 Cell growth, glucose utilization and ethanol production by *Issatchenkia* sp. S1 in 150 mL of YM medium with 0, 50, 100, 150, 200 and 250 g/L of glucose in 250 mL Erlenmeyer flask, 5% inoculum size, initial pH 6.0 and incubation temperature at 40 °C; (a) growth of the *Issatchenkia* sp. S1, (b) glucose utilization and (c) ethanol production; 0 g/L, 50 g/L, 100 g/L, 150 g/L, 200 g/L and 250 g/L of glucose.

(b)



(c)

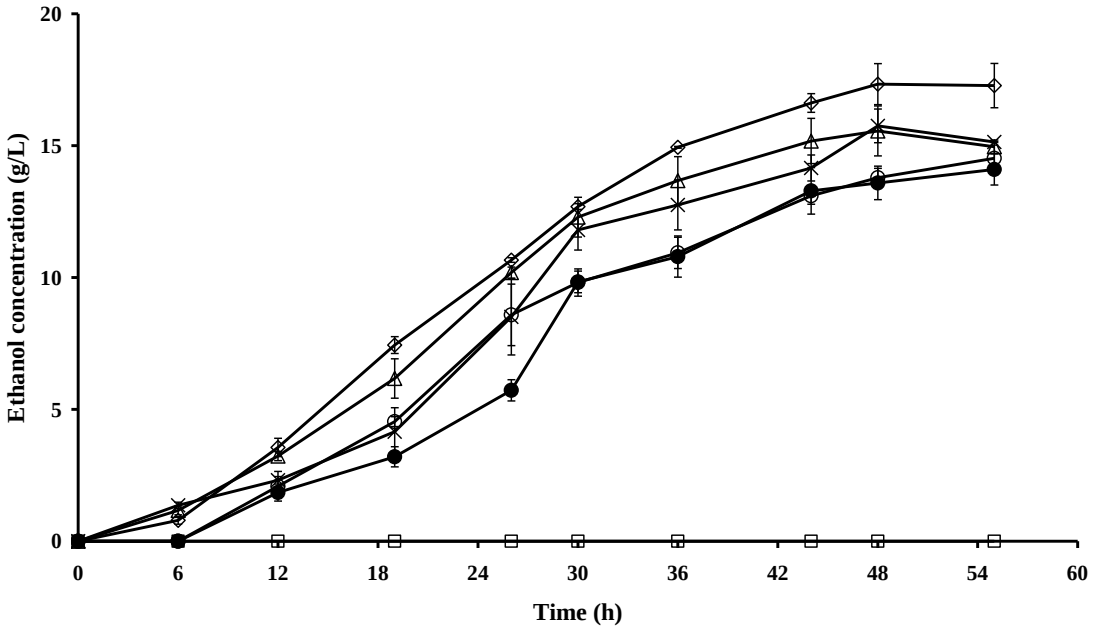


Figure 4.2 Continued.

(a)

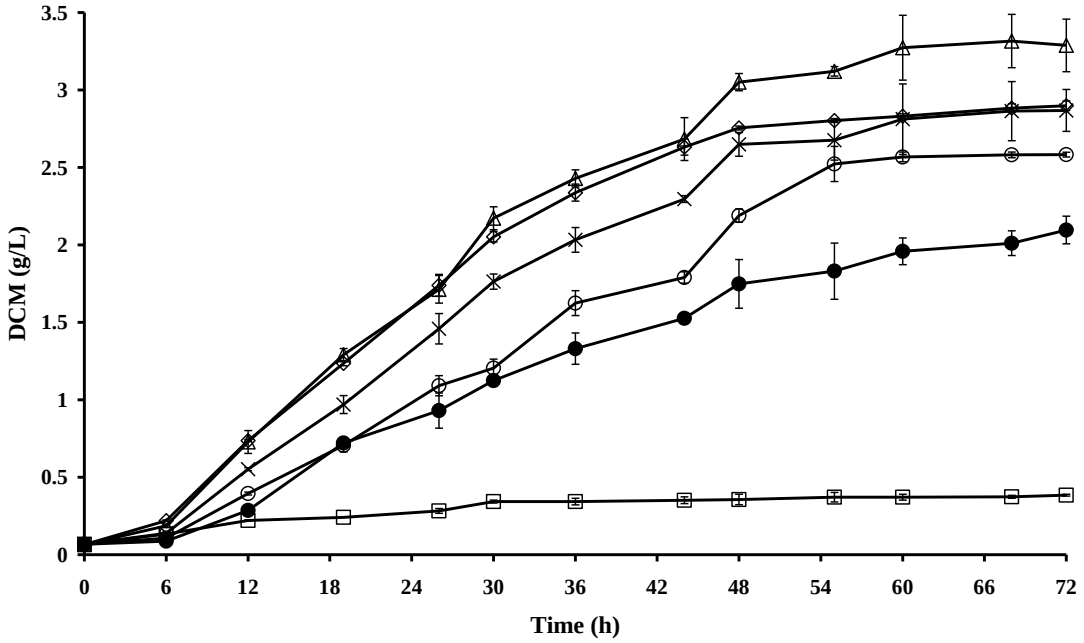
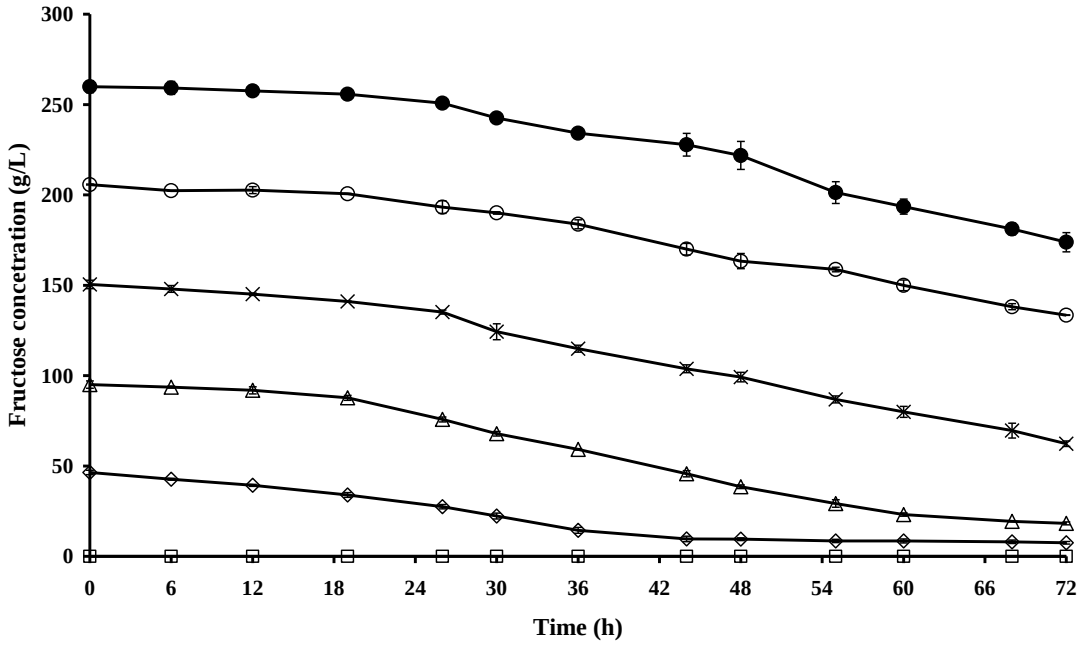


Figure 4.3 Cell growth, fructose utilization and ethanol production by *Issatchenkia* sp. S1 cultured in 150 mL of YM medium with 0, 50, 100, 150, 200 and 250 g/L of fructose in 250 mL Erlenmeyer flask, 5% inoculum size, initial pH 6.0 and incubation temperature at 40 °C; (a) growth of *Issatchenkia* sp. S1, (b) fructose utilization and (c) ethanol production; ⊟ 0 g/L, ◇ 50 g/L, △ 100 g/L, ✳ 150 g/L, ⊖ 200 g/L and ● 250 g/L of fructose.

(b)


Fructose concetration (g/L)
300
250
200
150
100
50
0
0
6
12
18
24
30
36
42
48
54
60
66
72
Time (h)

(c)

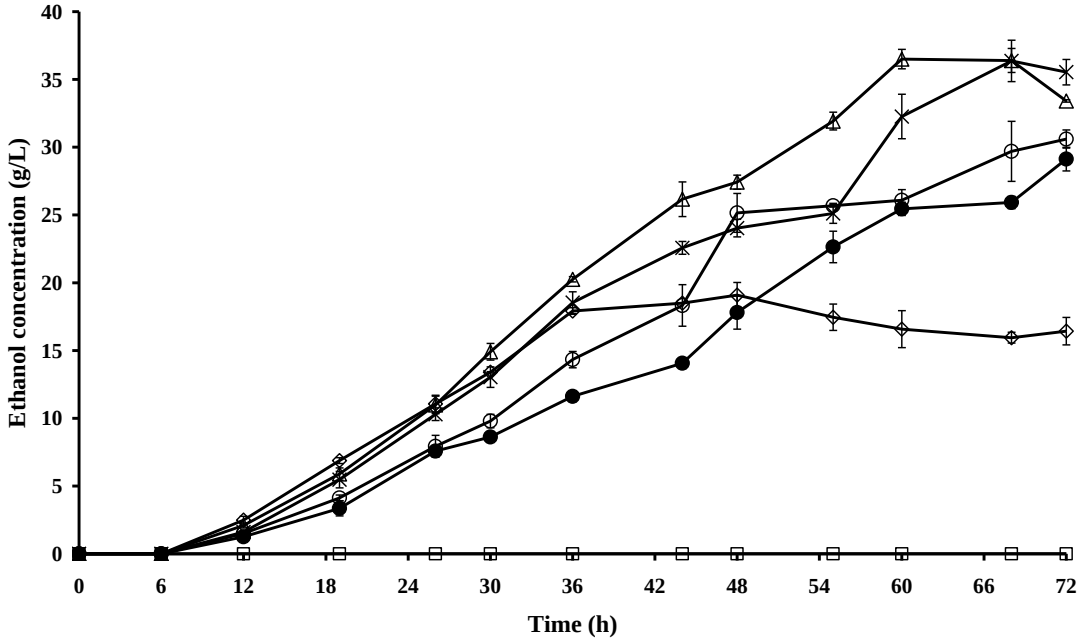


Figure 4.3 Continued.

Table 4.2 Ethanol yield (g/g), productivity (g/L/h) and specific growth rate of *Issatchenkia* sp. S1 cultured in 150 mL of YM medium with 0, 50, 100, 150, 200 and 250 g/L of glucose or fructose in 250 mL Erlenmeyer flask at 40 °C with static condition (means of two replicates, ± standard error).

Sugar concentration (g/L)	Specific growth rate (h^{-1})	Volumetric productivity (g/L/h)	Ethanol yield (g/g)
Glucose			
0	0.116 ± 0.001^{b}	0^{a}	0^{a}
50	0.221 ± 0.004^{g}	0.410 ± 0.003^{d}	0.339 ± 0.023^{cd}
100	0.216 ± 0.001^{g}	0.392 ± 0.008^{d}	0.397 ± 0.020^{ef}
150	0.186 ± 0.003^{e}	0.327 ± 0.056^{c}	0.370 ± 0.037^{de}
200	0.185 ± 0.006^{e}	0.330 ± 0.045^{c}	0.281 ± 0.017^{b}
250	0.143 ± 0.004^{d}	0.220 ± 0.015^{b}	0.304 ± 0.039^{bc}
Fructose			
0	0.103 ± 0.007^{a}	0^{a}	0^{a}
50	0.202 ± 0.005^{f}	0.497 ± 0.007^{e}	0.492 ± 0.016^{f}
100	0.202 ± 0.016^{f}	0.563 ± 0.005^{f}	0.477 ± 0.001^{f}

150	0.174 ± 0.005^{e}	0.515 ± 0.005^{ef}	0.412 ± 0.001^{ef}
200	0.133 ± 0.002^{cd}	0.398 ± 0.018^{d}	0.425 ± 0.010^{e}
250	0.122 ± 0.006^{bc}	0.323 ± 0.011^{c}	0.339 ± 0.018^{cd}

Values followed by the same letter are not significantly different at $P \leq 0.05$.

4.4 Effect of shaking on ethanol production

As results from the previously experiment, the ethanol production was examined in Erlenmeyer flask with glucose or fructose in YM medium. *Issatchenkia* sp. S1 was cultured in YM medium with glucose performed the highest ethanol production at 50 g/L of glucose concentration, and YM medium with fructose was shown the highest ethanol production at 100 g/L of fructose concentration. For studying the effect of shaking for cultivation, *Issatchenkia* sp. S1 cultured in YM medium with 50 g/L glucose and YM medium with 100 g/L fructose were used. Under shaking condition, it had a clear influence on the growth, sugar consumption and ethanol production by *Issatchenkia* sp. S1. The results were presented in Figure 4.4. Glucose was utilized rapidly and exhausted after 12 h of fermentation and ethanol production rate increased 3.06 times compared with static condition (Figure 4.4c). In this condition, *Issatchenkia* sp. S1 showed high ability of glucose utilization and ethanol production. If had more glucose in fermentation broth, the ethanol production might be increased. Additionally, the YM medium with fructose showed the same results of glucose. The consumption of fructose was decreased until after 24 h of operation, the consumption was very slowly decreased although fructose remained in the medium. However, the utilization of fructose was not completely as glucose. After fermentation stopped, especially in fructose, ethanol concentration was decreased (Figure 4.4c). At 60 h after operation, ethanol loss was about 30.7%

compared at 30 h after operation. The degradation of ethanol by evaporation in the water at 40 $^{\circ}$C with shaking was very low (2.63-4.07% loss). Indeed, after depletion of glucose (30 h after operation) 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).

Shaking is important for uniform mixing of the medium components and maintained yeast cells as homogeneous condition (Doran, 1997; Walker, 1998). Therefore, growth rate, consumption rate, and ethanol production rate of *Issatchenkia* sp. S1 in shaking condition were very higher than static condition (Figure 4.4).

Moreover, cell morphology of *Issatchenkia* sp. S1 was differenced in static and shaking condition. *Issatchenkia* sp. S1 cell cultured in static condition was formed psudomycelium and cell size was bigger than in shaking condition. In shaking condition, yeast cell was not found psudomycelium and cell was higher density than static condition. The morphological change of *Issatchenkia* sp. S1 due to shaking may be effected on cell activity and ethanol production as well.

(a)

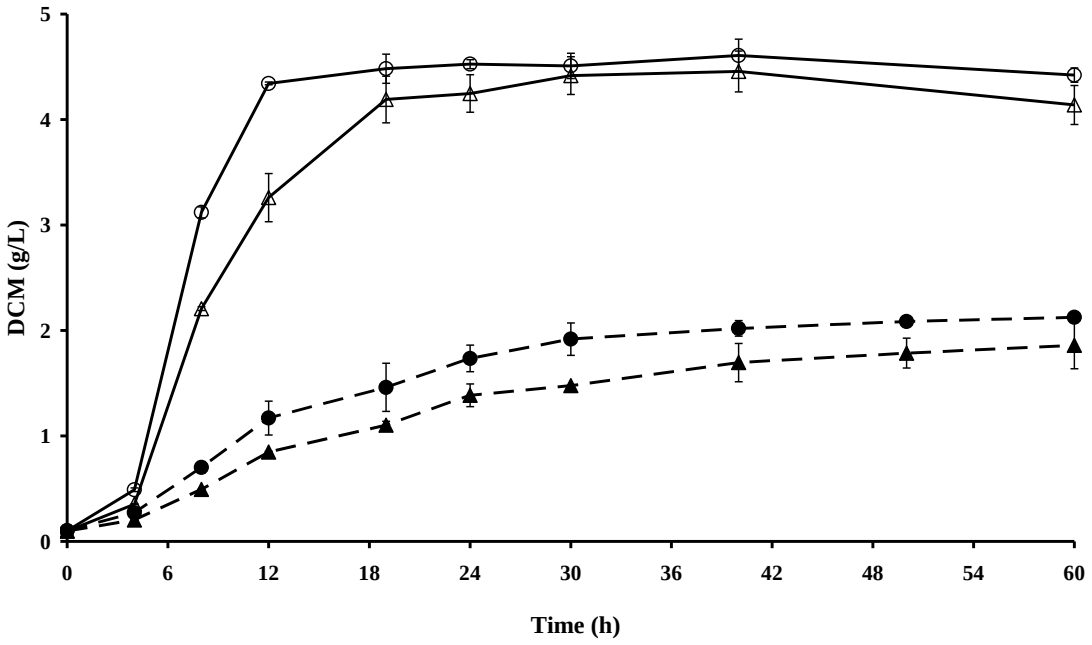
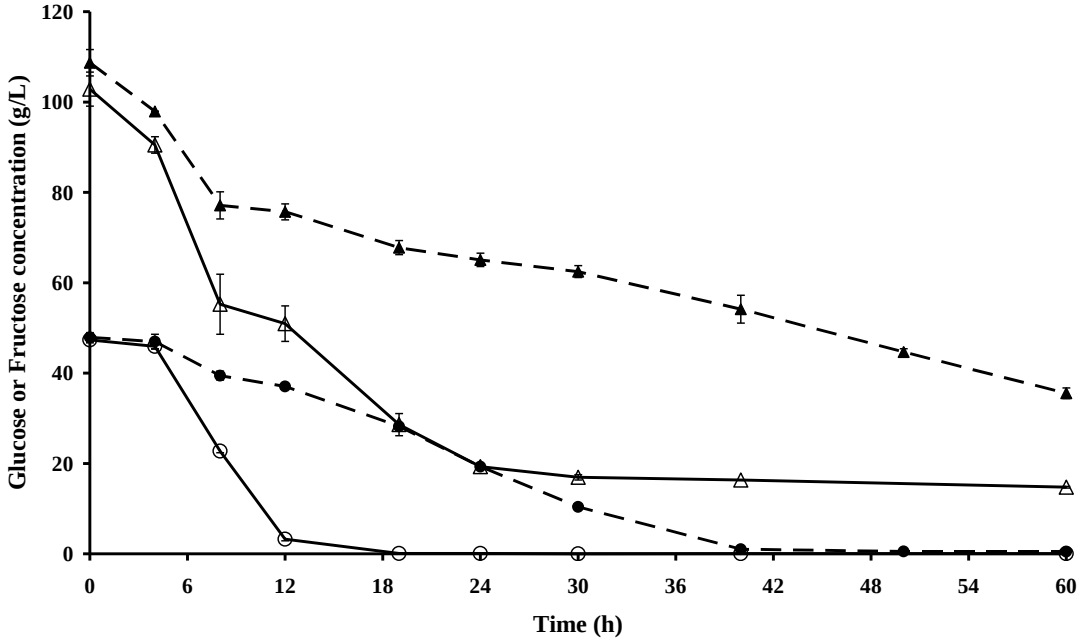


Figure 4.4 Cell growth, sugar utilization and ethanol production by *Issatchenkia* sp. S1 cultured in YM medium with 50 g/L glucose or 100 g/L fructose in 250 mL Erlenmeyer flask (150 mL reaction volume) with shaking speed at 200 rpm and static condition, 5% inoculum size, initial pH 6.0 and incubation temperature at 40 °C; (a) growth of *Issatchenkia* sp. S1, (b) glucose or fructose utilization and (c) ethanol production; —●— glucose concentration in shaking condition, - -⊖- - glucose concentration in static condition, —▲— fructose concentration in shaking condition and - -△- - fructose concentration in static condition.

(b)



(c)

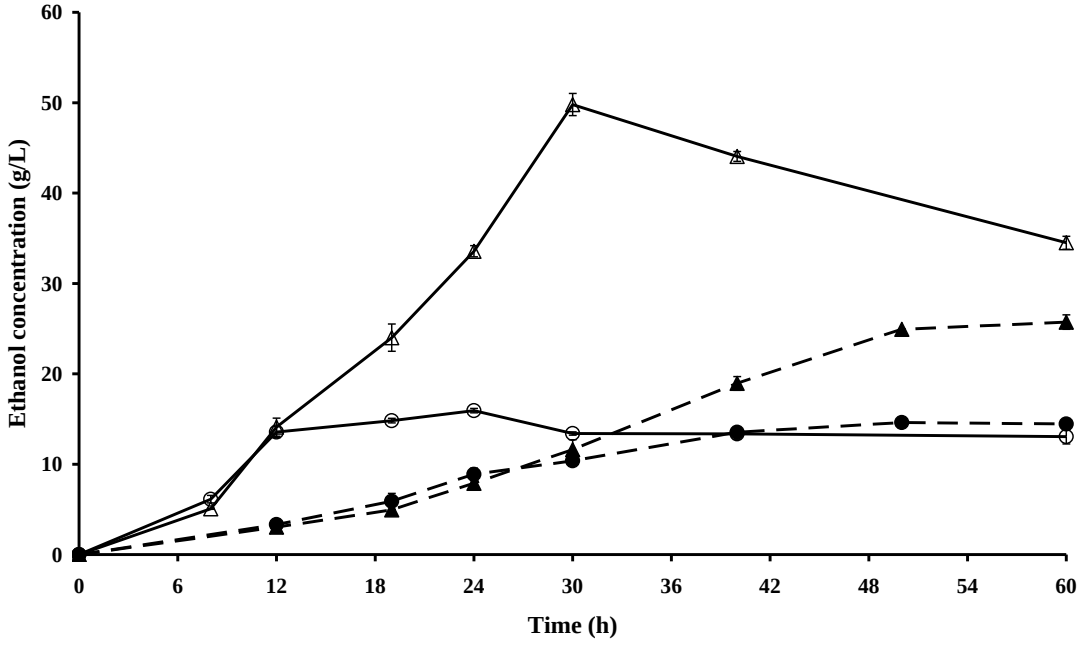


Figure 4.4 Continued.

Table 4.3 Ethanol yield (g/g), productivity (g/L/h) and specific growth rate of *Issatchenkia* sp. S1 cultured in YM medium with 50 g/L glucose or 100 g/L fructose in 250 mL Erlenmeyer flask at 40 °C with shaking speed at 200 rpm and static conditions (means of two replicates, ± standard error).

Conditions	Specific growth rate (h^{-1})	Volumetric productivity (g/L/h)	Ethanol yield (g/g)
Static			
50 g/L glucose	0.216 ± 0.011^{b}	0.370 ± 0.029^{a}	0.333 ± 0.002^{ab}
100 g/L fructose	0.183 ± 0.001^{a}	0.328 ± 0.023^{a}	0.360 ± 0.022^{b}
Shaking			
50 g/L glucose	0.349 ± 0.001^{d}	1.130 ± 0.051^{b}	0.319 ± 0.010^{a}
100 g/L fructose	0.309 ± 0.003^{c}	1.398 ± 0.026^{c}	0.484 ± 0.006^{c}

Values followed by the same letter are not significantly different at $P \leq 0.05$.

4.5 Effect of glucose and fructose concentrations on ethanol production in shaking condition

From Table 4.3, it was found that shaking condition had important effect on sugar utilization and ethanol production rate of *Issatchenkia* sp. S1. The concentration of glucose and fructose in previously experiment was rapidly decreased especially in glucose. *Issatchenkia* sp. S1 with shaking condition seemed to be more promoted glucose and fructose utilizations at higher concentration than those of static condition. Therefore, the concentration of glucose and fructose were optimized again with shaking condition.

Results of growth, consumption and ethanol production in glucose and fructose are shown in Figure 4.5 and 4.6, respectively. Growth of *Issatchenkia* sp. S1 in YM medium with 50 g/L glucose was shown the same curve of 100 g/L as shown

in Figure 4.5a. However, the growth of *Issatchenkia* sp. S1 was decreased when cultured in YM medium with 150 g/L glucose. Ethanol production rate of *Issatchenkia* sp. S1 was showed the highest rate cultured in YM medium with 100 g/L glucose (1.189 g/L/h, Table 4.4), which fructose also showed the same result as glucose. When compared the specific growth rate, volumetric productivity and ethanol yield of *Issatchenkia* sp. S1 in shaking condition (Table 4.4) with static condition (Table 4.2), there were dramatically increased in glucose and fructose also.

However, the growth of yeast cells and the production of ethanol was inhibits because of osmotic effects and/or the high concentration of glucose in medium as Ghose and Tyagi (1979) mentioned.

(a)

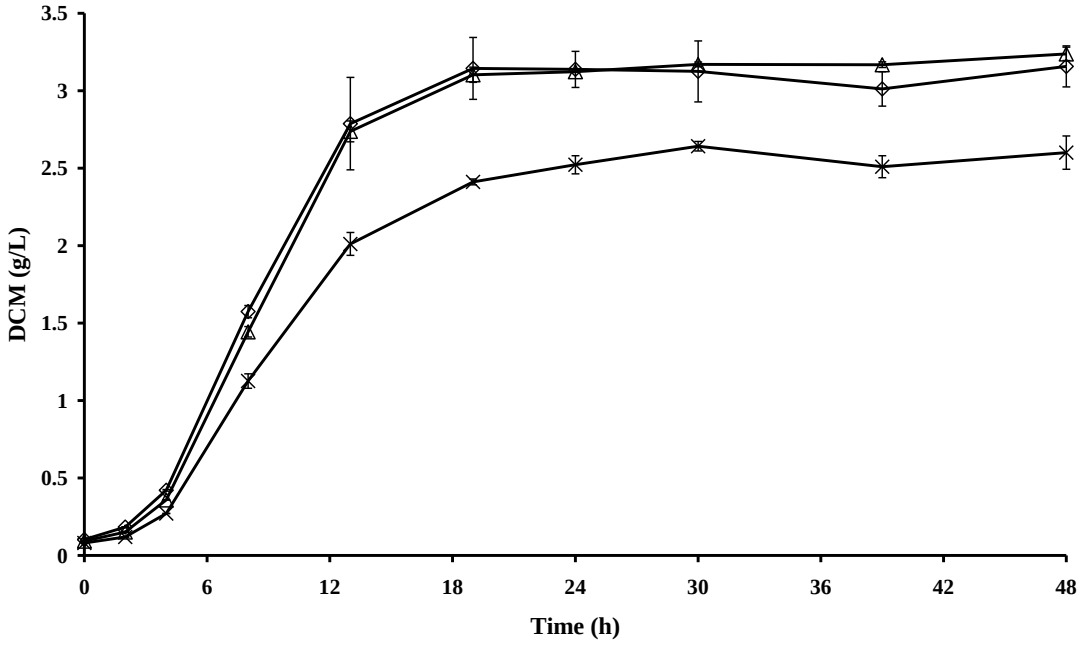
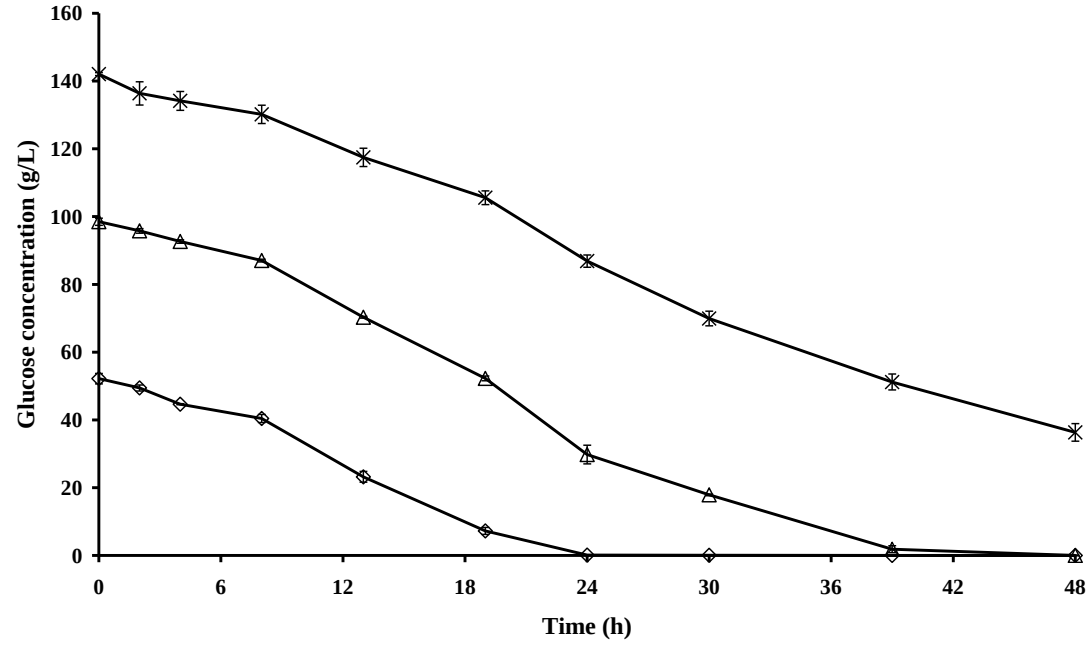


Figure 4.5 Cell growth, glucose utilization and ethanol production by *Issatchenkia* sp. S1 cultured in YM medium with 50, 100 or 150 g/L glucose in 250 mL Erlenmeyer flask (150 mL reaction volume) with shaking speed at 200 rpm, 5% inoculum size, initial pH 6.0 and incubation temperature at 40 °C; (a) growth of *Issatchenkia* sp. S1, (b) glucose utilization and (c) ethanol production; ◇ 50 g/L, △ 100 g/L and ✳ 150 g/L of glucose.

(b)



(c)

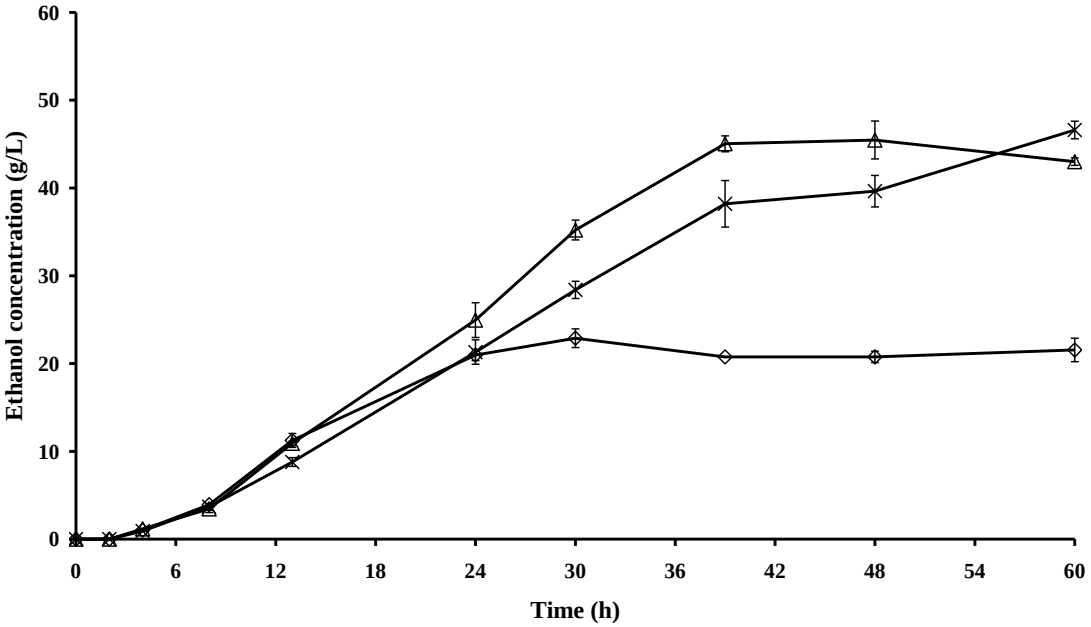


Figure 4.5 Continued.

(a)

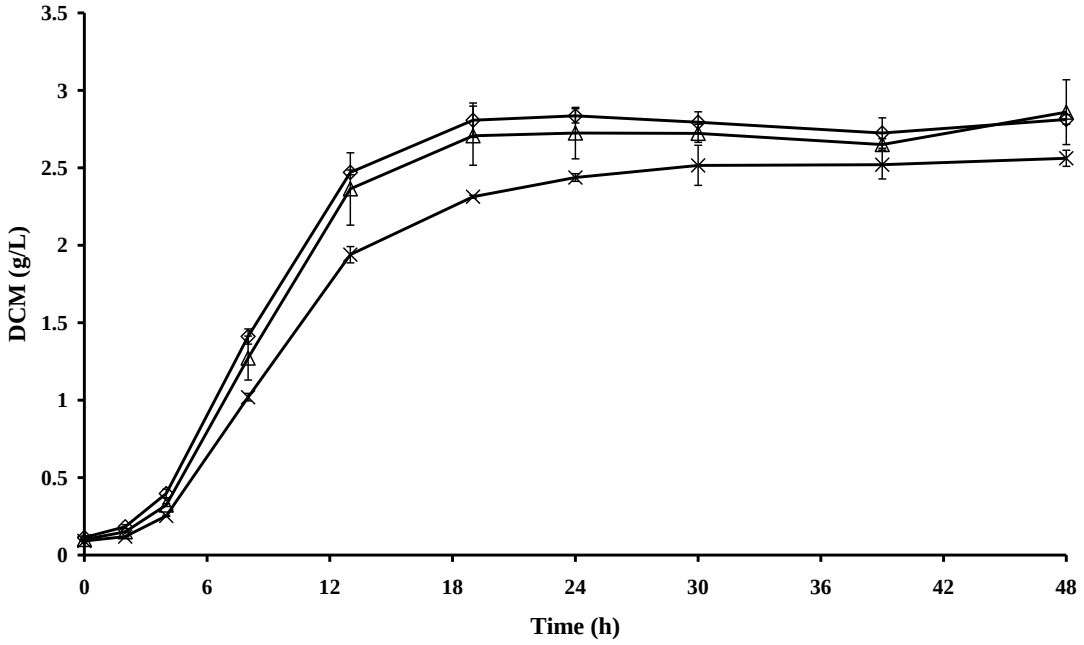
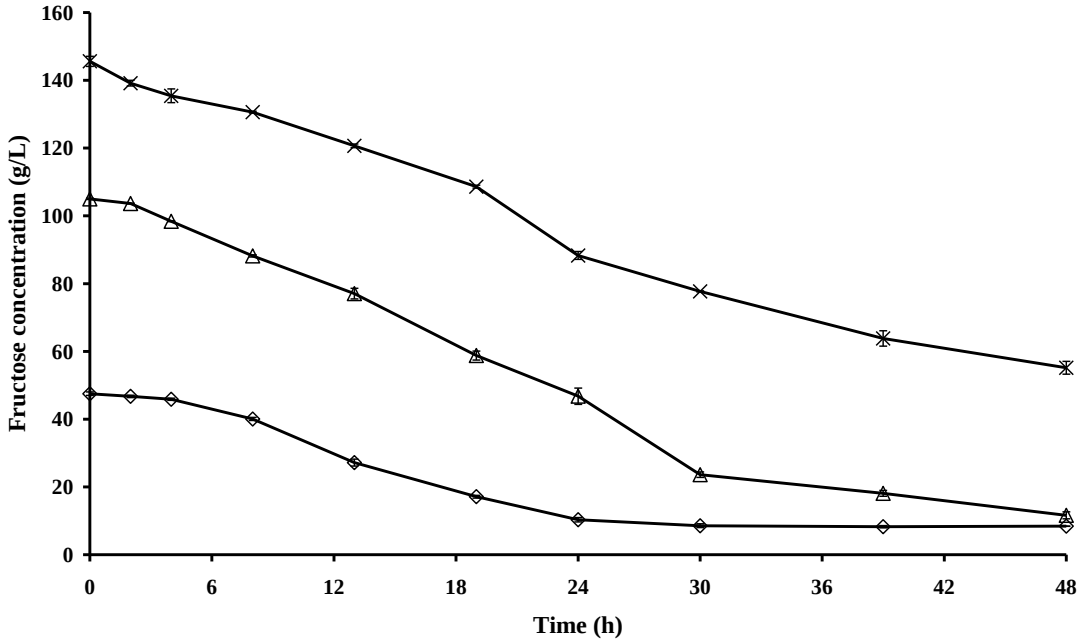


Figure 4.6 Cell growth, fructose utilization and ethanol production by *Issatchenkia* sp. S1 cultured in YM medium with 50, 100 or 150 g/L of fructose in 250 mL Erlenmeyer flask (150 mL reaction volume) with shaking speed at 200 rpm, 5% inoculum size, initial pH 6.0 and incubation temperature at 40 °C; (a) growth of *Issatchenkia* sp. S1, (b) fructose utilization and (c) ethanol production; ◇ 50 g/L, △ 100 g/L and ✳ 150 g/L of fructose.

(b)


Fructose concentration (g/L)
160
140
120
100
80
60
40
20
0
0
6
12
18
24
30
36
42
48
Time (h)

(c)

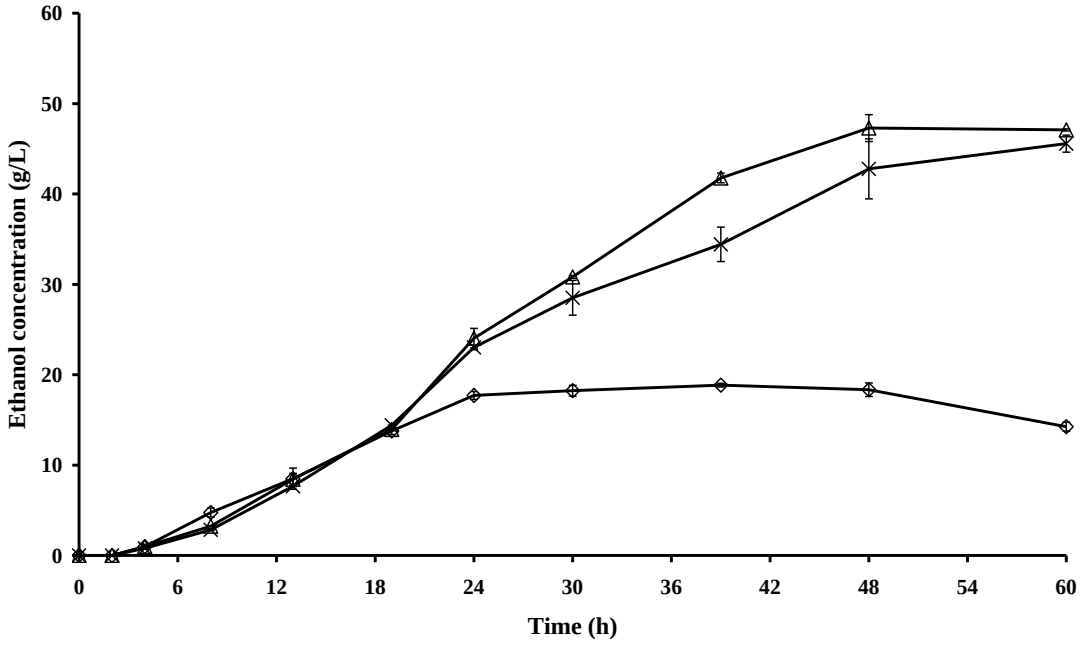

Ethanol concentration (g/L)
60
50
40
30
20
10
0
0
6
12
18
24
30
36
42
48
54
60
Time (h)

Figure 4.6 Continued.

Table 4.4 Ethanol yield (g/g), productivity (g/L/h) and specific growth rate of *Issatchenkia* sp. S1 cultured in YM medium with 50, 100 or 150 g/L of glucose or fructose in 250 mL flask with shaking speed at 200 rpm at 40 °C of incubation temperature (means of two replicates, ± standard error).

Sugar concentration (g/L)	Specific growth rate (h^{-1})	Volumetric productivity (g/L/h)	Ethanol yield (g/g)
Glucose			
50	0.355 ± 0.001^{ab}	0.999 ± 0.033^{b}	0.442 ± 0.003^{ab}
100	0.372 ± 0.001^{b}	1.189 ± 0.097^{d}	0.464 ± 0.024^{abc}
150	0.372 ± 0.008^{b}	1.019 ± 0.070^{bc}	0.422 ± 0.026^{a}
Fructose			
50	0.337 ± 0.001^{a}	0.832 ± 0.029^{a}	0.482 ± 0.001^{bc}
100	0.353 ± 0.017^{ab}	1.168 ± 0.086^{cd}	0.506 ± 0.009^{c}
150	0.356 ± 0.003^{ab}	1.111 ± 0.010^{bcd}	0.473 ± 0.039^{abc}

Values followed by the same letter are not significantly different at $P \leq 0.05$.

4.6 Order of glucose and fructose consumption

The results of order of glucose and fructose consumption showed in Figure 4.7. The YM medium with both glucose and fructose were cofermented to ethanol. The fermentation of fructose always lags behind that of glucose. Hence, *Issatchenkia* sp. S1 appeared to be generally glucophilic. The reasons for the preference for glucose are still unclear, but studies have recently been carried out to try to elucidate the physiological and molecular basis of the glucose to fructose discrepancy (Berthels *et al*., 2008; Guillaume *et al*., 2007).

A higher or lower fermentation rate is not associated with a corresponding change in the fermentation rate of fructose. The metabolic pathway of fructose fermentation is very similar to that of glucose. Even the transporters are shared although their affinity for glucose is higher than for fructose. Hence, the transport step is a first candidate for the cause of the discrepancy in glucose and fructose fermentation. After transport glucose is phosphorylated by glucokinase, hexokinase 1 and hexokinase 2, whereas fructose is only phosphorylated by the latter two enzymes (Barnett, 1997). The affinity of the hexokinases is higher for glucose. Hence, the phosphorylation step is a second candidate for the cause of the discrepancy in glucose and fructose fermentation. Fructose is a ketose sugar, nearly 30% of which is present in the furanose form in solution (Flood *et al*., 1996), whereas glucose is an aldose, nearly 99.9% of which is present in the pyranose form (Hyvönen *et al*., 1977). Since glucose and other sugars are transported in the pyranose rather than in the furanose form, the actual transport-competent concentration of fructose is below its total concentration (Bission, 1999). Differences in physicochemical properties like these may explain the lower affinity for fructose of the transport system (Reifenberger *et al*., 1997) and the hexokinases (Sols *et al*., 1958; Rose *et al*., 1991).

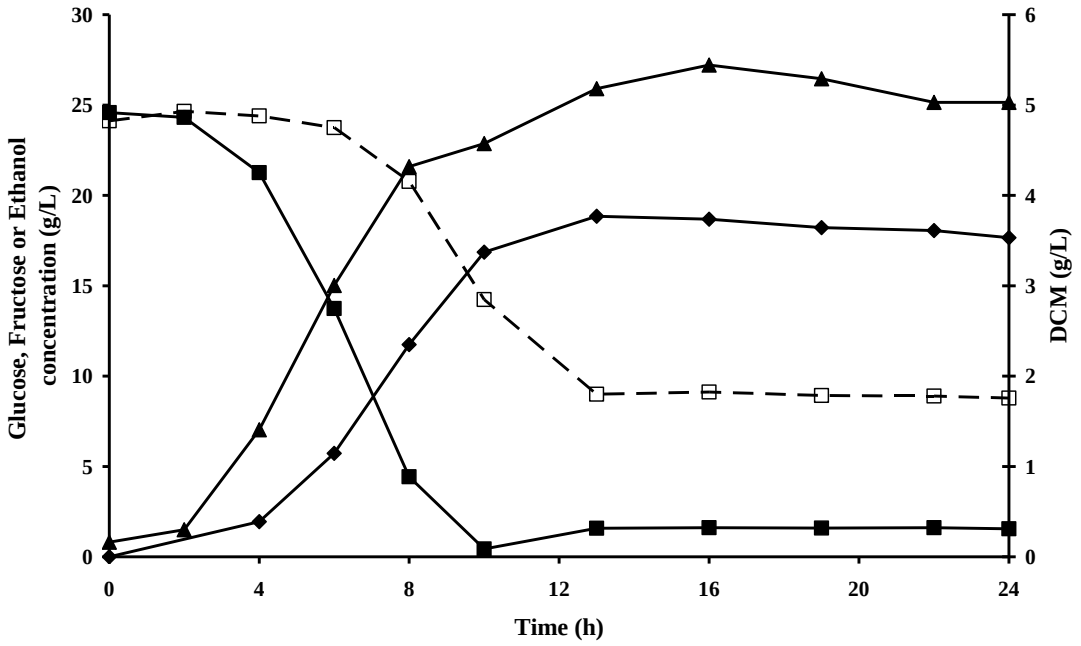


Figure 4.7 Cell growth, glucose and fructose utilization, and ethanol production by *Issatchenkia* sp. S1 cultured in YM medium with 25 g/L of glucose and 25 g/L fructose in 250 mL Erlenmeyer flask (150 mL reaction volume) with shaking speed at 200 rpm, 5% inoculum size, initial pH 6.0 and incubation temperature at 40 °C; ▲ growth of *Issatchenkia* sp. S1, ■ glucose utilization, □ fructose utilization and ◆ ethanol production.

4.7 Effect of nitrogen source on ethanol production

The different concentrations and sources, and there combination of nitrogen source on ethanol production were studied following in Table 3.1. Effect of nitrogen source, their combination and concentration was depicted in Figure 4.8 and Table 4.5. Growth, consumption rate and ethanol production of *Issatchenkia* sp. S1 had related with nitrogen source and their concentrations. *Issatchenkia* sp. S1 had some a bit

growth in trail 1 (no nitrogen). However, it did not produce ethanol and very low consumed glucose. *Issatchenkia* sp. S1 was very low activity when depletion of nitrogen sources. Moreover, Ammonium sulfate could not stimulated growth and ethanol production also (trail 2, 2.0 g/L $(NH_4)_2SO_4$) might be because of lack of some salts, some necessary nutrients, and/or some growth factors in medium. When yeast extract, malt extract and/or peptone were added as the nitrogen sources in medium, growth, consumption rate and ethanol production rate were increased following increased their concentration. Therefore, the addition organic nitrogen sources composed yeast extract, malt extract and peptone was necessary for stimulated yeast growth and their ethanol production in enrich medium. The highest of growth and specific growth rate in this experiment was shown in trail 17 (nitrogen source as YM medium) (Figure 4.8a and Table 4.5). However, when 2 g/L $(NH_4)_2SO_4$ (trail 18) was added, the ethanol production rate was increased about 1.4 time compared with trail 17 (Table 4.5). The addition of $(NH_4)_2SO_4$ was increased the nitrogen content in the medium and thus to enhance the ethanol productivity of *Issatchenkia* sp. S1.

Nitrogen is necessary sources for the growth and multiplication of yeasts and it also influences the ethanol productivity (Bafrncová *et al*., 1999). Deficiencies in the supply of nitrogenous compounds remain the most common causes of poor fermentative performance and sluggish or stuck fermentation (Bission, 1991; Bission, 1999).

Additionally, nitrogen, together with carbon, is one of the main elements found in many macromolecules of living organisms, playing a central role in structure and function, and most organisms have elaborate control mechanisms to provide a constant supply of nitrogen (Magananik, 2005; Magananik and Kaiser, 2002).

Similarly to carbon catabolite repression, a mechanism known as nitrogen catabolite repression (Hofman-Bang, 1999; Magananik, 2005) induces differential nitrogenous compound utilization. It has been observed that ammonia, asparagine, glutamine and glutamate are preferentially used by yeast (Magananik, 2005). When these primary nitrogen sources are absent, or present in concentrations low enough to limit growth, other nitrogen sources such as amino acids and peptides can be used. The utilization of secondary nitrogen sources requires the synthesis of specific-catabolic enzymes and permeases, the expression of which is highly regulated by nitrogen catabolite repression. Considering that carbon and nitrogen are the main nutrients in industrial fermentation substrates, it would imply that the mutual interaction of these nutrients may play an important role in yeast metabolism (Peter *et al.*, 2006; Júnior *et al.*, 2008).

(a)

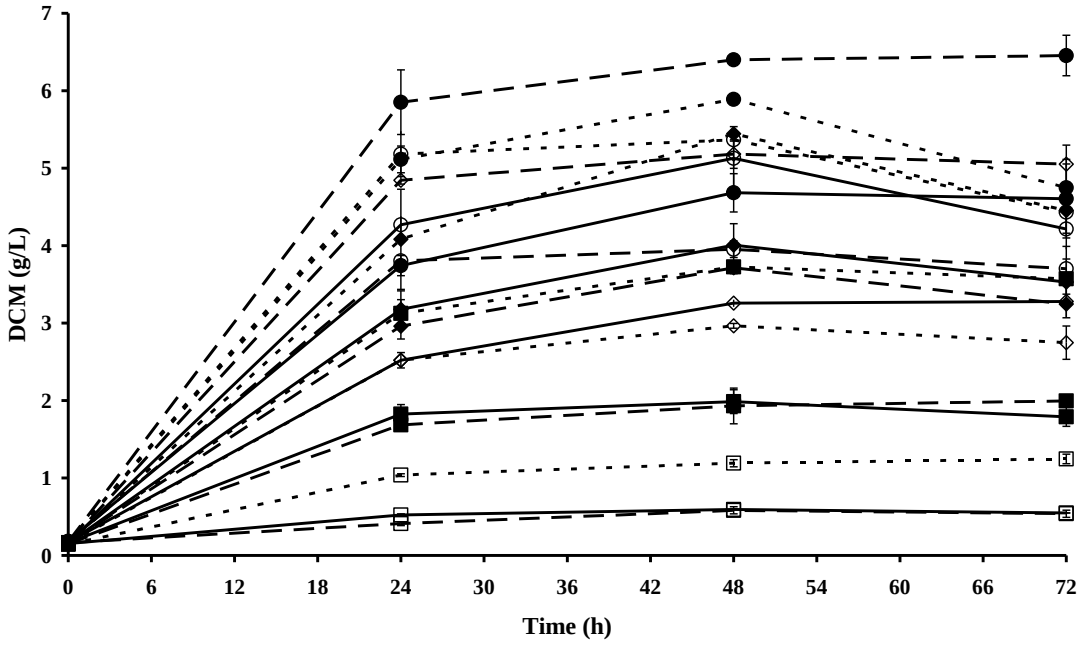
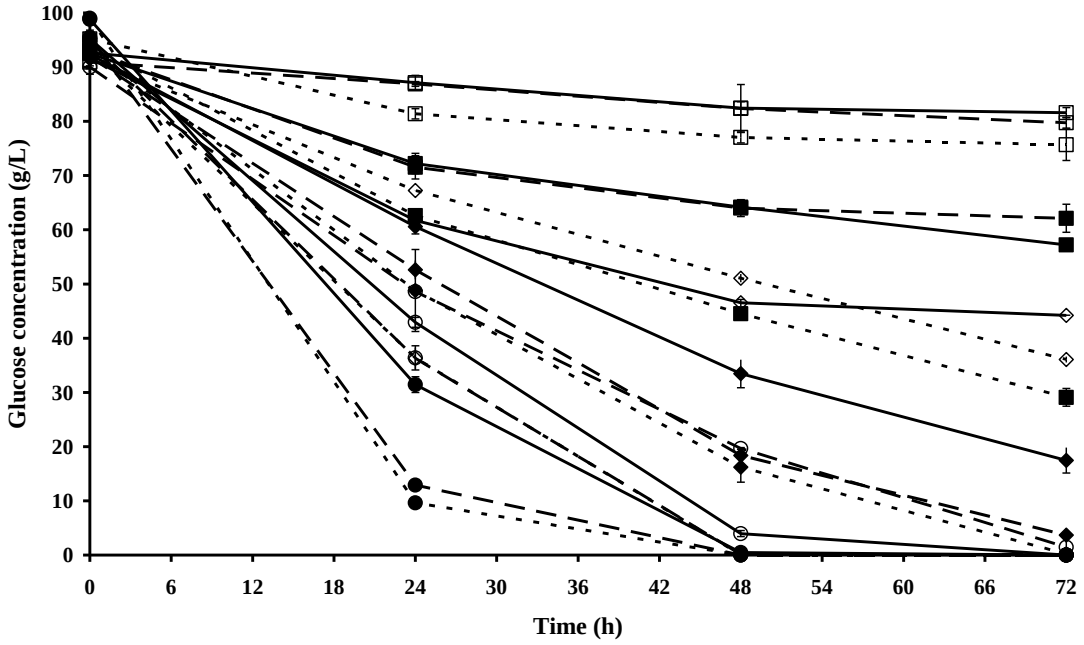


Figure 4.8 Cell growth, glucose utilization and ethanol production by *Issatchenkia* sp. S1 cultured in 150 mL of 100 g/L glucose with various nitrogen sources and concentrations in 250 mL Erlenmeyer flask with shaking speed at 200 rpm, 5% inoculum size, initial pH 6.0 and incubation temperature at 40 °C; (a) growth of *Issatchenkia* sp. strain S1, (b) glucose utilization and (c) ethanol production; trail 1, trail 2, trail 3, trail 4, trail 5, trail 6, trail 7, trail 8, trail 9, trail 10, trail 11, trail 12, trail 13, trail 14, trail 15, trail 16, trail 17 and trail 18.

(b)



(c)

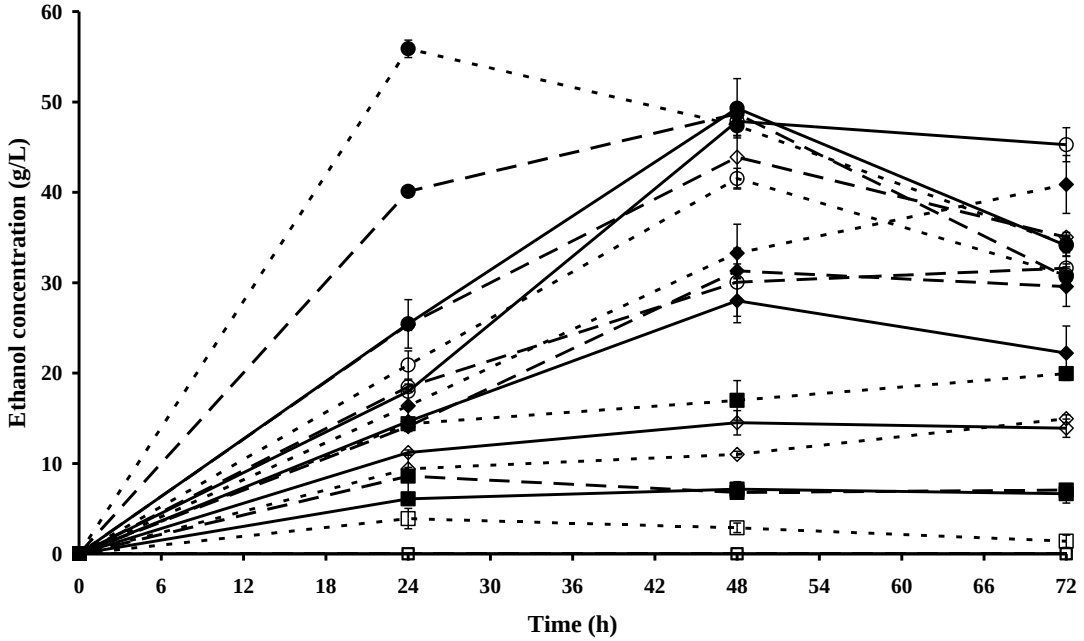


Figure 4.8 Continued.

Table 4.5 Ethanol yield (g/g), productivity (g/L/h) and specific growth rate of *Issatchenkia* sp. S1 cultured in 150 mL of 100 g/L glucose with various nitrogen sources and concentrations in 250 mL Erlenmeyer flask with shaking speed at 200 rpm at 40 °C (means of two replicates, ± standard error).

Trail	N sources	Specific growth rate (h^{-1})	Volumetric productivity (g/L/h)	Ethanol yield (g/g)
1	No	0.015 ± 0.001^{a}	0^{a}	0^{a}
2	2.0 g/L $(NH_4)_2SO_4$	0.011 ± 0.001^{a}	0^{a}	0^{a}
3	1.5 g/L malt extract 2.0 g/L $(NH_4)_2SO_4$	0.037 ± 0.001^{b}	0.162 ± 0.027^{b}	0.202 ± 0.047^{b}
4	3.0 g/L malt extract 2.0 g/L $(NH_4)_2SO_4$	0.069 ± 0.005^{c}	0.253 ± 0.024^{bc}	0.203 ± 0.010^{b}
5	1.5 g/L yeast extract 2.0 g/L $(NH_4)_2SO_4$	0.064 ± 0.003^{c}	0.357 ± 0.044^{cd}	0.280 ± 0.098^{c}
6	1.5 g/L yeast extract 1.5 g/L malt extract 2.0 g/L $(NH_4)_2SO_4$	0.124 ± 0.007^{d}	0.599 ± 0.003^{d}	0.307 ± 0.019^{cd}
7	3.0 g/L yeast extract 2.0 g/L $(NH_4)_2SO_4$	0.098 ± 0.004^{d}	0.392 ± 0.005^{d}	0.269 ± 0.007^{cd}
8	3.0 g/L yeast extract 3.0 g/L malt extract 2.0 g/L $(NH_4)_2SO_4$	0.195 ± 0.002^{fg}	1.056 ± 0.034^{fg}	0.463 ± 0.026^{de}
9	2.5 g/L peptone 2.0 g/L $(NH_4)_2SO_4$	0.098 ± 0.004^{e}	0.392 ± 0.005^{e}	0.269 ± 0.007^{cd}
10	2.5 g/L peptone 1.5 g/L malt extract 2.0 g/L $(NH_4)_2SO_4$	0.126 ± 0.010^{h}	0.610 ± 0.041^{h}	0.375 ± 0.009^{fg}
11	2.5 g/L peptone 1.5 g/L yeast extract 2.0 g/L $(NH_4)_2SO_4$	0.117 ± 0.007^{e}	0.587 ± 0.009^{e}	0.355 ± 0.022^{e}
12	2.5 g/L peptone 1.5 g/L yeast extract	0.164 ± 0.027^{h}	0.683 ± 0.035^{g}	0.438 ± 0.118^{f}

1.5 g/L malt extract

Table 4.5 Continued.

Trail	N sources	Specific growth rate (h^{-1})	Volumetric productivity (g/L/h)	Ethanol yield (g/g)
13	2.5 g/L peptone 1.5 g/L yeast extract 1.5 g/L malt extract 2.0 g/L $(NH_4)_2SO_4$	0.171 ± 0.004^{de}	0.749 ± 0.016^{e}	0.502 ± 0.002^{de}
14	5.0 g/L peptone 2.0 g/L $(NH_4)_2SO_4$	0.151 ± 0.001^{f}	0.770 ± 0.012^{h}	0.357 ± 0.013^{g}
15	5.0 g/L peptone 3.0 g/L malt extract 2.0 g/L $(NH_4)_2SO_4$	0.208 ± 0.012^{fg}	0.871 ± 0.018^{ef}	0.445 ± 0.065^{f}
16	5.0 g/L peptone 3.0 g/L yeast extract 2.0 g/L $(NH_4)_2SO_4$	0.148 ± 0.005^{i}	1.060 ± 0.112^{i}	0.499 ± 0.028^{g}
17	5.0 g/L peptone 3.0 g/L yeast extract 3.0 g/L malt extract	0.237 ± 0.017^{g}	1.671 ± 0.035^{f}	0.510 ± 0.027^{g}
18	5.0 g/L peptone 3.0 g/L yeast extract 3.0 g/L malt extract 2.0 g/L $(NH_4)_2SO_4$	0.205 ± 0.008^{h}	2.328 ± 0.040^{j}	0.511 ± 0.009^{g}

Values followed by the same letter are not significantly different at $P \leq 0.05$.

4.8 Ethanol production in 2 L Batch fermenter

4.8.1 Effect of aeration on ethanol production

The cell growth, glucose consumption and ethanol production during the fermentation of various aeration rates were shown in Figure 4.9. *Issatchenkia* sp. S1 showed the highest cell density (Figure 4.9a) and specific growth rate (Table 4.6)

when operated under 1.0 vvm of aeration rate. The addition of air into reactor was stimulated yeast cell growth. In contrast, ethanol was produced lower than no aeration. However, cell density and specific growth rate was decreased when decreased aeration rate but increased ethanol production and ethanol yield. Ethanol concentration and ethanol yield were highest when did not fed the air (Figure 4.9c and Table 4.6). Moreover, ethanol production in added air condition (0.2 and 1.0 vvm) (Figure 4.9c) were decreased after depletion of glucose. Because of diauxic shift was occurred after depletion of glucose and accumulation of ethanol. The metabolism in alcoholic fermentation was changed. The fermentation product, ethanol, becomes a substrate and is degraded (Pronk *et al*., 1996).

Wayman and Parekh (1990) reported that ethanol fermentation is not a wholly anaerobic process. In many instances, active starter cultures are grown under aerobic or semi-aerobic conditions to improve yeast yields and growth rates. Even if sufficient nutrients such as sugar, nitrogen and vitamins are presented, 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.

The role of oxygen in alcoholic fermentations has been reviewed (Markham, 1969). Oxygen acts primarily as a terminal acceptor of electrons from the respiratory chain. It also acts as a yeast growth factor. Oxygen appears to be involved in the synthesis of oleic acid and ergosterol, which stimulate yeast growth under anaerobic

conditions (Andreasen and Stier, 1954). A high degree of aeration results in an increase of biomass, i.e., multiplication and less ethanol production (Maxon and Johnson, 1953).

(a)

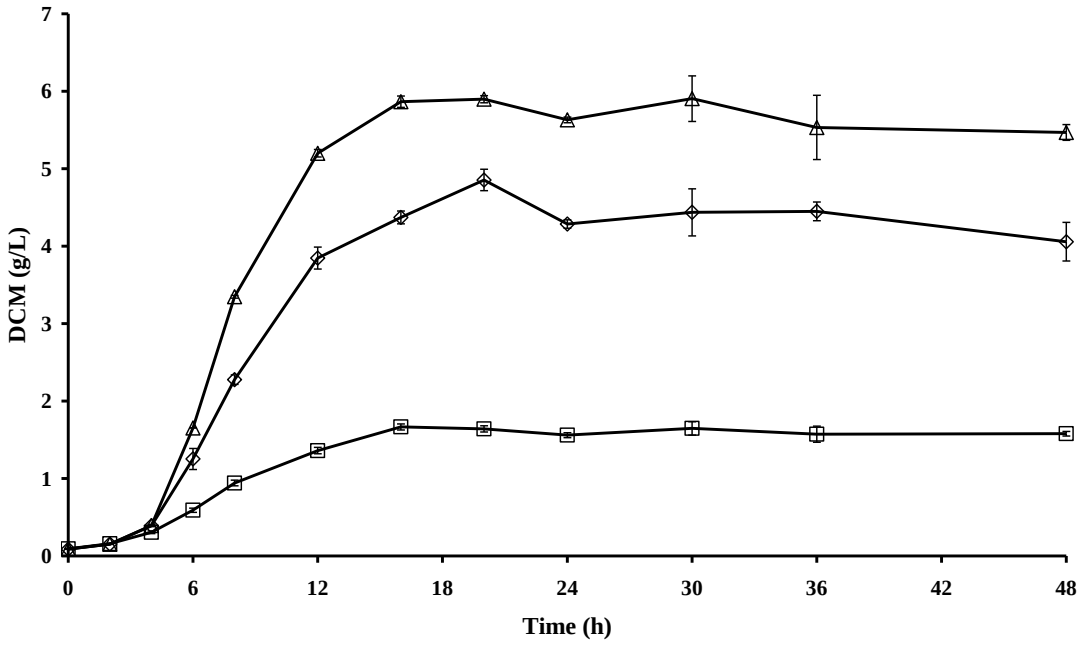
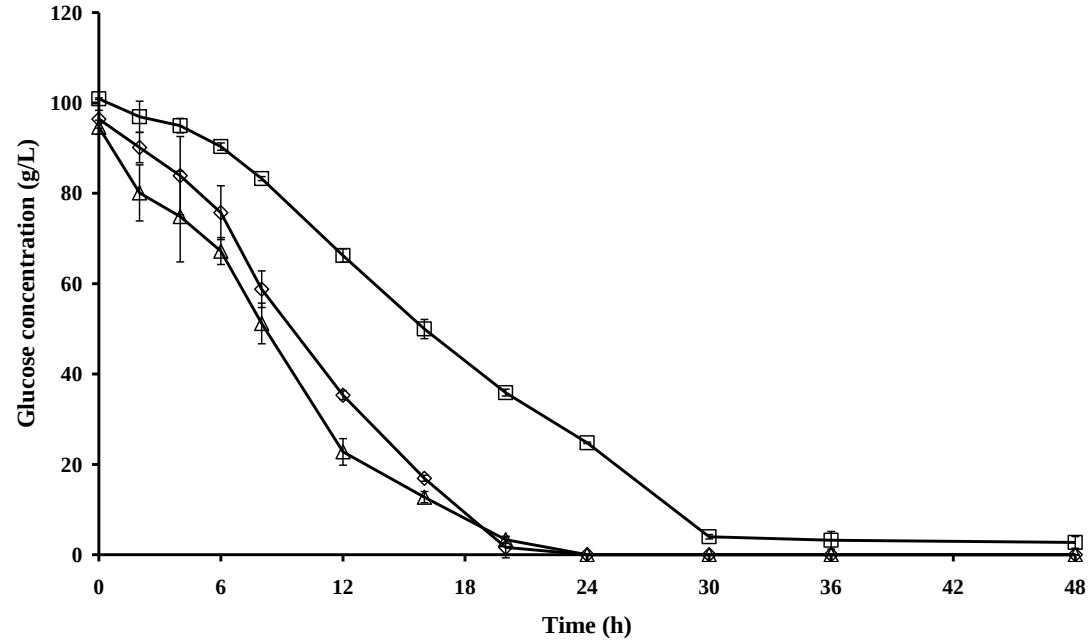


Figure 4.9 Cell growth, glucose utilization and ethanol production by *Issatchenkia* sp. S1 cultured with YM medium with 100 g/L glucose and 2 g/L $(NH_4)_2SO_4$ in 2 L fermenter with agitation speed at 100 rpm, 5% inoculum size, pH was controlled at 4.0, and operation temperature at 40 $^{\circ}$C; (a) growth of *Issatchenkia* sp. S1, (b) glucose utilization and (c) ethanol production; ⊞ 0 vvm, ◇ 0.2 vvm, and △ 1.0 vvm of aeration rate.

(b)


Glucose concentration (g/L)
120
100
80
60
40
20
0
0
6
12
18
24
30
36
42
48
Time (h)

(c)

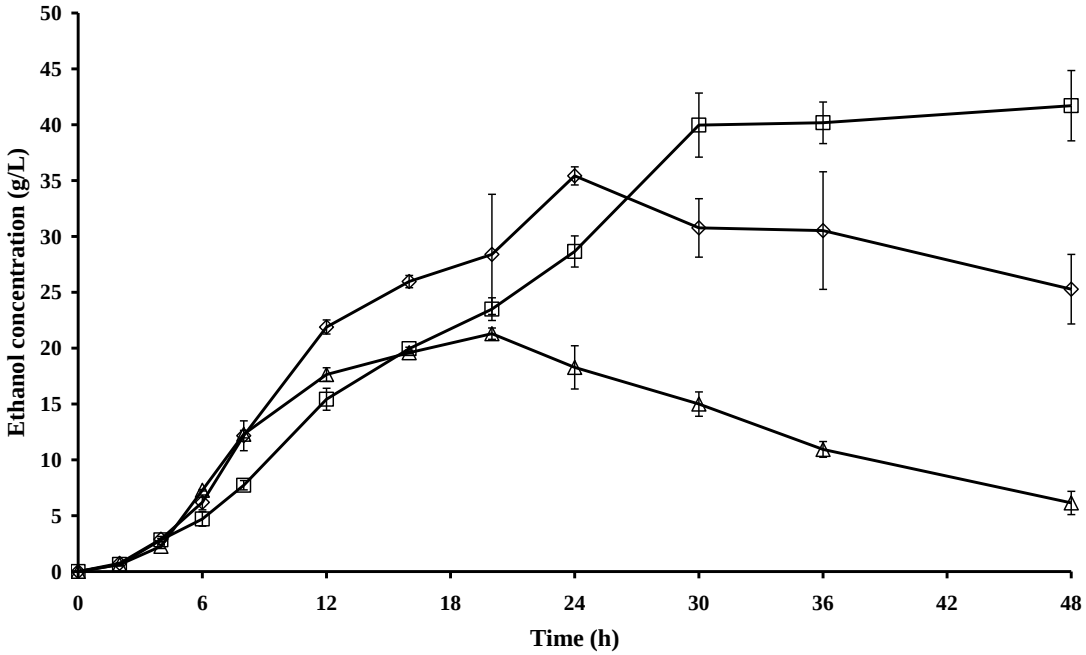

Ethanol concentration (g/L)
50
45
40
35
30
25
20
15
10
5
0
0
6
12
18
24
30
36
42
48
Time (h)

Figure 4.9 Continued.

Table 4.6 Ethanol yield (g/g), productivity (g/L/h) and specific growth rate of *Issatchenkia* sp. S1 cultured in YM medium with 100 g/L glucose and 2 g/L $(NH_4)_2SO_4$ in 2 L fermenter (1 L reaction volume) in various aeration rate, agitation speed at 100 rpm, 5% inoculum size, pH was controlled at 4.0, and operation temperature at 40 °C (means of two replicates, ± standard error).

Aeration rate (vvm)	Specific growth rate (h^{-1})	Volumetric productivity (g/L/h)	Ethanol yield (g/g)
0	0.297 ± 0.001^{a}	1.194 ± 0.058^{b}	0.437 ± 0.007^{c}
0.2	0.430 ± 0.004^{b}	1.476 ± 0.034^{c}	0.368 ± 0.016^{b}
1.0	0.482 ± 0.002^{c}	1.065 ± 0.025^{a}	0.225 ± 0.002^{a}

Values followed by the same letter are not significantly different at $P \leq 0.05$.

4.8.2 Effect of agitation rate on ethanol production

From previous results, *Issatchenkia* sp. S1 was cultivated in 2 L fermenter was shown the ethanol concentration and ethanol yield highest when did not aeration. The results of agitation rate are shown in Figure 4.10. The increasing of agitation speed in the batch fermenter could be increased growth, glucose consumption, and ethanol production as shown in Table 4.6. Agitation speed at 500 rpm was shown the great result of ethanol production rate (1.886 ± 0.056 g/L/h) and ethanol yield (0.516 ± 0.026 g/g). However, the agitation speeds at higher than 500 rpm was not studied because limitation of instrument and it could not used to operate at higher scale process.

Kongkiattikajorn *et al*. (2007) reported that agitation of fermentation broth normally satisfied the oxygen demand of a fermentation process. Among other factors having an impact on the operating conditions during fermentation in bioreactors is agitation and mixing. Agitation is important for uniform mixing of the medium components within the fermenter (dispersion of cells and nutrients) as well as mass transfer phenomena (e.g., oxygen transfer rates). The effect of agitation on ethanol production is important for the successful progress of the fermentation. Agitation is important for adequate mixing, mass transfer and heat transfer. It is not only assisted mass transfer between the different phases present in the culture, but also maintained homogeneous chemical and physical conditions in the culture by continuous mixing. Agitation creates shear forces, which affect microorganisms in several ways, causing morphological changes, variation in their growth and product formation and also damaging the cell structure (Mittal, 1992).

(a)

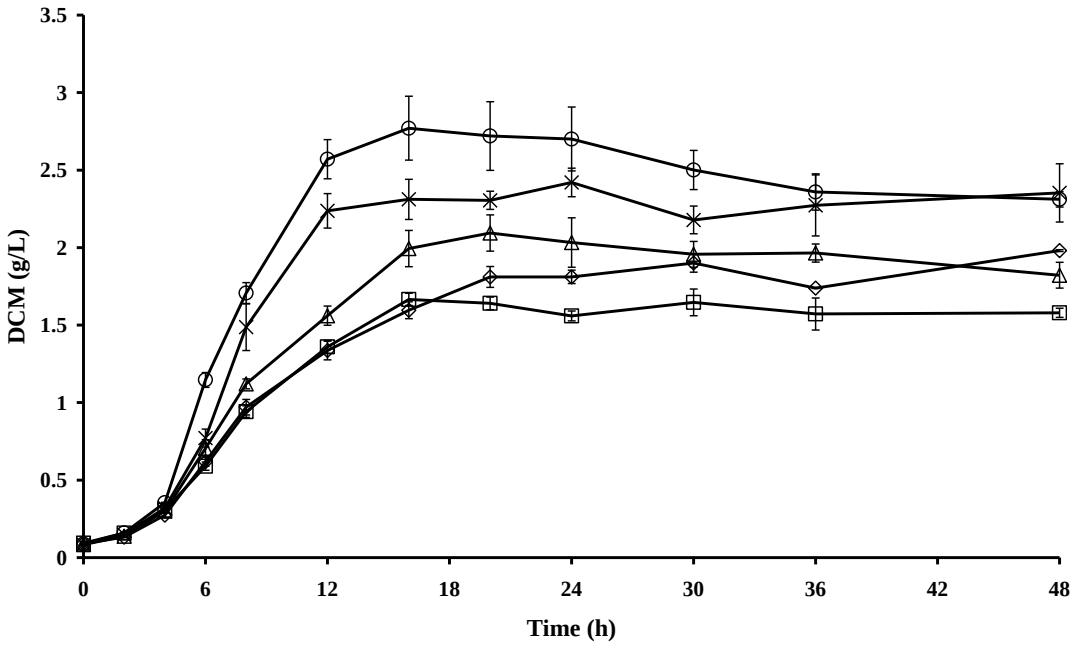
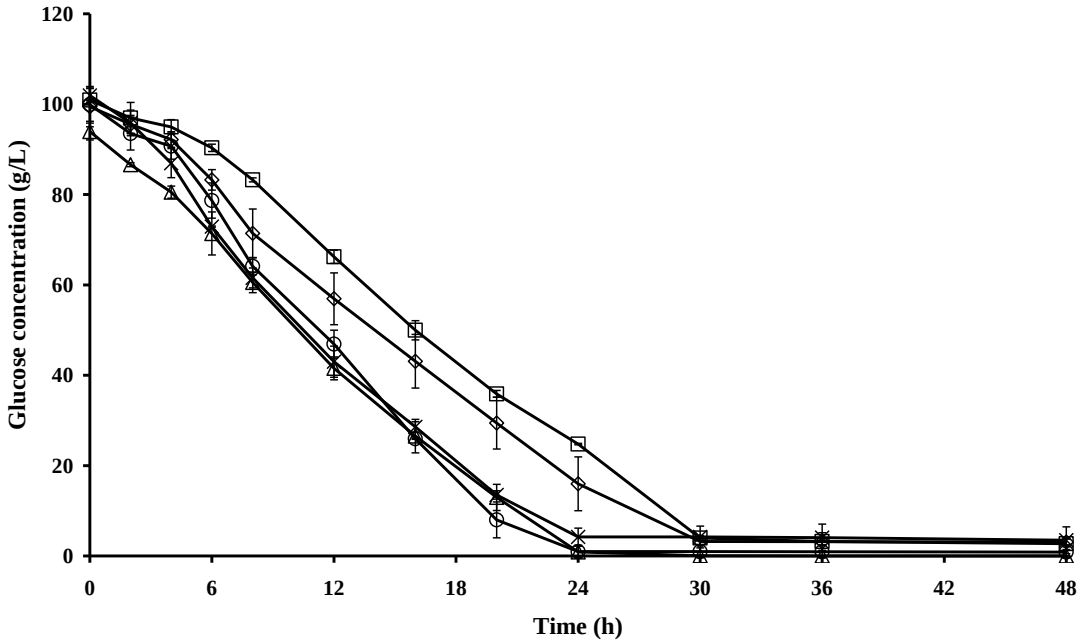


Figure 4.10 Cell growth, glucose utilization and ethanol production by *Issatchenkia* sp. S1 in 100 g/L glucose in YM medium added 2 g/L $(NH_4)_2SO_4$ in 2 L fermenter, 5% inoculum size, pH 4.0, and temperature at 40 °C. The agitation speed was varied at 100, 200, 300, 400 and 500 rpm; (a) growth of *Issatchenkia* sp. S1, (b) glucose utilization and (c) ethanol production; ⊟ 100 rpm, ◇ 200 rpm, △ 300 rpm, ✳ 400 rpm and ⊖ 500 rpm of agitation speed.

(b)


Glucose concentration (g/L)
120
100
80
60
40
20
0
0
6
12
18
24
30
36
42
48
Time (h)

(c)

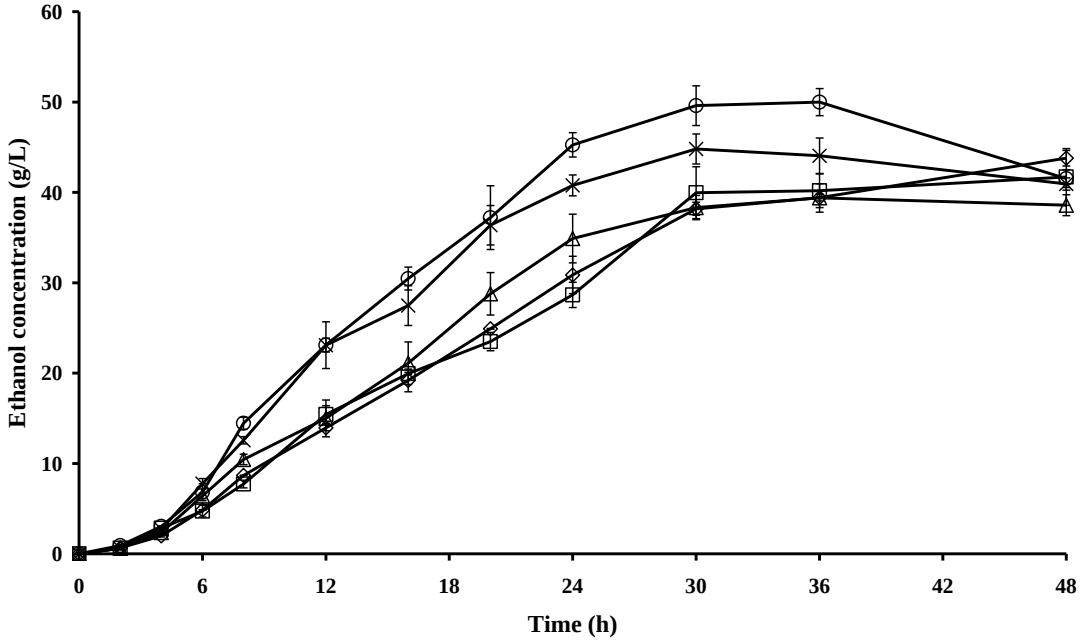


Figure 4.10 Continued.

Table 4.7 Ethanol yield (g/g), productivity (g/L/h) and specific growth rate of *Issatchenkia* sp. S1 cultured YM medium with 100 g/L glucose and 2 g/L $(NH_4)_2SO_4$ in 2 L fermenter (1 L reaction volume) with various agitation speed, 5% inoculum size, pH 4.0, and operation temperature at 40 $^{\circ}$C (means of two replicates, ± standard error).

Agitation rate (rpm)	Specific growth rate (h^{-1})	Volumetric productivity (g/L/h)	Ethanol yield (g/g)
100	0.297 ± 0.001^{a}	1.194 ± 0.058^{a}	0.437 ± 0.007^{a}
200	0.314 ± 0.010^{a}	1.286 ± 0.086^{ab}	0.455 ± 0.033^{a}
300	0.340 ± 0.007^{b}	1.454 ± 0.112^{b}	0.425 ± 0.001^{a}
400	0.368 ± 0.012^{c}	1.699 ± 0.048^{c}	0.464 ± 0.011^{a}
500	0.370 ± 0.009^{c}	1.886 ± 0.056^{c}	0.516 ± 0.026^{b}

Values followed by the same letter are not significantly different at $P \leq 0.05$.

4.9 Ethanol production in 10 L Batch fermenter

Ten liters of batch fermentation were operated for determined the ethanol production by thermotolerant *Issatchenkia* sp. Cell growth, glucose consumption and ethanol production during fermentation process was shown in Figure 4.11. Cell was grown rapidly and reached to stationary after 12 h after start fermentation. Ethanol production was increased and slightly slow after 30 h after start fermentation because of depletion of glucose in medium (4.780 ± 1.490 g/L)

Moreover, 10 L batch fermentation showed lower specific growth rate, productivity and ethanol yield (Table 4.6 and Table 4.8) than 2 L batch fermentation. Since the scale-up of microbial fermentation processes is complicated and unpredictable process performance can result. Indeed it was often observed that the biomass yield and any growth-associated products are often decreased on the scale-up of an aerobic process (Hewitt and Nienow, 2007). Due to the above, in a growing cell damping of the amplitude, phase shift, and time pattern deformation of the metabolic rhythms considered were detected, depending on the volume change character (Pawlowski and Zielenkiewicz, 2004).

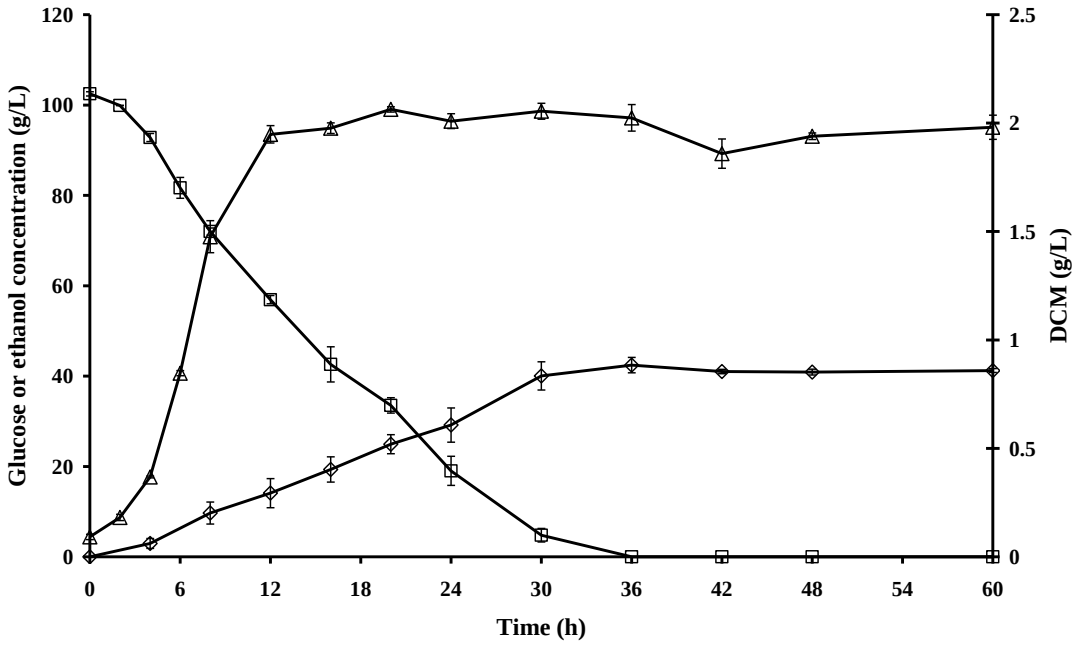


Figure 4.11 Cell growth, glucose utilization and ethanol production by *Issatchenkia* sp. S1 cultured in YM medium with 100 g/L glucose and 2 g/L $(NH_4)_2SO_4$ in 10 L batch fermenter, 5% inoculum size, pH was controlled at 4.0, agitation speed at 500 rpm and operation temperature at 40 °C; ⊟ glucose concentration (g/L), ◇ ethanol concentration (g/L) and △ DCM (g/L).

4.10 Ethanol production in 10 L Fed-batch fermenter

Ten liters of fed-batch fermentation were operated for increasing the ethanol production by *Issatchenkia* sp. S1. The 10 L of fed-batch was operated the same conditions of 10 L batch fermentation. And the concentrate glucose was added into the fermenter as substrate for increasing the ethanol production in the same reactor.

4.10.1 Fed-batch A

The first fed-batch called “fed-batch A”. The result of 10 L batch operation found that glucose depleted at 30 h after operation. In this operation was added 350 g glucose (1 L of volume) before depletion of glucose (28 h after fermentation) which expected the final glucose concentration in the medium about 50 g/L. The results of fed-batch A was shown in Figure 4.12. Addition of glucose could increase the ethanol production. In 10 L batch culture, the highest ethanol concentration was 42.43 ± 1.69 g/L. Moreover, when glucose was added in fed batch A, the ethanol concentration was increased up to 63.60 ± 1.45 g/L or increased about 1.6 times

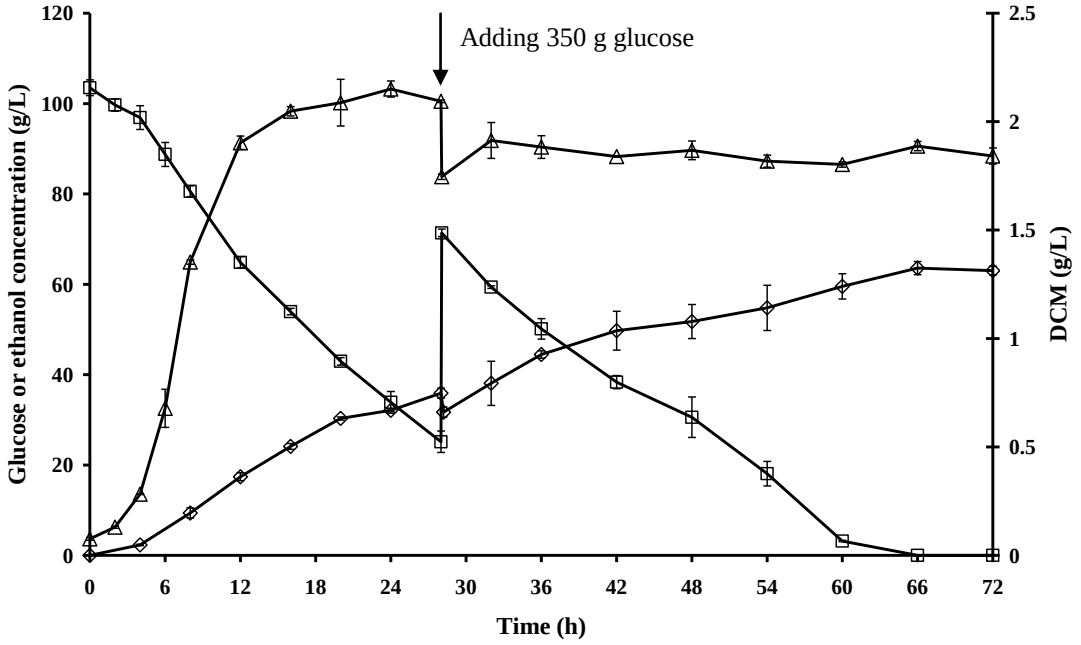


Figure 4.12 Cell growth, glucose utilization and ethanol production by *Issatchenkia* sp. S1 cultured in YM medium with 100 g/L glucose and 2 g/L

$(NH_4)_2SO_4$ in 10 L fed-batch fermenter, 5% inoculum size, pH was controlled at 4.0, agitation speed at 500 rpm and operation temperature at 40 °C. Glucose was added at 28 h after fermentation; ⊟ glucose concentration (g/L), ◇ ethanol concentration (g/L) and △ DCM (g/L).

4.10.2 Fed-batch B

From the results of fed-batch A, the ethanol production was increased by adding glucose syrup and all of glucose was utilized within 66 h after fermentation. The ethanol production could increase by increasing of glucose. Therefore, the ethanol production was expected to produce at higher concentration when adding more glucose syrup. The second fed-batch was operated by adding glucose syrup more than fed-batch A called "fed-batch B". The first addition, 350 g glucose (1 L of volume) was added at the same time of fed-batch A (28 h after fermentation). And then, the second addition, 400 g glucose (1 L of volume) was added before glucose depletion (52 h after fermentation). The results of fed-batch B were shown in Figure 4.13. The ethanol production of fed-batch B was very low after the second glucose added may be because of low activity of old cell, and yeast cell reached to stationary phase.

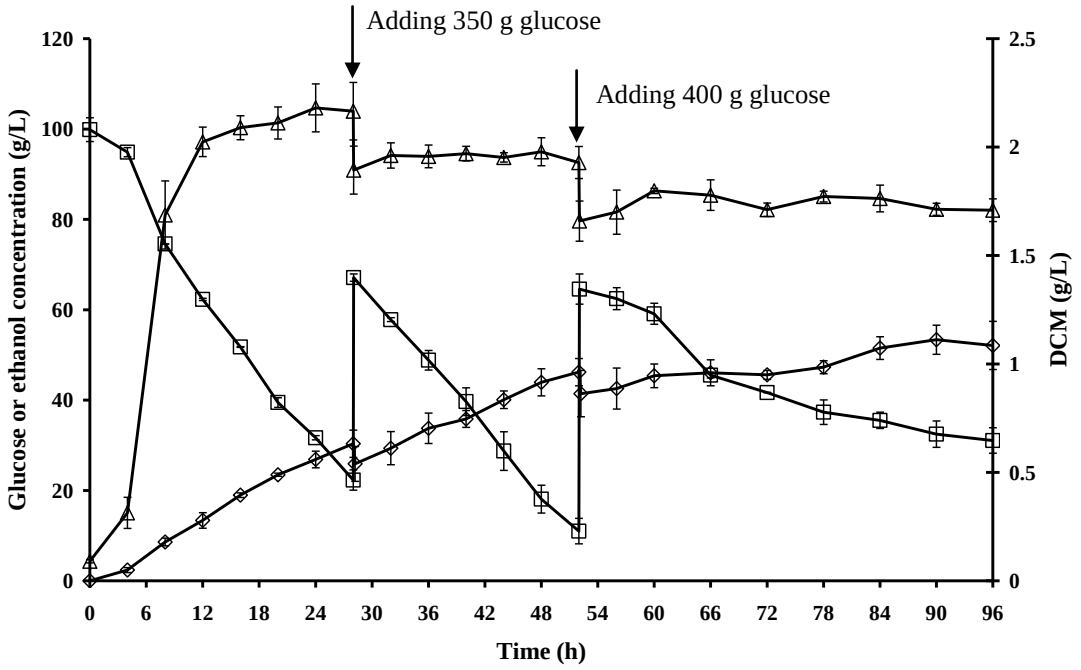
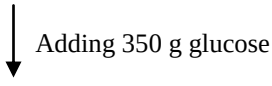


Figure 4.13 Cell growth, glucose utilization and ethanol production by *Issatchenkia* sp. S1 cultured in YM medium with 100 g/L glucose and 2 g/L $(NH_4)_2SO_4$ in 10 L fed-batch fermenter, 5% inoculum size, pH controlled at 4.0, agitation speed at 500 rpm and operation temperature at 40 °C. A litter of 350 g and 400 g glucose were added at 28 h and 52 h after fermentation, respectively; ⊟ glucose concentration (g/L), ◇ ethanol concentration (g/L) and △ DCM (g/L).

4.10.3 Fed-batch C

For improving of ethanol production, addition of glucose was changed the adding time from 28 h after fermentation (Figure 4.12) to exponential phase of yeast growth. This fed-batch call "fed-batch C". The fed-batch C was added 350 g glucose (1 L of volume) at 12 h after fermentation. The results of growth, glucose consumption and ethanol production of fed- batch C showed in Figure 4.14. The

ethanol productivity of fed-batch C (1.33 ± 0.069 g/L/h) was higher than those of fed-batch A and fed-batch B (1.28 ± 0.04; 1.04 ± 0.12 g/L/h, respectively) (Table 4.8). The addition of glucose at exponential phase could enhance the ethanol production.



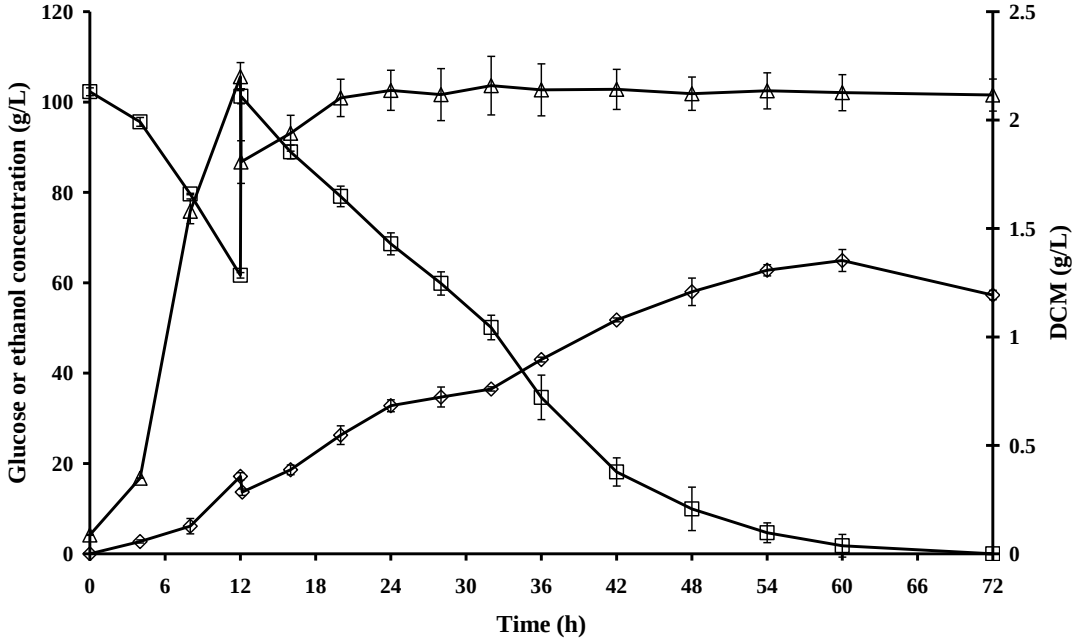


Figure 4.14 Cell growth, glucose utilization and ethanol production by *Issatchenkia* sp. S1 cultured in YM medium with 100 g/L glucose and 2 g/L $(NH_4)_2SO_4$ in 10 L fed-batch fermenter, 5% inoculum size, pH controlled at 4.0, agitation speed at 500 rpm and operation temperature at 40 °C. A litter of 350 g glucose was added at 12 h after fermentation; ⊟ glucose concentration (g/L), ◇ ethanol concentration (g/L) and △ DCM (g/L).

4.10.4 Fed-batch D

From results of fed-batch C, glucose was utilized rapidly after adding of glucose syrup at 12 h after fermentation. For increasing the ethanol production, the addition of glucose was added 2 times between exponential to early stationary phase of yeast growth. This fed-batch call "fed-batch D". The first addition was added at the same time of fed-batch C. After that 400 g glucose (1 L of volume) was added at

24 h after fermentation. The results of fed-batch D are showed in Figure 4.15. Ethanol concentration in fed-batch was highest concentration of 77.81 ± 1.88 g/L among all studied fed-batch types. Moreover, fed-batch showed the high productivity and high yield of ethanol (Table 4.8).

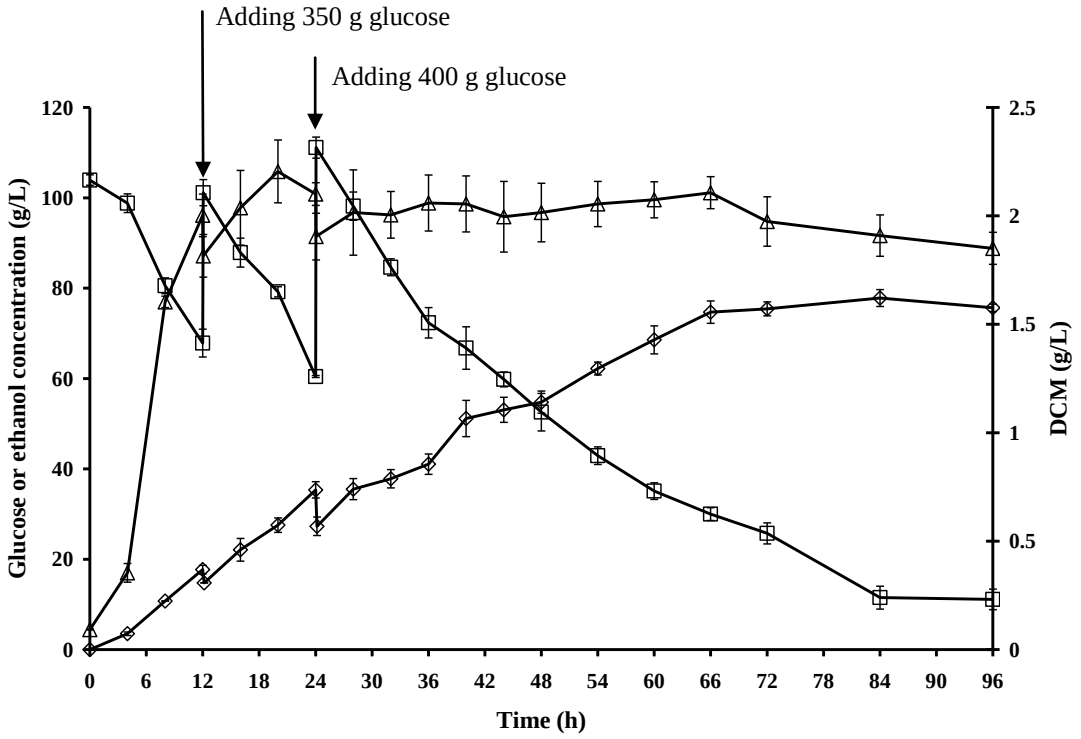


Figure 4.15 Cell growth, glucose utilization and ethanol production by *Issatchenkia* sp. S1 cultured in YM medium with 100 g/L glucose and 2 g/L $(NH_4)_2SO_4$ in 10 L fed-batch fermenter, 5% inoculum size, pH controlled at 4.0, agitation speed at 500 rpm and operation temperature at 40 °C. A litter of 350 g/L and 1 L of 400 g/L glucose were added at 12 h and 24 h after fermentation, respectively; ⊟ glucose concentration (g/L), ◇ ethanol concentration (g/L) and △ DCM (g/L).

Table 4.8 Ethanol yield (g/g), productivity (g/L/h) and specific growth rate of *Issatchenkia* sp. S1 cultivated in YM medium with 100 g/L glucose and 2 g/L $(NH_4)_2SO_4$ in 10 L fermenter in batch and fed-batch operation, agitation speed at 500 rpm, 5% inoculum size, pH at 4.0, and temperature at 40 °C (means of two replicates, ± standard error).

Type	specific growth rate (1/h)	Volumetric Productivity (g/L/h)	Ethanol yield (g/g)
Batch	0.355 ± 0.012^{a}	1.335 ± 0.104^{f}	0.431 ± 0.005^{bcd}
Fed-batch A			
I. 0-28 h	0.371 ± 0.016^{a}	1.283 ± 0.040^{ef}	0.458 ± 0.018^{cd}
II. 28-72 h		0.914 ± 0.116^{bc}	0.508 ± 0.001^{d}
Fed-batch B			
I. 0-28 h	0.366 ± 0.001^{a}	1.084 ± 0.108^{cd}	0.393 ± 0.063^{abc}
II. 28-52 h		0.846 ± 0.035^{b}	0.324 ± 0.033^{a}
III. 52-96 h		0.314 ± 0.049^{a}	0.360 ± 0.079^{ab}
Fed-batch C			
I. 0-12 h	0.363 ± 0.002^{a}	1.427 ± 0.068^{f}	0.412 ± 0.009^{bc}
II. 12-72 h		1.068 ± 0.036^{cd}	0.507 ± 0.010^{d}
Fed-batch D			
I. 0-12 h	0.357 ± 0.005^{a}	1.474 ± 0.079^{f}	0.490 ± 0.001^{d}
II. 12-24 h		1.716 ± 0.150^{g}	0.506 ± 0.011^{d}
III. 24-96 h		1.132 ± 0.005^{de}	0.507 ± 0.001^{d}

Values followed by the same letter are not significantly different at $P \leq 0.05$.

4.11 Some organic acid produced by thermotolerant *Issatchenkia* sp. S1

As indicated in Figure 2.5, 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, acetic acid, succinic acid, oxalic acid and oxaloacetic acid by using HPLC technique (Figure 4.16a) in 10 L batch fermentation. The major peaks were found at the retention time of 9.15 and 12.57 minutes (Figure 4.16b). The retention time of 9.15 minutes was some component in medium. It was not metabolized or increased during fermentation. However, the retention time of 12.80 minutes represents was constituted until 30 h after operation as showed in Figure 4.16c. At 30 h after operation of 10 L batch fermentation, glucose concentration in the YM medium added 2 g/L $(NH_4)_2SO_4$ came to depletion (Figure 4.17). Therefore, after 30 h of operation in 10 L batch fermenter, oxaloacetic acid was decreased might be because S1 was utilized oxaloacetic acid as carbon source for cell maintenance due to the depletion of glucose in the medium (Figure 4.17).

In anaerobic conditions, oxaloacetate is the means of entry of pyruvate into the cytosolic citric acid. Although the mitochondria are no longer functional, the enzymes of the tricarboxylic acids cycle are present in the cytoplasm. Pyruvate carboxylase (PC) catalyzes the carboxylation of pyruvate into oxaloacetate. The prosthetic group of this enzyme is biotin; it serves as a CO_2 transporter. The reaction makes use of ATP molecule (Ribéreau-Gayon *et al*, 2000):

$$\text{biotin-PC} + \text{ATP} + CO_2 \rightleftharpoons CO_2\text{-biotin-PC} + \text{ADP} + [\text{Pi}] \quad \text{(a)}$$

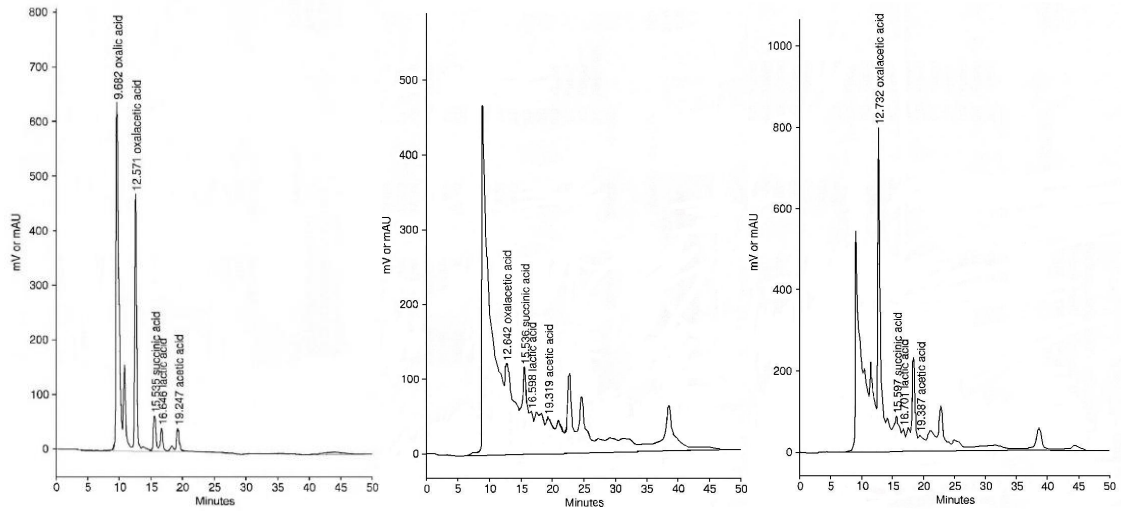
$$CO_2\text{-biotin-PC} + \text{pyruvate} \rightleftharpoons \text{biotin-PC} + \text{oxaloacetate} \quad \text{(b)}$$

Or (Mukhopadhyay *et al*, 2001; Devlin, 2006):

$$\text{Pyruvate} + HCO_3^- + \text{ATP} \xrightleftharpoons{\text{PC}} \text{oxaloacetate} + \text{ADP} + \text{Pi} \qquad \text{(c)}$$

Another reaction for synthesis of oxaloacetate, it was synthesized from phosphoenolpyruvate by phosphoenolpyruvate carboxylase (PPC) (Mukhopadhyay *et al*, 2001):

$$\text{Phosphoenolpyruvate} + HCO_3^- \xrightleftharpoons{\text{PPC}} \text{Oxaloacetate} + \text{Pi} \qquad \text{(d)}$$



(a) (b) (c)

Figure 4.16 Organic acid peak detection by HPLC in 10 L batch fermenter performed by *Issatchenkia* sp. S1 in YM medium with 100 g/L glucose and 2 g/L $(NH_4)_2SO_4$ in 10 L batch fermenter, 5% inoculum size, pH was controlled at 4.0, agitation speed at 500 rpm and operation temperature at 40 $^{\circ}$C; (a) peak detection of 2 g/L of oxalic acid, oxaloacetic acid, succinic acid, lactic acid and acetic acid, (b) peak detection of fermentation broth at start fermentation and (c) peak detection of fermentation broth at 30 h after fermentation.

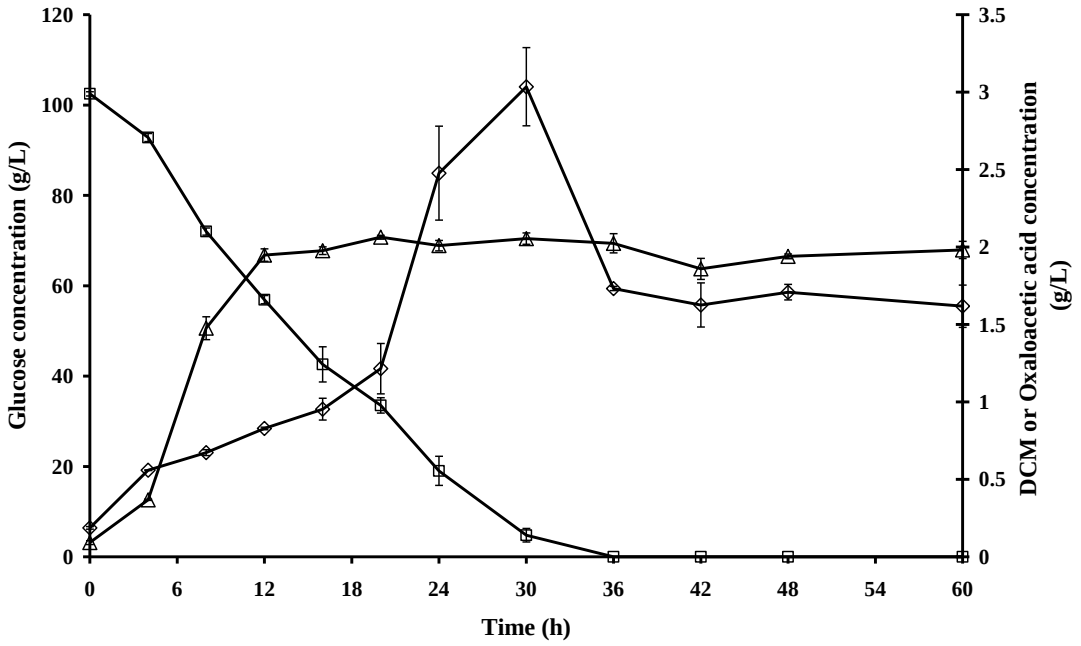


Figure 4.17 Cell growth, glucose concentration and oxaloacetic acid formation by *Issatchenkia* sp. S1 cultured in YM medium with 100 g/L glucose and 2 g/L $(NH_4)_2SO_4$ in 10 L batch fermenter, 5% inoculum size, pH was controlled at 4.0, agitation speed at 500 rpm and operation temperature at 40 $^{\circ}$C; ⊟ glucose concentration (g/L), ◇ oxaloacetic acid concentration (g/L) and △ DCM (g/L).

CHAPTER V

CONCLUSION

Issatchenkia sp. S1 shows high capability of glucose and fructose utilization as carbon sources in YM medium but weak in sucrose, lactose, glycerol, manital, maltose, cassava starch and potato starch. Under static condition, glucose concentration for growth and ethanol production are 50 g/L (μ = 0.221 h^{-1}, Y_{ps} = 0.397 g/g and Q_p = 0.410 g/L/h), and fructose showed the highest growth and ethanol production at 100 g/L (μ = 0.202 h^{-1}, Y_{ps} = 0.477 g/g and Q_p = 0.563 g/L/h). The shaking effect had dramatically affected on growth, sugar consumption and ethanol production. The concentrations of glucose and fructose were optimized again under shaking condition. *Issatchenkia* sp. S1 was cultured in YM medium with glucose under shaking condition showed the highest growth and ethanol production at 100 g/L (μ = 0.352 h^{-1}, Y_{ps} = 0.464 g/g and Q_p = 1.189 g/L/h), and in fructose at 100 g/L showed the highest growth and ethanol production (μ = 0.526 h^{-1}, Y_{ps} = 0.506 g/g and Q_p = 1.168 g/L/h). When 2 g/L $(NH_4)_2SO_4$ was added in YM medium with 100 g/L glucose, Q_p was increased 1.4 time (2.328 g/L/h).

Results of growth, glucose consumption and ethanol production were observed in 2 L fermenter. *Issatchenkia* sp. S1 cultured in YM medium with 100 g/L glucose and 2 g/L $(NH_4)_2SO_4$ was conducted. The yeast showed high growth and cell concentration when cultured under aerotion at 1.0 vvm (μ =0.482 h^{-1}). Yeast growth was decreased when decreased the aeration rate. In contrast, ethanol concentration

and ethanol yield were increased when decreased the aeration rate. *Issatchenkia* sp. S1 cultured in 2 L fermenter with no aeration showed the highest ethanol yield (0.437 g/g).

Agitation rate could stimulate growth and ethanol production. Agitation rate at 500 rpm was shown that the highest values of μ, Q_p and Y_{ps} were 0.37 h^{-1}, 1.886 g/L/h and 0.516 g/g, respectively.

Issatchenkia sp. S1 was cultured in 6 L of 10 L fermenter using YM medium with 100 g/L glucose and 2 g/L $(NH_4)_2SO_4$ at 500 rpm of agitation. The scale up affect on growth and ethanol production, which the μ, Q_p and Y_{ps} were decreased from 0.370 h^{-1}, 1.886 g/L/h and 0.516 g/g to 0.355 h^{-1}, 1.335 g/L/h and 0.431 g/g, respectively. The ethanol production in 10 L fermenter was improved by fed-batch operation. The best result of fed-batch operation was fed-batch D. The ethanol concentration was up to 77.8 ± 1.88 g/L (about 1.83 times higher that of in 10 L batch fermenter) within 72 h (Q_p = 1.132 - 1.716 g/L/h, and Y_{ps} = 0.490 - 0.507 g/g).

Furthermore, the oxaloacetic acid produced as by-product of *Issatchenkia* sp. S1 was investigated in 10 L batch fermenter. The production of oxaloacetic acid was increased following with the production of ethanol. After glucose came to depletion, some of oxaloacetic acid was used as carbon source for cell maintenance. The ethanol production by *Issatchenkia* sp. S1 in enrich medium are interesting because it produced high amount of ethanol and other compounds that might be involved in some metabolisms of this yeast during ethanol fermentation. Especially, the production of oxaloacetic acid is very interesting to study in the future for using as substrate for polymerization reaction to make a new product of bioplastic or biopolymer.

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APPENDIX

1. Dry cell mass equation calculation

Cell mass concentrations were also calculated from OD measurements using a correlation factor developed between gravimetric dry cell mass (g/L) and OD (absorbance units), as shown in Figure 3.2. This factor was obtained as the slope of the linear correlation (DCM = 0.435 OD + 0.0058; R^2 = 0.99) between the cell mass concentration and absorbance at 660 nm developed using whole broth samples collected during the course of several fermentations.

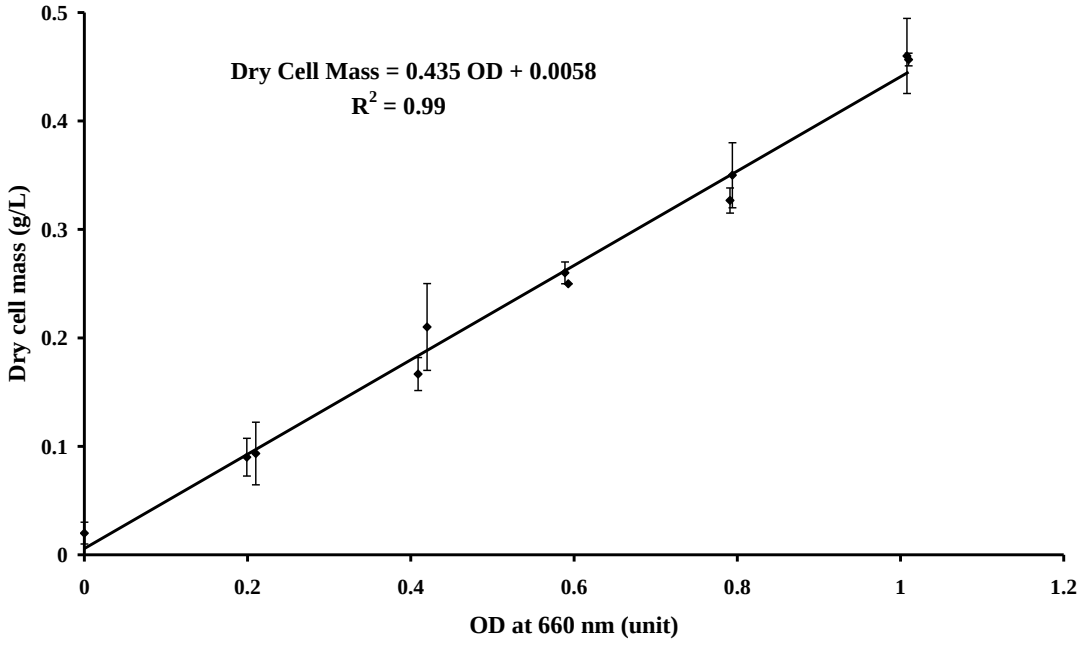

Figure A Linear correlation between DCM and OD at 660 nm of *Issatchenkia* sp. S1 cultured in YM medium with 20 g/L glucose at 40 °C.

2. Determination of organic carbon, organic matter and total nitrogen in fermentation medium and their compositions

Fermentation medium (YM medium with 100 g/L glucose and 2 g/L $(NH_4)_2SO_4$), yeast extract, malt extract and meat peptone was used for determined organic carbon, organic matter and total nitrogen. Determination of organic carbon and organic matter was used titration method (Black, 1965). And total nitrogen was determined by Kjeldahl method (Kjeldahl, 1883). The quantity of organic carbon, organic matter and total nitrogen showed in Table A.

Table A Organic matter, organic carbon and total nitrogen in fermentation medium and their composition.

Source	Organic matter (%)	Organic carbon (%)	Organic nitrogen (%)
Fermentation medium	8.75 ± 0.04	5.09 ± 0.02	0.13 ± 0.01
Yeast extract	62.38 ± 0.95	36.27 ± 0.55	7.83 ± 0.00
Malt extract	81.18 ± 6.29	47.20 ± 3.66	1.34 ± 0.05
Meat peptone	70.10 ± 0.47	40.76 ± 0.28	10.37 ± 0.06

3. Evaporation of ethanol

Ethanol was diluted in water at various concentrations. After 24 h of shaking (200 rpm) in 250 mL Erlenmeyer flask (150 mL of diluted ethanol) at 40 $^{\circ}$C, the ethanol concentrations were determined compared at the beginning concentrations. The results showed in Table B.

Table B. The evaporation of ethanol in shaking condition (200 rpm) at 40 °C.

Trail	Ethanol concentration (g/L)		Ethanol loss (%)
	t = 0 h	t = 24 h	
1	18.814 ± 0.302	18.046 ± 0.057	4.072 ± 1.846
2	38.142 ± 0.152	36.976 ± 0.156	3.054 ± 0.795
3	58.527 ± 0.045	56.154 ± 0.257	4.055 ± 0.366
4	77.680 ± 0.116	75.638 ± 0.241	2.628 ± 0.165

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BIOGRAPHY

Tapanawat Muaenjang was born in Satun, Thailand. In 2000, he studied in Department of General Science, Faculty of Science, Prince of Songkla University, Songkla. He graduated the Bachelor's of Science in General Science in 2004. After graduation, in 2004, he was Master's student in School of Biotechnology at Suranaree University of Technology. During Master's student, he had an experience on poster presentation in title "The study of ethanol production by thermotolerant yeast S1 strain" at the 11th Biological Science Graduated Congress, December 15-17, 2006, Faculty of science, Chulalongkorn University, Thailand.