Bioethanol Production Using Raw Glycerol from Biodiesel Process

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ชื่อวิทยานิพนธ์ การผลิตเอทานอลโดยใช้กลีเซอรอลดิบจากกระบวนการผลิตไบโอดีเซล

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บทคัดย่อ

เนื่องจากปัญหาราคาน้ำมันปิโตรเลียมและปัญหาสิ่งแวดล้อมที่เพิ่มขึ้น ส่งผลให้มีการวิจัยและพัฒนาการใช้พลังงานทดแทนซึ่งรวมถึงน้ำมันไบโอดีเซลที่นำมาใช้ทดแทนน้ำมันดีเซล จากกระบวนการผลิตไบโอดีเซลจะมีกลีเซอรอลเป็นวัสดุเศษเหลือเกิดขึ้นในปริมาณร้อยละ 10 (น้ำหนักต่อปริมาตรของน้ำมันพืชที่ใช้) งานวิจัยนี้ศึกษาการใช้กลีเซอรอลดิบจากกระบวนการผลิตไบโอดีเซลเพื่อเป็นทรัพยากรทางเลือกสำหรับการผลิตเอทานอล ในบรรดาแบคทีเรีย 5 สายพันธุ์ที่ทดสอบเพื่อคัดเลือกสายพันธุ์ที่ผลิตเอทานอลได้สูงสุด พบว่า *Enterobacter aerogenes* TISTR1468 ผลิตเอทานอลจากกลีเซอรอลที่ความเข้มข้น 20 กรัมต่อลิตร สูงกว่า *Klebsiella oxytoca* TISTR556, *Klebsiella terrigena* SU3, *Klebsiella pneumoniae* SU32 และ*Citrobacter freundii* SU17 ตามลำดับ โดยให้ค่าเอทานอลสูงสุดเท่ากับ 7.24 กรัมต่อลิตรจากการใช้กลีเซอรอลดิบเปรียบเทียบกับ 7.54 กรัมต่อลิตร จากกลีเซอรอลบริสุทธิ์ (ชุดควบคุม) แสดงว่าเชื้อใช้กลีเซอรอลดิบได้อย่างมีประสิทธิภาพ ดังนั้น จึงคัดเลือก *E. aerogenes* TISTR1468 สำหรับการทดลองต่อไป

จากการศึกษาหาค่าที่เหมาะสมแบบดั้งเดิม โดยศึกษาหาสูตรอาหารเลี้ยงเชื้อที่เหมาะสมต่อการผลิตเอทานอลของ *E. aerogenes* TISTR1468 ที่อุณหภูมิ 37 องศาเซลเซียส โดยเลี้ยงเชื้อบนเครื่องเขย่า (อัตรา 120 รอบต่อนาที) พบว่า สูตรอาหารเลี้ยงเชื้อที่เหมาะสมประกอบด้วย กลีเซอรอลดิบ 30 กรัมต่อลิตร แอมโมเนียมฟอสเฟต 2.0 กรัมต่อลิตร และยีสต์สกัด 5.0 กรัมต่อลิตร ผลิตเอทานอลได้สูงสุด 14.53 กรัมต่อลิตร ผลผลิตของผลิตภัณฑ์ 0.478 กรัมต่อกรัม และอัตราการผลิตเอทานอล 0.73 กรัมต่อลิตรต่อชั่วโมง เมื่อใช้หลักการพื้นผิวตอบสนอง (RSM) เพื่อออกแบบการทดลองสำหรับทำนายและหาสภาวะที่เหมาะสมเพื่อเพิ่มการผลิตเอทานอล พบว่า ปัจจัยที่เหมาะสม คือ กลีเซอรอลดิบ 38.28 กรัมต่อลิตร แอมโมเนียมฟอสเฟต 2.1 กรัมต่อลิตร และ ยีสต์สกัด 7.2 กรัมต่อลิตร โดยให้การผลิตเอทานอลสูงสุด 16.19 กรัมต่อลิตร ผลผลิตของผลิตภัณฑ์ 0.670 กรัมต่อกรัม และอัตราการผลิตเอทานอล 0.80 กรัมต่อลิตรต่อชั่วโมง เมื่อเปรียบเทียบผลที่ได้กับการใช้สูตรอาหารเลี้ยงเชื้อเริ่มต้น พบว่า การผลิตเอทานอลและอัตราการผลิตเพิ่มขึ้น 2.24 และ 1.50 เท่า ตามลำดับ หลังจากนั้นศึกษาสภาวะแวดล้อมที่เหมาะสมต่อการผลิตในถังปฏิกรณ์ขนาด 3

ลิตร โดยควบคุมอุณหภูมิการเลี้ยงที่ 37 องศาเซลเซียส พบว่าสภาวะแวดล้อมที่เหมาะสมได้แก่ พีเอชเริ่มต้นที่ 8.0 โดยไม่มีการควบคุมพีเอชระหว่างการหมัก อัตราการให้อากาศที่ 0.5 ลิตรอากาศต่อลิตรอาหารต่อนาที และอัตราการกวนที่ 60 รอบต่อนาที โดยให้การผลิตเอทานอลได้สูงสุด 14.0 กรัมต่อลิตร ผลผลิตของผลิตภัณฑ์ 0.60 กรัมต่อกรัม อัตราการผลิตเอทานอล 0.70 กรัมต่อลิตรต่อชั่วโมง และอัตราการใช้กลีเซอรอล 1.21 กรัมต่อลิตรต่อชั่วโมง

เมื่อเปรียบเทียบการผลิตเอทานอลภายใต้สภาวะที่เหมาะสม โดยผลิตในรูปแบบการหมักแบบกะ แบบกึ่งกะ แบบต่อเนื่อง และแบบสองขั้นตอน (สภาวะมีอากาศเล็กน้อยและสภาวะไร้อากาศ) เมื่อศึกษาผลของความเข้มข้นของกลีเซอรอลเริ่มต้นในการหมักแบบกะ พบว่าเมื่อความเข้มข้นของกลีเซอรอลเริ่มต้นเพิ่มขึ้นทำให้อัตราการผลิตเอทานอลเพิ่มขึ้นและมีค่าลดลงเมื่อกลีเซอรอลมีค่ามากกว่า 20 กรัมต่อลิตร มีการพัฒนาแบบจำลองทางคณิตศาสตร์เพื่ออธิบายอัตราการใช้สารตั้งต้น (กลีเซอรอล) และการยับยั้งโดยสารตั้งต้น พบว่าอัตราการเจริญจำเพาะสูงสุดของเชื้อ *E. aerogenes* TISTR1468 เท่ากับ 0.708 ต่อชั่วโมง ค่าคงที่อิ่มตัวของสารตั้งต้น (K_s) เท่ากับ 6 กรัมต่อลิตร และค่าคงที่ของการยับยั้ง (K_I) เท่ากับ 57 กรัมต่อลิตร กลีเซอรอลเริ่มต้นที่ความเข้มข้น 50 กรัมต่อลิตร เชื้อผลิตเอทานอลสูงสุดเท่ากับ 22.97 กรัมต่อลิตร อัตราการผลิตเอทานอลเท่ากับ 1.0 กรัมต่อลิตรต่อชั่วโมง และผลผลิตของผลิตภัณฑ์เท่ากับ 0.52 กรัมต่อกรัม อัตราการใช้สารตั้งต้นสูงสุดเท่ากับ 2.34 กรัมต่อลิตรต่อชั่วโมง จากการนำแบบจำลองที่ได้มาทดลองเพื่อยืนยันผลพบว่าค่าที่ได้จากการทดลองและค่าที่ได้จากแบบจำลองมีค่าใกล้เคียงกัน เมื่อศึกษาการผลิตเอทานอลแบบกึ่งกะ เพื่อลดผลการยับยั้งของสารตั้งต้น โดยเปรียบเทียบรูปแบบการเติมสารอาหาร 2 รูปแบบ พบว่าการเติมสารอาหารแบบครั้งคราวที่มีการถ่ายอาหารออกและเติมใหม่เพื่อให้ปริมาตรในถังปฏิกรณ์คงที่ ให้การผลิตเอทานอลสูงสุดเท่ากับ 19.97 กรัมต่อลิตร และการเติมแบบครั้งคราวโดยปริมาตรของอาหารเพิ่มขึ้น ได้เอทานอลสูงสุดเท่ากับ 19.21 กรัมต่อลิตร เมื่อศึกษาผลของระยะเวลากักเก็บสาร (HRT) ต่อการผลิตเอทานอลจากการหมักแบบต่อเนื่อง พบว่าระยะเวลากักเก็บสารที่ 30 ชั่วโมง ให้การผลิตเอทานอลสูงสุด 15 กรัมต่อลิตร การผลิตเอทานอลแบบสองขั้นตอนให้การผลิตเอทานอลสูงกว่าการผลิตในขั้นตอนเดียวภายใต้สภาวะมีอากาศเล็กน้อยและไร้อากาศ การผลิตเอทานอลแบบสองขั้นตอนให้การผลิตเอทานอลสูงสุด 24.5 กรัมต่อลิตร และอัตราการผลิต 0.68 กรัมต่อลิตรต่อชั่วโมง ดังนั้น การผลิตเอทานอลแบบสองขั้นตอนจึงให้การผลิตเอทานอลได้ดีกว่าการผลิตแบบกะ แบบกึ่งกะ และแบบต่อเนื่อง เมื่อผลิตเอทานอลในถังหมักขนาด 20 ลิตร (ปริมาตรเลี้ยงเชื้อ 15 ลิตร) โดยใช้สภาวะที่เหมาะสมเช่นเดียวกับการผลิตในระดับห้องปฏิบัติการ (ขนาด 3 ลิตร) พบว่าจุลินทรีย์มีรูปแบบการเจริญและการผลิตเอทานอลที่คล้ายคลึงกันทั้งในสภาวะมีอากาศเล็กน้อย, สภาวะไร้อากาศ และการผลิตแบบสองขั้นตอน นอกจากนี้ การ

ผลิตแบบสองขั้นตอนให้การเจริญสูงสุด (3.14 กรัมน้ำหนักเซลล์แห้งต่อลิตรที่เวลา 30 ชั่วโมง) รองลงไปคือ ภายใต้สภาวะมีอากาศเล็กน้อยและสภาวะไร้อากาศ ให้การเจริญสูงสุด 2.82 กรัมต่อลิตร ที่เวลา 24 ชั่วโมง และ 1.17 กรัมต่อลิตร ที่เวลา 42 ชั่วโมง ตามลำดับ การผลิตแบบสองขั้นตอนให้การผลิตเอทานอลสูงสุด 18.84 กรัมต่อลิตร โดยให้ผลผลิตของจุลินทรีย์และผลิตภัณฑ์ 0.83 กรัมเซลล์ต่อกรัมกลีเซอรอลที่ใช้ไป และ 0.45 กรัมเอทานอลต่อกรัมกลีเซอรอลตามลำดับ เมื่อศึกษาการผลิตเอทานอลโดยใช้เซลล์อิสระและเซลล์ที่ถูกตรึงพบว่า การตรึงเซลล์โดยใช้ฟองน้ำเป็นตัวตรึงให้การผลิตเอทานอลสูงสุด (5.88 กรัมต่อลิตร) และสามารถนำเซลล์ตรึงกลับมาใช้ซ้ำได้มากกว่า 10 ครั้ง นอกจากนี้เซลล์ตรึงสามารถทนต่อความเข้มข้นของเอทานอลที่ความเข้มข้นสูงได้ดีกว่าเซลล์อิสระ จากการศึกษาครั้งนี้แสดงให้เห็นว่ากลีเซอรอลดิบที่ได้จากกระบวนการผลิตไบโอดีเซลสามารถนำมาใช้เป็นสารตั้งต้นสำหรับการผลิตเอทานอลโดยใช้เซลล์อิสระและเซลล์ที่ถูกตรึงของ *Enterobacter aerogenes* TISTR1468

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ABSTRACT

The increase in petroleum price and environmental problem resulted in research and development on renewable energy and biodiesel is being substituted for diesel oil. The main by–product of biodiesel production is the crude glycerol, which is about 10 (%w/v) of vegetable oil. This research aims to use raw glycerol from biodiesel production process as an alternative resource for ethanol production from bacteria. Among five bacterial strains tested, *Enterobacter aerogenes* TISTR1468 was selected as the strain exhibited higher ethanol production from 20 g/L raw and pure glycerol than those from *Klebsiella oxytoca* TISTR556, *Klebsiella terrigena* SU3, *Klebsiella pneumoniae* SU32 and *Citrobacter freundii* SU17, respectively. The highest ethanol concentration was 7.24 g/L using raw glycerol compared to 7.54 g/L using pure glycerol (the control). This indicated that the strain could use raw glycerol efficiently. Therefore, *E. aerogenes* TISTR1468 was selected for further studies.

Medium optimization studies for ethanol production from *E. aerogenes* TISTR1468 was conducted at 37 °C on a shaker (120 rpm shaking speed) using conventional method. The optimum medium contained 30 g/L raw glycerol, 2.0 g/L $(NH_4)_2HPO_4$ and 5.0 g/L yeast extract. Under this condition, the highest ethanol production, yield and productivity increased to 14.53 g/L, 0.478 g/g and 0.73 g/L/h, respectively. Response surface methodology (RSM) was used to design experiment for prediction and optimization to enhance ethanol production. The optimum parameters were 38.28 g/L raw glycerol, 2.10 g/L $(NH_4)_2HPO_4$ and 7.20 g/L yeast extract, giving the highest ethanol concentration, yield and productivity of 16.19 g/L, 0.670 g/g and 0.80 g/L/h, respectively, By comparing the results obtained under the optimal condition with that of the original medium, it was found that the ethanol

production and productivity increased 2.24 and 1.50 folds, respectively. Optimization on environmental factors was carried out in a 3 L-fermenter at 37 °C. The optimum conditions were the initial pH at 8.0 without controlled-pH during cultivation, 0.5 vvm aeration rate and 60 rpm agitation speed. It gave the highest values of 14.0 g/L ethanol, 0.60 g/g yield, 0.70 g/L/h productivity and 1.21 g/L/h glycerol consumption rate.

The optimum condition was used to enhance ethanol production in batch, fed-batch, continuous and two-stage (combination of micro-aerobic and anaerobic) fermentation. In batch process, the effect of initial glycerol concentration on the rate of ethanol formation was studied. The rate of ethanol formation increased with the increase of glycerol concentration up to 20 g/L and decreased thereafter. A kinetic model describing the rate of substrate utilization and inhibition was developed. The maximum specific growth rate (μ_m) was 0.708 h^{-1} with the substrate saturation constant (K_s) of 6 g/L and the substrate inhibition constant (K_I) of 57 g/L. Highest ethanol production of 22.97 g/L was achieved at 50 g/L raw glycerol. The maximum value of substrate consumption rate was 2.34 $g_{glycerol}$/L/h whereas ethanol yield and ethanol production rate were 0.52 $g_{ethanol}/g_{glycerol}$ and 1.00 $g_{ethanol}$/L/h, respectively. The results of the model simulations showed good agreement with the experimental data obtained at varying initial glycerol concentrations. To overcome substrate inhibition, the ethanol was produced under fed-batch. Two feeding strategies were tested, the fixed volume and variable volume intermittent fed-batch fermentation gave the maximum ethanol production of 19.97 and 19.21 g/L, respectively. Under continuous process, hydraulic retention time (HRT) was varied and HRT of 30 h gave the highest ethanol production of 15 g/L. For two-stage process, ethanol production was higher than the one-stage process with the highest ethanol concentration of 24.5 g/L and productivity of 0.68 g/L/h. This strategy gave better results for ethanol production than batch, fed-batch and continuous fermentation. The optimum condition from bench scale (3 L fermenter) was used in larger scale (20 L fermenter with 15 L working volume). The cell growth and ethanol production in 3 L and 20 L reactors exhibited similar trend under anaerobic, micro-aerobic and two-stage fermentation. Moreover, two-stage fermentation in 20 L fermentation gave the highest biomass production (3.14 g DCW/L at 30 h), followed by under micro-aerobic and

anaerobic condition, giving the maximum cell concentration of 2.82 g/L at 24 h and 1.17 g/L at 42 h, respectively. For ethanol production, two-stage condition gave the highest ethanol production of 18.84 g/L, in which the cell yield was 0.83 g/g and ethanol yield of 0.45 g/g, respectively. Immobilized cells for ethanol production were compared with free cells. Sponge was found to be the best supporting material for cell immobilization. It gave the highest ethanol production (5.88 g/L) and could be reused more than ten times. In addition, the immobilized cells could tolerate higher ethanol concentration than the free cells. Therefore, crude glycerol from the biodiesel production could be used directly to produce ethanol by free and immobilized cells of *Enterobacter aerogenes* TISTR1468.

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Kanokrat Saisa-ard

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LIST OF ABBREVIATIONS AND SYMBOLS

TSA	=	Trypticasein soy agar
DCW	=	Dry cell weight
°C	=	Degree celcious
OD	=	Optical density
nm	=	Nanometer
L	=	Liter
mL	=	Milliliter
g	=	Gram
g/L	=	Gram per liter
g/L/h	=	Gram per liter per hour
h	=	Hour
g/g	=	Gram per gram
w/w	=	Weight by weight
rpm	=	Revolutions per minute
vvm	=	volume per volume per minute
RSM	=	Response surface methodology
CCD	=	Centered Composite Design
ΔS	=	Amount of substrate
ΔP	=	Amount of product
ΔX	=	Amount of cell
X	=	Cell concentration
X_0	=	Initial cell concentration
μ	=	Specific growth rate
μ_{max}	=	Maximum specific growth rate
ρ	=	Specific production rate
K_S	=	Saturation constant

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

K_I	=	Substrate inhibition constant
$Y_{P/S}$	=	Ethanol yield
$Y_{X/S}$	=	Cell yield
HRT	=	Hydraulic retention time

CHAPTER 1

INTRODUCTION

Introduction

Due to environmental concerns and decreasing fuel reserves, biodiesel is being increasingly used as substitute for diesel oil (Jitwung and Yargeau, 2011) as well as bioethanol. Biodiesel and bioethanol have various advantages such as an alternative to petroleum-based fuel, renewable fuel, a favorable energy balance, lower harmful emission and nontoxic fuel. Because of these environmental advantages, biodiesel and bioethanol can be expected as a substitute for conventional fuel. The Thai government has planned to increase the national renewable energy share from 0.5% presently to 8% by the year 2011 (Sailasuta, 2005). Thai government's biodiesel development strategy is to replace 10% of pretodiesel in transport sector by biodiesel by 2012. The plan is to increase the use of biodiesel from 365 million liters in the 2007 to 3100 million liters by 2012 (Sajjakulnukit, 2005)

Biodiesel produced by chemical and enzymatic method generate glycerol as the by-product (Ito *et al*., 2005). The rapid development of biodiesel synthesis in recent years has produced a tremendous amount of byproduct, glycerol, which is about 10 %w/v of vegetable oil (Dasari *et al*., 2005; Wu *et al*., 2011; Oh *et al*., 2012). This surplus of raw glycerol has deleteriously affected the traditional market for glycerol and has created a significant environmental problem because glycerol cannot be discharged directly into the environment without treatment (da Silva *et al*., 2009). One of the promising applications of glycerol is the production of valuable products such as 1,3-propanediol and 2,3-butanediol (Sattayasamitsathit *et al*., 2010), ethanol, hydrogen, succinic acid, 1,2-propanediol, dihydroxyacetones, and polyglycerols (Pachauri and He, 2006) by the group of bacteria including *Bacillus*, *Clostridium*, *Enterobacter*, *Klebsiella* and *Lactobacillus* species (Biebl *et al*., 1998; Deckwer, 1995; Zheng *et al*., 2008). Ethanol and hydrogen from crude glycerol could be produced by *Enterobacter aerogenes* HU-101 (Ito *et al*., 2005). Besides ethanol, formic acid is also produced during glycerol fermentation by mixed cultures (Temudo

et al., 2008). A mutant strain of *Klebsiella pnemoniae* GEM167 could produce higher ethanol concentration (21.5 g/L) than the wild type strain using raw glycerol as a substrate (Oh *et al*., 2011).

Conversion of the low-price crude glycerol to higher value products could increase the economic viability for the biofuel industry. The cost of ethanol production from glycerol is almost 40% less than that of production from corn-derived sugar, when both feedstock demand and operational cost are considered (Yazdani and Gonzalez, 2007). Ethanol is clean energy and can be used as a raw material and supplement to gasoline or used as a resource for biodiesel production instead of methanol which is usually produced from natural gas (Ito *et al*., 2005).

Therefore, this research investigated the utilization of raw glycerol from biodiesel plant for production of ethanol. This process development involves strain selection, optimization on medium composition and environmental conditions, kinetics study in the fermenter, scale-up for efficient production of ethanol.

Literature Review

1. Biodiesel production and generation of crude glycerol

Due to the depletion of the world's petroleum reserves and the increasing environmental concerns, there is a great demand for alternative sources of petroleum-based fuel, including diesel and gasoline fuels. Biodiesel, a clean renewable fuel, has recently been considered as the best candidate for a diesel fuel substitution because it can be used in any compression ignition engine without the need for modification (Xu and Wu, 2003). Biodiesel is a low-emissions diesel substitute fuel made from renewable resources and waste lipid. The most common way to produce biodiesel is through transesterification, especially alkali-catalyzed transesterification (Leung *et al*., 2010). Biodiesel fuels are defined as fatty acid methyl ester or ethyl ester from vegetable oils or animal fats and they are used as fuels in diesel engines and heating systems (Marchetti *et al*., 2007). Today, most of the biodiesel is produced by the alkali-catalyzed process. Fig. 1 shows a simplified flow chart of the alkali-catalyst process. As described earlier, feedstocks with high free fatty acid will react undesirably with the alkali catalyst thereby forming soap. The maximum amount of free fatty acids acceptable in an alkali-catalyzed system is below 2.5 wt% free fatty acid. If the oil or fat feedstock has free fatty acid content over 2.5 wt%, a pretreatment step is necessary before the transesterification process (Leung *et al*., 2010). The transesterification of triglycerides with alcohol is a balanced and catalyzed reaction (Bournay *et al*., 2005), as illustrated in Fig. 2. Among suitable alcohols (methanol, ethanol, propanol, butanol, and amyl alcohol) methanol and ethanol are utilized most frequently especially methanol because of its low cost and its physical and chemical advantages. There are different types of catalysts; base such as sodium or potassium hydroxides, acids such as sulfuric acid and lipases (Fukuda *et al*., 2001).

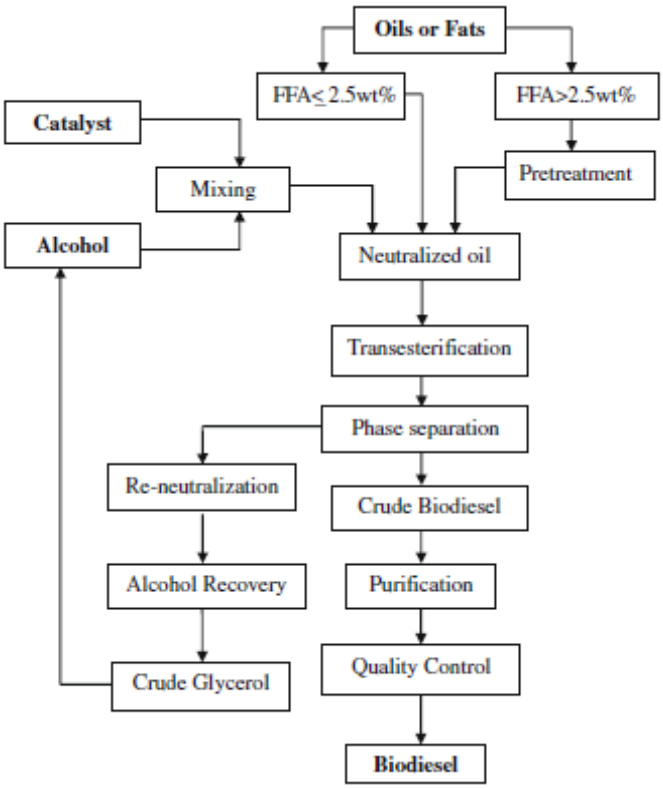


Figure 1. Simplified process flow chart of alkali-catalyzed biodiesel production
Source: Leung *et al*., (2010)

Alkalis used for transesterification include NaOH, KOH, carbonates, and alkoxides such as sodium methoxide, sodium ethoxide, sodium propoxide, and sodium butoxide. Alkali-catalyzed transesterification proceeds approximately 4000 times faster than that catalyzed by the same amount of an acidic catalyst and is thus most often used commercially (Fukuda *et al.*, 2001).

For every 9 kg of biodiesel produced, about 1 kg of a crude glycerol by-product is formed, which is about 10 %w/v of vegetable oil (Dasari *et al*., 2005; Wu *et al*., 2011; Oh *et al*., 2012). The applications of crude glycerol which is obtained from biodiesel production are found in food, drug, cosmetic and tobacco industries. However, crude glycerol derived from biodiesel production possesses very low value because of the impurities such as methanol which is used as a substrate for biodiesel production (Thompson and He, 2006). Moreover, crude glycerol may contain high concentration of salts such as sodium chloride since an alkali which is used as catalyst

is neutralized with an acid (Ito *et al*., 2005). The make-up of crude glycerol varies depending on the parent of feed stock and the biodiesel production process (Thompson and He, 2006). The glycerol content of the crude glycerol obtained from biodiesel process using rapeseed oil was produced at least 98% and neither ash, nor inorganic compounds are detected in the glycerol produced. The major impurities of the glycerol are water, methanol and matter organic non-glycerol (MONG, such as methyl ester) (Bounay *et al*., 2005). The usage of low-grade quality of glycerol obtained from biodiesel production is a big challenge as this glycerol cannot be used for direct food and cosmetic uses. Therefore, if the crude glycerol is used in food, cosmetics, and drugs, further purifications are needed such as bleaching, deodoring, and ion exchange to remove trace properties.

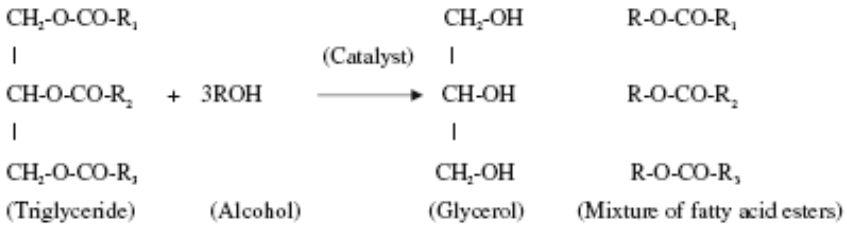


Figure 2. Transesterification of triglyceride with alcohol; R_1, R_2, R_3 and R' represent alkyl groups

Source: Fukuda *et al*., 2001

2. Fermentation of glycerol to valuable products

The glycerol molecule (1,2,3-propanetriol), molecular formula ($C_3H_8O_3$), molecular weight of 92.09 g/mol. Glycerol is a highly reactive tri-alcohol which has two-primary and secondary hydroxyl groups. Glycerol is water soluble, colorless, odorless, viscous and hygroscopic liquid with a specific gravity of 1.26 g/mL, melting temperature of 18.2°C, and boiling temperature of 290°C (accompanied by decomposition). Chemically, glycerol is available for reacting with a stable alcohol under most operation conditions, and it is basically non-toxic to human health and to the environment. The key feature of its usefulness is the particular combination

among its physiochemical properties, compatibility with other substances, and easy handling. Due to these particular properties, glycerol has found more than 1500 end-uses or large volume applications. Glycerol is abundant in nature as the structural component of many lipids. Wide glycerol occurrence in nature allows different kind of microorganisms to metabolize it as a sole carbon and energy source (Posada and Cardoona, 2010). Glycerol can substitute traditional carbohydrate, such as sucrose, glucose, and starch, in some industrial fermentation process (Solomon *et al*., 1995; Barbirato *et al*., 1997; Menzel *et al*., 1997). Although crude glycerol can be burnt, with the consequent energetic advantages, the setting up of biorefineries that co-produce products of higher economic value along with biofuels has been proposed as a solution for the economic viability of this product. Several strategies based on chemical and biological transformations are being pursued to convert glycerol into more valuable products (Yazdani and Gonzalez, 2007). An example of some of these includes (Lopez *et al*., 2009):

- The conversion of glycerol into propylene glycol and acetone through thermo-chemical process (Chiu *et al*., 2006; Dasari *et al*., 2005).

- The etherification of glycerol with either alcohols (methanol or ethanol) or alkenes (isobutene) and production of oxygen-containing components, which could have suitable properties use in fuel or solvents (Karinen and Krause, 2006).

-The microbial conversion (fermentation) of glycerol to 1,3-propanediol, which can be used as a basic ingredient of polyesters (Barbirato *et al*., 1998; Ito *et al*., 2005).

- Other products such as butanol (Biebl, 2001), propionic acid (Bories *et a*l., 2004), ethanol and formate (Jarvis *et al*., 1997), succinic acid (Lee *et al*., 2001), dihydroxyacetone (Bories *et al*., 1991), polyhydroxyalkanoates (Koller *et al*., 2005), or hydrogen and ethanol (Ito *et al*., 2005) were also obtained using glycerol as a carbon source.

Biochemical pathway for glycerol fermentations is given in Fig. 3, glycerol is dehydrogenated to dihydroxyacetone by glycerol dehydrogenase. Dihydroxyacetone is phosphorylated by dihydroxyacetone kinase to dihydroxyacetone phosphate which after can be converted to pyruvate in the course of the known sequence of glycolytic reactions involving another dehydrogenation and two ATP-forming steps. Glycolytic

pathway is the metabolic pathway which converts glucose via a series of reactions to 2 molecules of pyruvate. The first step in glycolysis is the phosphorylation of glucose by ATP to form glucose 6-phosphate catalyzed by the enzyme hexokinase. The second step is the isomerization of glucose 6-phosphate to fructose 6-phosphate by phosphoglucose isomerase enzyme. The third step is a second phosphorylation to form fructose 1,6-bisphosphate catalyzed by the enzyme phosphofructokinase. The last part of glycolysis involves the formation of pyruvate and more molecules of ATP. This is accomplished by a rearrangement of 3-phosphoglycerate to form 2-phosphoglycerate followed by a dehydration to form phosphoenolpyruvate (PEP). The final nearly irreversible reaction is the formation of ATP and pyruvate catalyzed by the enzyme pyruvate kinase. The reductive glycerol conversion consists of a vitamin B12-mediated dehydration to 3-hydroxypropionaldehyde and a reduction of the aldehyde to 1,3-PD by glycerol dehydratase and 1,3-propanediol dehydrogenase, respectively. In the enterobacteria pyruvate is cleaved to acetyl-CoA and formate in a reaction catalyzed by the enzyme pyruvate formate-lyase. From acetyl-CoA, acetate is formed via acetyl-phosphate. Acetaldehyde and ethanol are formed from acetyl-CoA by aldehyde dehydrogenase and alcohol dehydrogenase, respectively. Formate is usually cleaved to hydrogen and carbon dioxide by a formate lyase. As in sugar fermentation, pyruvate can also be condensed to α -acetolactate to give acetoin finally and 2,3-butanediol. Lactic acid, a reduction product of pyruvate, and succinic acid, which originates from phosphoenolpyruvate, also appears among the end-products of the enterobacterial fermentation. In *C. butyricum* and related strains, virtually two products are formed in addition to 1,3-PD: acetic and butyric acids. Butyric acid is formed after condensation of two molecules of acetyl-CoA in a reaction chain that involves two NADH-oxidizing steps and generation of ATP. Small amounts of ethanol are also found. *C. pasteurianum* forms butanol in addition, which sometimes becomes the predominating product (Biebl *et al*., 1999).

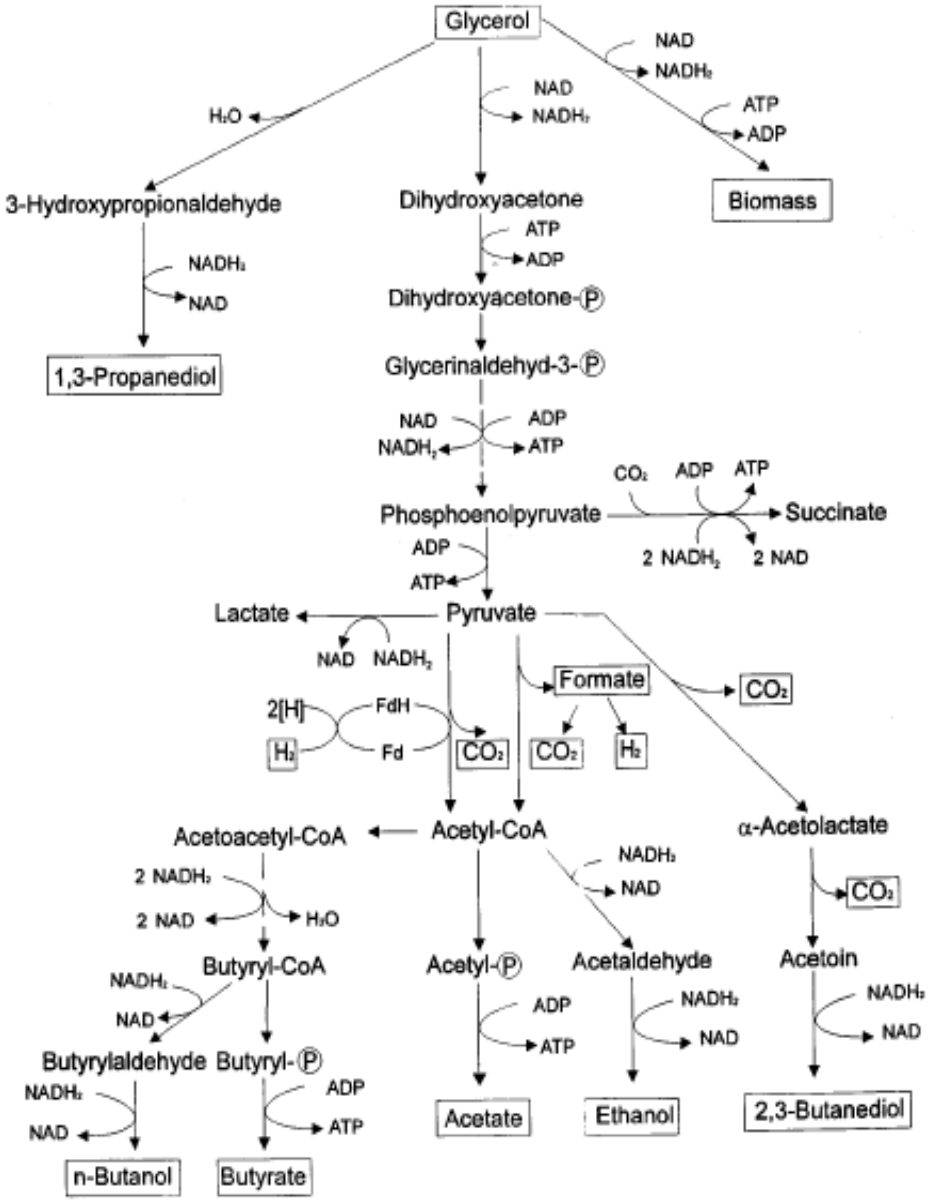


Figure 3. Biochemical pathways of glycerol fermentation, pyruvate utilization is indicated for different organisms. Butyrate and n-butanol are produced by clostridia, while 2,3-butanediol is only formed by enterobacteria. Acetate and ethanol are produced by both bacterial groups

Source: Biebl *et al*., 1999

3 Bioethanol production

Ethanol, also known as ethyl alcohol, molecular formula CH_3CH_2OH or C_2H_6O with the molecular weight of 46.07 g/mol. Ethanol is a flammable, colorless, mildly toxic chemical compound with a distinctive perfume-like odor with a specific gravity of 0.789 g/mL, melting temperature of -114°C, and boiling temperature of 78°C. Ethanol is used as a solvent in dissolving medicines, food flavorings and colorings that do not dissolve easily in water.

3.1 Microorganism for ethanol production

Among many microorganisms that have been exploited for ethanol production, *Saccharomyces cerevisiae* still remains as the prime species. *Zymomonas mobilis* has also been intensively studied over the past three decades and repeatedly claimed by some researchers to replace *S. cerevisiae* in ethanol production, because this species possesses some "superior characteristics" compared to its counterpart *S. cerevisiae* (Bai *et al*., 2008).

3.1.1 *Saccharomyces cerevisiae*

Although many researchers studied the ethanol fermentation with *S. cerevisiae,* in some cases a lack of recognition of its metabolic pathway led to approaches that are unlikely to yield significant improvements. The main metabolic pathway involved in the ethanol fermentation is glycolysis (Embden–Meyerhof–Parnas or EMP pathway), through which one molecule of glucose is metabolized, and two molecules of pyruvate are produced (Madigan *et al*., 2000). Under anaerobic conditions, the pyruvate is further reduced to ethanol with the release of CO_2. Theoretically, the yield is 0.511 for ethanol and 0.489 for CO_2 on a mass basis of glucose metabolized. Two ATPs produced in the glycolysis are used to drive the biosynthesis of yeast cells which involves a variety of energy-requiring bioreactions.

3.1.2 *Zymomonas mobilis*

Z. mobilis is an anaerobic, gram-negative bacterium which produces ethanol from glucose via the Entner–Doudoroff (ED) pathway in conjunction with the enzymes pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) (Conway, 1992). This microorganism was originally discovered in fermenting sugar-rich plant

saps, e.g. in the traditional pulque drink of Mexico, palm wines of tropical African, or ripening honey (Swings and Deley, 1977).

Compared with the EMP pathway of *S. cerevisiae*, which involves the cleavage of fructose-1, 6-bisphosphate by fructose bisphosphate aldolase to yield one molecule each of glyceraldehydes-3-phosphate and dihydroxyacetone phosphate, the ED pathway forms glyceraldehyde-3-phosphate and pyruvate by the cleavage of 2-keto-3-deoxy-6-phosphogluconate by 2-keto-3-deoxy-gluconate aldolase, yielding only one molecule ATP per glucose molecule. As a consequence, *Z. mobilis* produces less biomass than *S. cerevisiae*, and more carbon is funneled to the ethanol fermentation. It was reported that the ethanol yield of *Z. mobilis* could be as high as 97% of the theoretical yield of ethanol to glucose (Sprenger, 1996), while only 90–93% can be achieved for *S. cerevisiae*. Also, as a consequence of the low ATP yield, *Z. mobilis* maintains a higher glucose metabolic flux, and correspondingly, guarantees its higher ethanol productivity, normally 3–5 folds higher than that of *S. cerevisiae* (Sprenger, 1996).

3.1.3 *Aerobacter aerogenes*

A. aerogenes strains 1033 and 1041 were isolated from patients at the Boston City Hospital. They found that only strain 1033 was capable to degraded glycerol in the absence of oxygen, though both strains could oxidize this substrate. The adaptive patterns and the end products of glycerol oxidation in the two strains suggested that glycerol was metabolized in strain 1033 via dihydroxyacetone and in strain 1041 via L-a-glycerophosphate. The end products of glycerol fermentation in strain 1033 were known as ethanol and formic acid in equimolar amounts. But in the presence of arsenious oxide, the fermentation rate was greatly depressed. Lactic acid was identified as an end product, and the fixation of CO_2 was observed; no other acid accumulated. (Magasanik *et al.*, 1953).

3.1.4 *Enterobacter aerogenes*

E. aerogenes HU-101 was studied to convert purified glycerol and crude glycerol of biodiesel wastes (Ito *et al.* 2005). *E. aerogenes* HU-101 was isolated from a methanogenic sludge developed in their laboratory. Cultures were maintained at −80°C with 15% glycerol. They used a synthetic medium and a complex medium with adding the desired concentrations of yeast extract and tryptone to the synthetic

medium. They found that glycerol from biodiesel wastes should be diluted with a synthetic medium to increase the rate of glycerol utilization and the addition of yeast extract and tryptone to the synthetic medium accelerated the production of H_2 and ethanol. The yields of H_2 and ethanol decreased with an increase in the concentrations of biodiesel wastes and commercially available glycerol (purified glycerol).

Furthermore, the rates of H_2 and ethanol production from biodiesel wastes were much lower than those at the same concentration of purified glycerol, partially due to a high salt content in the wastes. This study compared continuous culture with a packed-bed reactor using self-immobilized cells and using porous ceramics as a support material to fix cells in the reactor. The result is, the maximum rate of H_2 production from pure glycerol was 80 mmol/L/h yielding ethanol at 0.8 mol/mol-glycerol, while that from biodiesel wastes was only 30 mmol/L/h in self immobilized cells and for the adding support material could yielded the maximum H_2 production rate from biodiesel wastes reached 63 mmol/L/h obtaining an ethanol yield of 0.85 mol/mol glycerol (Ito *et al*. 2005).

3.2.5 *Kluyvera cryocrescens*

K. cryocrescens S26 was carried out in RG minimal medium supplemented with 25 g/L of crude glycerol (80%, w/v) under anaerobic fermentation. The glycerol content in the medium was equivalent to 20 g/L. Ethanol was accumulated as a major product at the rate of 0.052 g/L/h with molar yield of 84.8% per consumed glycerol. Formic acid was primarily generated as byproduct and negligible amount of lactic and succinic acid was observed. There were no other organic acids such as acetic acid and no 1,2- or 1,3-propanediol detected in the culture broth. In a gas phase, H_2 and CO_2 were generated gradually, resulting in final cumulative amount of 95 and 90 mmol, respectively (Choi *et al*., 2011)

3.2 Ethanol from agricultural, industrial and urban residues

Nowadays bioethanol is the most widespread biofuel. Currently, the bioethanol production is primarily from sugarcane, maize (corn) and sugar beets and there's discussion about whether it is a sustainable energy resource that may offer environmental and long-term economic advantages over fossil fuels but, the technology to make it economically competitive to produce ethanol from cellulosic

feedstock is in development (Hernandez and Kafarov, 2009). Therefore, lignocellulosic substances such as agricultural wastes are attractive feedstocks for bioethanol production. Agricultural wastes are cost effective, renewable and abundant. Bioethanol from agricultural waste could be a promising technology though the process has several challenges and limitations such as biomass transport and handling, and efficient pretreatment methods for total delignification of lignocellulosics (Sarkar *et al*., 2012).

Bioethanol fuel is mainly produced by the sugar fermentation process. The main sources of sugar required to produce ethanol come from fuel or energy crops or biomass or industrial waste. Some of the most important issue for the production of ethanol as a biofuel on commercial scale is the production costs, the availability of carbon source and other nutrients required for the growth of microorganisms used (Takana *et al*., 1999). Therefore, the utilization of agro-industrial wastes could help to reduce the production cost of ethanol (Ruanglek *et al*., 2006). *Zymomonas mobilis* ATCC10988 produced 59.0 g/L ethanol in undiluted pineapple juice and pineapple waste without nutritional supplementation (Takana *et al*., 1999). Moreover, cassava waste can be utilized to produce ethanol using yeast *Saccharomyces cerevisiae* TISTR5596 with the maximum ethanol production of 3.62% (w/v) at 24 h fermentation (Srinorakutara *et al*., 2006). Complex nutritional source for *Zymomonas mobilis* NRRL-B-14023 could be substituted by many such as hydrolysate of fish soluble waste, ami-ami solution from glutamate-synthesizing process and autolysate of brewer's yeast. Researchers observed that the fish soluble was the best applicable source of nutritional replacement for ethanol production since it could significantly promote both specific growth rate and ethanol productivity compared with the yeast extract (Ruanglek *et al*., 2006). Whey was used as a substrate for the ethanol fermentation with yeast *Saccharomyces cerevisiae* and β-galactosidase and 5.6% (v/v) of ethanol concentration was produced at 24 h of fermentation (Staniszewski *et al*., 2007). Bread residues were converted into a suitable fermentation feed via a two-step starch hydrolysis using amylolytic enzyme using *Saccharomyces cerevisiae*, which results in an overall yield of 0.35 g ethanol/g of initial bread dry matter (Ebrahimi *et. al*., 2008). *Enterobacter aerogenes* HU-101 produced ethanol from glycerol-containing waste discharged after manufacturing process for biodiesel fuel with the

ethanol yield of 0.85 mol/mol glycerol (Ito *et al*., 2005). Table 1 show the microorganisms used in ethanol production, with different substrate.

Yazdani and co-workers (2007) demonstrated the ethanol production from glycerol. This approach would result in a decrease in bioethanol production cost by about 37% when compared to conventional bioethanol production from corn (Fig. 4) Calculations are based on the 2003–2005 period, except for glycerol prices, which are based on 2007. Operating cost was assumed to be similar to that estimated for molasses, raw, and refined sugar. Feedstock cost is 'net' for corn-derived ethanol (includes revenue from co-products), but not for glycerol-derived ethanol (does not include revenue from co-products H_2 or formic acid). Same cost advantages can be realized in the production of other fuels and reduced chemicals from glycerol (Yazdani and Gonzalez, 2007).

4. Factors affecting ethanol production from glycerol

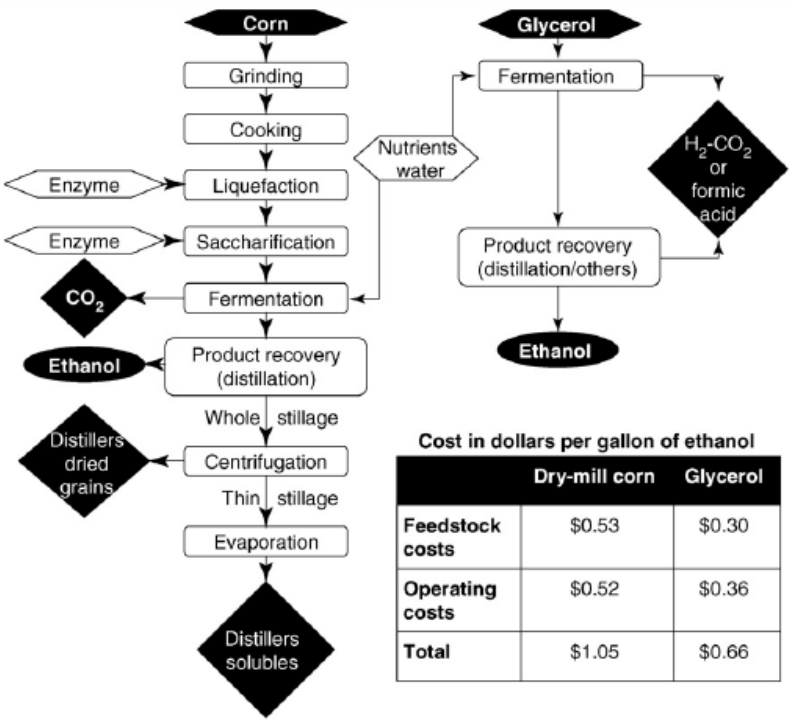
4.1 Microbial strain

The microorganisms that can convert glycerol to ethanol and various compounds include *Aerobacter aerogenes* strain 1033 and 1041 which were isolated originally from patients of Boston City Hospital. The end products of glycerol fermentation in strain 1033 were ethanol and formic acid in equimolar amounts (Makasanik *et al*., 1953). *Citrobacter freundii*, selected from enrichment cultures with glycerol, produced mainly acetate and yields more 1,3-propanediol. In contrast, glycerol conversion by *KIebsiella oxytoca* leads mainly to 1,3-propanediol and ethanol, implying a lower 1,3-propanediol production (Homann *et al*., 1990). *Enterobacter agglomerans* CNCM 1210 and *Clostridium butyricum* CNCM 1211 which were isolated from a distillery waste-water anaerobic digester gave different composition of the end products yield. *E. agglomerans* produce large amounts of formate, which is not a metabolite for the other enterobacteriaceae as well as ethanol and lactate but with different yields (Barbirato *et al*., 1995). *Klebsiella planticola* DR3, isolated from the rumen contents of red deer (*Cervus elaphus*), dissimilated glycerol for formate and ethanol (Jarvis *et al*., 1997). *Clostridium butyricum* AT1, a newly isolated thermophilic strain, converted glycerol to end-products such as 1,3-

propanediol, n-butyrate and ethanol (Wittlich *et al*., 2001). *Enterobacter aerogenes* HU-101, isolated as a high-rate H_2 producer from methanogenic sludge, can convert various carbohydrates, such as sugars and sugar alcohols, to mainly H_2 and ethanol with a minimal production of 1,3-propanediol, lactate and acetate when glycerol was used as the substrate (Ito *et al*., 2005).

Table 1. Examples of microorganism for ethanol production with different substrate

Microorganisms	Substrate	Ethanol (g/L)	Ethanol yield (g/g)	Reference
Z. mobilis + *C. tropicalis*	Fruit and vegetable residues	50	-	Patle and Lal, 2008
Z. mobilis	Sugar cane molasses	55.8	0.34	Cazetta *et al*., 2007
K. marxianus	Cheese whey powder	-	0.35-0.54	Kargi and Ozmihci, 2006
S. cerevisiae	Citrus waste pulp	6.84	0.25	Raposo *et al*., 2009
S. cerevisiae	Beet molasses	9.21	0.46	Raposo *et al*., 2009
S. cerevisiae	Carob pod extract	10.30	0.34	Raposo *et al*., 2009
E. aerogenes HU-101	Waste glycerol	-	0.48	Ito *et al*., 2005
A. aerogenes 1033	Waste glycerol	-	0.43	Makasanik *et al*., 1953
Engineering *E. coli* SY03	Waste glycerol	-	0.51	Yanzalez and Gonzalez, 2008
Klebsiella sp. HE1	Waste glycerol	-	0.40	Wu *et al*.,2011



Cost in dollars per gallon of ethanol

	Dry-mill corn	Glycerol
Feedstock costs	$0.53	$0.30
Operating costs	$0.52	$0.36
Total	$1.05	$0.66

Figure 4. Comparing pathway on ethanol production cost from corn and glycerol
Source: Yazdani and Gonzalez, 2007

4.2 Glycerol concentration

Batch culture with *Enterobacter agglomerans* regurated at pH 7 with initially 20 g/L glycerol, 0.15 mol ethanol/mol glycerol was produced. The same experiment was performed with initially 70 g/L glycerol, the formation of ethanol was significantly lowered (0.02 mol ethanol/mol glycerol), resulting in the higher 1,3-propanediol yield (Barbirato *et al*., 1998). Ethanol production of *Enterobacter aerogenes* HU-101 was carried out with biodiesel waste. 0.96, 0.83, 0.67 and 0.56 mol ethanol/mol glycerol were produced from 1.7, 3.3, 10 and 25 g/l glycerol as initial glycerol concentration, respectively. The yields of ethanol decreased with the increase in the concentration of biodiesel waste (Ito *et al*., 2005). The specific growth rate of *Kluyvera cryocrescens* S26 was gradually decreased when the glycerol was added at level up to 100 g/L. The growth inhibition effect was more evident when the medium contained more than 100 g/L of glycerol, leading to significantly suppressed

cell growth. The result indicated that a high concentration of glycerol decreased the yields of ethanol and cell growth (Choi *et al*., 2011).

4.3 Supplement nutrients

Some nutritional components present in complex nutrient sources can be essential for cell growth and metabolite production. Glycerol from biodiesel waste was diluted with deionized water, glycerol was not completely consumed and no growth was observed. This indicated that some nutrients should be added to ferment glycerol waste (Ito *et al*., 2005). This result similar with the ethanol production using crude glycerol by *Kluyvera cryocrescens* could produce ethanol about 11 g/L using yeast extract as supplement nutrient and higher than polypeptone and tryptone. Since yeast extract is known to contain nitrogen and carbohydrate, it may be utilized as carbon source to synthesized biomass or ethanol (Choi *et al*., 2011). Moreover, yeast extract has a buffering capacity and this might contribute to high productivity using the media containing yeast extract (Gaudreau *et al*., 1997).

4.4 Influence of pH

The role of pH was investigated in batch cultures of *Clotridium pasteurianum* DSM 525 controlled at values between 4.5 and 7.5 using glycerol at a concentration of 50 g/l. At the pH 4.5, a greater part of the glycerol was converted to butanol as a main product that was produced 38.8 mol/100 mol glycerol, 2.4 mol ethanol/100 mol glycerol was produced as a by-products. The maximum of ethanol yield was obtained from the cultivation at pH 7.5, 26.5 mol ethanol/100 mol glycerol was produced as a main product and 22.2 mol butanol/100 mol glycerol was produced in same condition (Biebl, 2001). Therefore, the suitable pH for ethanol production from glycerol was neutral pH (6.5-8.0), *Enterobacter aerogenes* HU-101 was pH 6.8 (Ito *et al*., 2005), pH 7 for *Klebsiella pneumoniae* (Mu *et al*., 2006) and *Klebsiella oxytoca* (Homann *et al*., 1990), pH 8.0 for mixed culture from a distillery wastewater treatment plant (Temudo *et al*., 2007), pH 6.5 for *Aerobacter aerogenes* (Magasanik *et al*. 1953).

4.5 Aeration rate and agitation speed

Klebsiella pneumoniae was studied in batch cultures under N_2 flow and four levels of air flow rates (0.1, 0.2, 0.4 and 0.6 vvm). The highest concentration of ethanol was found in anaerobic batch fermentation about 5 g/l, with increase of air flow the ethanol formation decrease (lower than 5 g/l), that indicated by-products formation depended on the aeration condition (Cheng *et al*., 2004). Since the dissolved oxygen activates faster cell growth of the gene expression system for enzymes of the citric acid cycle, which provides energy for cell growth (Chen *et al*., 2003). In addition, oxygen supply at high level can enhance the generation of ATP by reducing NADH, which is then used for biomass synthesis.

4.6 Impurities in crude glycerol

Effect of crude glycerol from biodiesel derived from alkali (KOH) and lipase catalyzed were studied to glycerol fermentation by *Klebsiella pneumoniae*, the final ethanol concentration (11.9 g/L) on crude glycerol derived from the alkali-catalyzed reaction was more than those on pure glycerol and crude glycerol from the lipase-catalyzed (10.3 and 4.3 g/L, respectively). The impurities such as salt (KCl) in glycerol derived from the alkali-catalyzed were more than in crude glycerol from the lipase-catalyzed, which affected products synthesis (Mu *et al*., 2006). Ito and coworkers (2005) studied the effect of sodium chloride on ethanol production using *Enterobacter aerogenes* HU-101 on pure glycerol, the ethanol concentration (80 mM ethanol) at 1% of sodium chloride was produced higher than at without sodium chloride (70 mM ethanol). When studied the effect of sodium chloride on glycerol from biodiesel waste, the maximum ethanol (55 mM) was obtained from the glycerol without sodium chloride added. The ethanol production decreased when sodium chloride increased (40 mM ethanol was produced from 1% sodium chloride) that caused by the presence of sodium chloride in the crude glycerol from biodiesel waste (Ito *et al*., 2005).

5. Response surface methodology for ethanol production

Selection of appropriate carbon, nitrogen and other nutrients is one of the most critical stages in the development of an efficient and economic bioprocess. Classical and statistical methodologies are available for screening nutrients in bioprocess optimization studies (Ramesh *et al*., 2004). The traditional 'one-factor at a time' technique used for optimizing a multi-variable system is not only time consuming but also often easily misses the alternative effects between components. These drawbacks of single factor optimization process can be eliminated by optimizing all the affecting parameters using response surface methodology (RSM). Recently, many statistical experimental design methods have been employed in bioprocess optimization (Maddox and Reichert, 1977). RSM is a combination of mathematical and statistical technique that is useful for analyzing the effects of several independent variables on the system response without the need a predetermined relationship between the objective function and the variables (Wang *et al*., 2008). There are various advantages in using statistical methodologies in terms of rapid and reliable short listing of nutrients, understanding interactions among the nutrients at varying concentrations and a tremendous reduction in total number of experiments, resulting in saving time, glassware, chemicals and manpower (Poorna and Neelesh, 2001). The step in medium optimization is to determine the optimum level of each key independent variable as identified by the screening stage using response surface methodology technique. This technique is practical for up to five variables. A major contour plot may be generated by determining the linear, interaction and quadratic effects of a key variable (i.e. nutrients, medium pH, etc.). The quadratic general polynomial model represent the relationship for the variables is given below;

$$Y = b_0 + \Sigma b_i X_i + \Sigma\Sigma b_{ij} X_i X_j + e \qquad (1)$$

Where Y is the independent variables (such as predicted yield), b_0 is the regression coefficient at center point; b_i is linear coefficients, b_{ij} is the quadratic coefficients

when i = j, and the first order interaction coefficient; X_i and X_j are levels of the different of variables and e is the random error (Sreekumar *et al*., 1999).

Response surface methodology (RSM) was applied to optimize ethanol production using by *Zymomonas mobilis*, glucose and yeast extract are the key media that influence ethanol production, followed by the inoculum concentration and ammonium sulfate, with phosphate showing little or no effect. Comparison on RSM optimized media with the media conventionally, the RSM media showed a high ethanol yied of 0.5 g/g with the 99% sugar utilization, whereas the conventional media showed 96% sugar utilization and less ethanol yield (Sreekumar *et al*., 1999). The optimization of critical medium components for the production of ethanol from cellulose by *Clostridium thermocellum* SS19 in anaerobic submerged fermentation was carried out using RSM. The design was employed by selecting filter paper, corn steep liquor, cysteine hydrochloride, magnesium chloride and ferrous sulphate as model factors, which have been found to be optimal for ethanol production, were 45, 8.0, 0.25, and 0.01 g/l, respectively. Among the five independent variables studied, all the nutrients were found significant, except magnesium chloride, it has also proved to be useful in increasing ethanol yield from 0.32 to 0.41 g/g (Balusu *et al*., 2005). Not only the optimization for ethanol production using free cell by RSM, but also the immobilized cells are optimized for ethanol production. The statistical experimental design was used to optimize the conditions of simultaneous saccharification and fermentation (SSF), viz. temperature, pH and time of fermentation of ethanol from sago starch with co-immobilized amyloglucosidase (AMG) and *Zymomonas mobilis* MTCC 92 by submerged fermentation. Maximum ethanol concentration of 55.3 g/l was obtained using a starch concentration of 150 g/L. The optimum conditions were found to be a temperature of 32.4 °C, pH of 4.93 and time of fermentation of 17.24 h. Thus, the central composite design (CCD) was found to be the most favourable strategy investigated with respect to ethanol production and enzyme recovery (Bandaru *et al*., 2006). A culture media for glycerol to ethanol biotransformation by *E. coli* under anaerobic condition was formulated and optimized by full factorial 2^2. The result shows the final components in media culture were glycerol 10, Na_2SO_4 0.0806, NaCl 0.0152, $MgSO_4 \cdot 7H_2O$ 0.0310 and peptone 4.25 g/L, respectively with the biomass productivity, ethanol specific productivity and glycerol to ethanol yield

were 0.165 g/L/d, 212 g/kg/h of cell mass and 59 g/kg of glycerol, respectively (Cofŕe *et al*., 2012). It is evident that the use of statistical media optimization approach, response surface methodology has helped to locate the most significant nutrients optimum levels with minimum effort and time, and it could also prove the ethanol production.

6. Fermentation type for ethanol production

A fermentation process has been receiving much attention for many years as it can be widely applied for the production of many bioproducts including pharmaceutical and agricultural products (Arpornwichanop and Shomchoam. 2006). Ethanol can be produced by four types of industrial operations; batch, continuous, fed-batch, and semi-continuous (Keim, 1983).

6.1 Batch fermentation

Batch fermentation process refers to the process that starts with the inoculation and end with the retrieval of the product happens inside a single fermenter with no intermediate steps. Most of the ethanol produced today is done by the batch operation because the investment costs are low, do not require much control, can be accomplished with unskilled labor and the greater flexibility that can be achieved by using a bioreactor for various product specifications.

The rate of cell growth, ethanol production and glycerol consumption were related to the cell concentration (X), ethanol concentration (P) and glycerol concentration (S). Specific growth rates (μ) of each initial glycerol concentration were calculated following Monod equation (2). Equation (3) was used to calculate the substrate inhibition on cell growth (Yalcin and Ozbas, 2004);

$$\text{Cells:} \quad \mu = \frac{\mu_{\max} S_0}{K_s + S_0} \tag{2}$$

$$\text{Cells:} \quad \mu = \frac{\mu_{\max} S_0}{K_s + S_0 + \frac{{S_0}^2}{K_I}} \tag{3}$$

Here, μ is the specific growth rate, μ_{max} the maximum specific growth rate, S_0 initial substrate concentration, K_s the substrate saturation constant of the Monod model, and K_I the substrate dissociation constant. The kinetics parameters; the maximum specific growth rate (μ_{max}) and substrate saturation constant (K_s) were determined using Lineweaver-Burk plot between $1/[S_0]$ and $1/\mu$ (Song *et al*., 2008).

The rate of cell growth, ethanol production and glycerol consumption relate to the cell concentration (X), ethanol concentration (P) and glycerol concentration (S). The specific production rate (ρ) and substrate consumption rate were calculated as described in equation (4) and (5), respectively (Phisalaphong *et al*., 2006);

$$\text{Ethanol:} \quad \frac{dP}{dt} = \rho X \tag{4}$$

$$\text{Substrate:} \quad -\frac{dS}{dt} = \frac{1}{Y_{x/s}}\left(\frac{dX}{dt}\right) + \frac{1}{Y_{p/s}}\left(\frac{dP}{dt}\right) + mX \tag{5}$$

Here, the cellular yield coefficient ($Y_{x/s}$), yield of substrate to product ($Y_{p/s}$) and the maintenance constant (m) (in this case assumed no maintenance in cell).

Qureshi and coworkers (2006) studied the kinetics parameters on the effect of xylose and ethanol concentration by *E. coli* FBR5. As the concentration of xylose increased (50-250 g/L), ethanol specific productivity (ρ) decreased from 0.98 to 0.70 h^{-1}. Ethanol inhibition studies suggested that the maximum tolerance of the culture was 50 g/L ethanol. However, the maximum ethanol that could be produced was 43.5 g/L. The value of K_s (Michaelis-Menten constant) was evaluated to be 4.38 g/L. The ethanol formation from cheese whey powder (CWP) solution was investigated as function of substrate concentration (52-312 g/L with total sugar content of 25-150 g/L) using batch experiment by *Kluyveromyces marxianus* DSMZ-7239. Sugar utilization was almost completed within 72 when CWP concentration less than 156 g/L and took longer time thereafter. The specific rate of sugar utilization increased with sugar concentration up to 75 g/L indicating substrate limitations at lower sugar concentration and the rate decreased thereafter due to substrate inhibition at high sugar concentration. Similar trends were also observed in the specific of ethanol

formation. Moreover, this using kinetic model for describe the rate of sugar utilization; the kinetic constants were determined using the experimental data. The results show that the model predictions of sugar utilization rates were in good agreement with the experimental data (Ozmihci and Kargi, 2007). The kinetics values of maximum specific growth rate (μ_{max}) and Monod constant (K_s) of *Saccharomyces cerevisiae* using agro-industrial by-products as a carbon source in batch process shown in table 2. The saturation constant (K_s) reflects the fact that large values of K_s imply that there is a weak affinity for the bacterial strain to 'bind' the substrate. For these studies, found that *S. cerevisiae* culture presents more affinity for the beet molasses extract because it shows lower K_s, comparatively with others carbon sources. Probably is due to the high content of carbohydrates, mostly sucrose (90%) and this substrate also evidences a high affinity to this yeast strain culture. (Roposo *et al*., 2009)

Table 2. Values of maximum specific growth rate (μ_{max}) and Monod constant (K_s) for *Saccharomyces cerevisiae*

Substrate	μ_{max} (h^{-1})	K_s (g/L)
Citrus waste pulp	0.35	10.69
Carob pod extract	0.33	12.47
glucose	0.38	9.40
sucrose	0.55	8.98
beet molasses	0.35	5.66

6.2 Fed-batch fermentation

A fed-batch is a biotechnological batch process which is based on feeding of a growth limiting nutrient substrate to a culture. The fed-batch strategy is typically used in bio-industrial processes to reach a high cell density in the bioreactor. Mostly the feed solution is highly concentrated to avoid dilution of the bioreactor. The fed-batch operation, which may be regarded as a combination of the batch and continuous operations, the feed solution, which contains substrate, culture, and the required minerals and vitamins, are fed at constant intervals while effluent is removed

discontinuously. The main advantage of the fed-batch system is that inhibition and catabolite repression are prevented by intermittent feeding of the substrate. If the substrate has an inhibitory effect, intermittent addition improves the productivity of the fermentation by maintaining a low substrate concentration. It is essential to keep the culture volume constant in continuous operation, whereas there is volume variation in the fed-batch processes (Stanbury *et al*., 1995; Yoshida *et al*., 1973). Two basic approaches to the fed-batch fermentation can be used: the constant volume fed-batch culture (fixed volume fed-batch) and the variable volume fed-batch as described below;

6.2.1 Fixed volume fed-batch

In this type of fed-batch, the limiting substrate is fed without diluting the culture. The culture volume can also be maintained practically constant by feeding the growth limiting substrate in undiluted form, for example, as a very concentrated liquid or gas (ex. oxygen). Basically, once the fermentation reaches a certain stage, (for example, when aerobic conditions cannot be maintained anymore) the culture is removed and the biomass is diluted to the original volume with sterile water or medium containing the feed substrate. The dilution decreases the biomass concentration and result in an increase in the specific growth rate. Subsequently, as feeding continues, the growth rate will decline gradually as biomass increases and approaches the maximum sustainable in the vessel once more, at which point the culture may be diluted again.

6.2.2 Variable volume fed-batch

As the name implies, a variable volume fed-batch is one in which the volume changes with the fermentation time due to the substrate feed. The way this volume changes it is dependent on the requirements, limitations and objectives of the operator. The feed can be provided according to one of the following options: (i) the same medium used in the batch mode is added; (ii) a solution of the limiting substrate at the same concentration as that in the initial medium is added; and (iii) a very concentrated solution of the limiting substrate is added at a rate less than (i), (ii) and (iii). The former means that once the fermentation reached a certain stage after which

is not effective anymore, a quantity of culture is removed from the vessel and replaced by fresh nutrient medium. The decrease in volume results in an increase in the specific growth rate, followed by a gradual decrease as the quasi-steady state is established. The latter type refers to a type of fed-batch in which supplementary growth medium is added during the fermentation, but no culture is removed until the end of the batch. This system presents a disadvantage over the fixed volume fed-batch and the repeated fed-batch process: much of the fermenter volume is not utilized until the end of the batch and consequently, the duration of the batch is limited by the fermenter volume

Ethanol production by recombinant *Saccharomyces cerevisiae* YPG/AB from starch in fed-batch increase by 200% compared to batch culture (Ülgen *et al*., 2002). Tomás-Pejó *et al*., (2009) studied the feeding strategies for ethanol production from wheat straw by *Kluyveromyces marxianus* CET10875 on fed-batch. Different initial substrate loading of 10% and 12% (w/v) and adding time (12, 24 and 40 h) were studied. The highest ethanol concentration (36.2 g/L) was reached with initial wheat straw of 10% and 12 h of addition time, whereas 12% (w/v), substrate addition at 12 h was also reflected in higher ethanol production (29.2 g/L) compared to the pulse at 40 h (27.6 g/L). It could be due to the loss of cell stability for long time thus, shorter addition time were reflected in higher ethanol yield and indicated that earlier pulse addition improved fed-batch experiment, this study 20% more ethanol when compared with batch process.

Ethanol production from glycerol by mutant *Klebsiella pnuemoniae* GEM167 (created by γ-ray irradiation) in fed-batch fermentation, when pure glycerol was used as the substrate the maximum level ethanol production was 21.5 g/L, followed by 20.5 and 19.9 g/L using untreated and pretreated glycerol, respectively (Oh *et al*., 2011). Therefore, it shows that fed-batch fermentation can enhance the bioproduct productions when compare with batch process. Table 3 shows the comparison of quantitative data from ethanol production process on glycerol using batch and fed-batch process.

Table 3. Comparison of ethanol production from glycerol by different microorganisms

Organism	Fermentation Type	Ethanol Production (g/L)	Productivity (g/L/h)	References
Enterobacter aerogenes HU-101	Batch	10.0	0.83	Ito *et al*., 2005
Klebsiella oxytoca M5a1	Fed-batch	19.5	0.56	Yang *et al*., 2007
Escherichia coli EH05	Batch	20.7	0.22	Durmin *et al*., 2009
Klebsiella pneumonia GEM167	Fed-Batch	21.5	0.93	Oh *et al*., 2011
Klebsiella pneumonia GEM167	Batch	8.6	0.72	Oh *et al*., 2011
Kluyvera cryocrescens S26	Batch	27.0	0.78	Choi *et al*., 2011

6.3 Continuous fermentation

Exponential growth in batch culture may be prolonged by the addition of fresh medium to the vessel. Provided that the medium has been designed such that the growth is substrate limited (i.e. by some component of the medium), and not toxin limited, exponential growth will proceed until the additional substrate is exhausted. This exercise may be repeated until the vessel is full. However, if an over flow device were fitted to the fermenter such that the added medium displaced an equal volume of culture from vessel then continuous production of cells could be achieved. If medium is fed continuously to such a culture at a suitable rate, a steady state is achieved eventually, that is formation of new biomass by the culture is balanced by the loss of cells from the vessel (Stanbury *et al*., 1995). In the continuous process, feed, which contains substrate, culture medium and other required nutrients, is pumped

continuously into an agitated vessel where the microorganisms are active. The culture broth contains ethanol, cells, and residual sugar (Maiorella *et al.*, 1981).

Continuous ethanol fermentation offer special advantages over batch and fed-batch operation by providing constant effluent quality, high productivity and control over the product by hydraulic retention time (HRT) adjustments. Effect of HRT (12.5-60 h) was studied for ethanol production from cheese whey powder (CWP) solution by *Kluyveromyces marxianus* DSMZ7239 in continuous process. Sugar utilization, ethanol and biomass formation increased with the increase of HRT, gave maximum ethanol productivity of 0.745 g/L/h at HRT 43.2 h. (Ozmihci and Kargi, 2007a). Moreover, effect of CWP concentration (55-200 g/L) was studied with constant HRT at 54 h, % sugar utilization and biomass formation decreased with increase feed sugar concentration due to high sugar concentration and other dissolved solid increased the osmotic pressure of fermentation broth which resulted in considerable activity loss in the yeast cells, and gave the maximum ethanol concentration and productivity of 3.7% v/v and 0.54 g/L/h, respectively at 100 g/L feed sugar concentration (Ozmihci and Kargi, 2007b).

Continuous culture of *Clostridium pasteurianum* on glycerol medium by varied the dilution rates between 0.1 and 0.4 h^{-1}. The culture was limited by glycerol only at the lowest dilution rate, at faster medium flow, an increasingly smaller fraction was fermented, probably due to inhibition by the products. 1,3-Propanediol was the main product at 0.1 $h^{-1}$of dilution rate, while butanol and ethanol were highest at dilution rate of 0.2 h^{-1} (Biebl. 2001). Continuous cultures using packed-bed reactor with self-immobilized cell of *Enterobacter aerogenes* HU101, the maximum rate of hydrogen production from pure glycerol was 80 mmol/L/h yielding ethanol at 0.8 mol/mol-glycerol, while that from biodiesel waste was only 30 mmol/L/h. However using porous ceramics as a support material to fix cells in the reactor, the maximum hydrogen production rate from biodiesel waste reached 63 mmol/L/h obtaining an ethanol yield of 0.85 mol/mol-glycerol (Ito *et al*., 2005).

6.4 Cell immobilization

Immobilization offers several potential advantages of a process engineering nature to the fermentation system. These include ease of handling and of cell

separation, and lowering of bulk viscosity, as well as the obvious potential benefits of increased cell concentration (Webb. 1989). Cell immobilization approaches have been applied to produce ethanol continuously in bioreactors. Various carriers have been applied to ethanol fermentation with immobilized cells (Liu *et al*., 2009), including calcium alginate (Najafpour *et al*., 2000), k-carrageenan (Nigam, 2000a), delignified cellulosic material (Kourkoutas *et al*., 2002), orange peel (Plessas *et al*., 2007) in different types of bioreactors, such as packed bed reactor (Nigam, 2000a), fluidized bed reactor (Shindo *et al*., 2001; Baptista *et al*., 2006). Immobilized cells of *S. cerevisiae* ATCC24553 in k-carrageenan and packed in trapped glass column reactor for ethanol production from pineapple cannery waste, the ethanol productivity of immobilized cell was 11.5 times higher than the free cells and the reactor was operated effectively under steady state for 87 day, slightly decline in productivity thereafter, due to a decrease in cell viability (Nigam, 2000b). Ethanol yield from mahula (Madhuca *latifolia* L.) flowers using immobilized cell of *S. cerevisiae* (in agar-agar and calcium alginate) was 3.5% higher than from free cell. Moreover, the immobilized could be reused at least three cycles of ethanol production without apparently lowering the productivity (Behera *et al*., 2010). Immobilized cells of *S. cerevisiae* C12 produced high level of ethanol (85-96 g/L) for more than 42 days without significant loss of ethanol productivity (Ivanova *et al*., 2011). Table 4 shows the ethanol production using immobilized cell.

Table 4. Ethanol production using immobilized cells

Organism	Material	Substrate	Ethanol production	References
S. cerevisiae ATCC24553	k-carrageenan	pineapple cannery waste	70 g/L	Nigam, 2000b
S. cerevisiae	agar-agar	mahula flower	151.2 g/kg	Behera *et al*., 2010
S. cerevisiae	calcium-aginate	mahula flower	154.5 g/kg	
S. cerevisiae	magnetic particle	glucose	60 g/L	Liu *et al*., 2009

7. Scale-up for the ethanol production

Scale-up means increasing the scale of fermentation, for example from the laboratory scale to the pilot plant scale or from the pilot scale to the production scale. Increase in scale means an increase in volume and the problems of process scale-up are due to the different ways in which process parameters are affected by the size of the unit. It is the task of the fermentation technologist to increase the scale of fermentation without a decrease in yield or, if a yield reduction occurs, to identify the factor which gives rise to the decrease and to rectify it. The major factors involved in scale-up are: (i) Inoculum development, an increase in scale may mean that extra stages have to be incorporated into the inoculum development program. (ii) Sterilization is a scale dependent factor because the number of contaminating microorganisms in a fermenter must be reduces to the same absolute number regardless of scale. Thus, when the scale of scale a process is increases the sterilization regime must result in a change in the quality of the medium after sterilization. (iii) Environmental parameters, the increase in scale may results in changed environment for the organism. These environmental parameters such as pH, temperature, dissolve oxygen, shear conditions and foam production (Stanbury *et al*., 1995).

Scaling-up of a microbial culture from laboratory work conducted in shake flasks requires translation to the kind of equipment used in a production plant, such as stirred vessel (Nimcevic and Gapes, 2000). Bioprocesses are usually developed in three stages or scales: (1) Bench or laboratory scale, where basic screening procedures are carried out; (2) pilot plant, where the optimal operating conditions are ascertained; and (3) plant scale, where the process is brought to economic fruition. Scale-up means reproducing in plant-scale equipment the results from a successful fermentation made in laboratory- or pilot-scale equipment. The scale-up process thus directly influences the production capacity and efficiency of a bioprocess. Fermentations in general can be evaluated by the following physical characteristics: mixing time, shear, heat transfer and mass transfer. Although the physical characteristics are inter-related, in scale-up and reactor design the mixing time, shear and mass transfer aspects are coupled while pH and heat transfer are considered separately. Unlike mass transfer

which takes place throughout the broth, heat transfer occurs only at the boundary surfaces of heat exchange. It is possible to provide the required heat transfer capacity of a large-scale fermenter by methods independent of process scale-up, such as by using a refrigerant rather than cooling water or by using an external heat exchanger. Consequently, equal heat transfer capacity traditionally has not been used as a basis for translating experimental results between different scales of operation. Similarly, proper pH control can be achieved independently by automatic addition of concentrate acids and bases in fermentation systems with an adequate dispersing mechanism (Ju and Chase, 1992).

The ethanol production was scaled-up from laboratory, semi-pilot, pilot plant and industrial scales (100 ml, 13, 125 and 45000 L, respectively), by *Saccharomyces cerevisiae* from concentrated apple juice, the same parameters have been monitored during cider fermentation. Small differences observed between cell growth when fermentations were carried out in flasks, semipilot and pilot reactors and gave the ethanol yields of 0.88, 0.99, 0.93 and 0.95 mol ethanol/mol sugar, The behavior was similar except for fermentations carried out in Erlenmeyer flasks which ethanol yield factor was slightly lower due to differences in the agitation power and in the particular geometry of each system (Roza *et al*., 2003). Sharma and coworkers (2004) studied the ethanol production using sunflower hulls as a substrate by *Trichoderma reesei* Rut C30 cellulase and *Saccharomyces cerevisiae* under optimized conditions in two fermenters, the results presented as revealed similar ethanol yield of 0.449 g/g and 0.446 g/g after 18 h of fermentation in 1 and 15 L fermentors, respectively (Sharma *et al*., 2004). Moreover, ethanol production yield after the scale up using optimum condition at laboratory scale of *Saccharomyces cerevisiae* (LPB-SC) from soybean molasses were no significant difference at laboratory (8 L), pilot scale (1 m^3) and industrial scale (10 m^3), with 169.8 L, 163.3 L and 162.7 L of absolute ethanol per dry molasses, respectively (Siqueira *et al*., 2008). Also the results from ethanol production by immobilized cells of *Saccharomyces cerevisiae* IR2 from sugar beet juice by comparing in 2 L and 50 L bubble bioreactors, there were no significant different in ethanol productivity (11.0 and 11.5 g/L/h, respectively) and yield (0.41 and 0.44 g/g, respectively) (Ogbona *et al*., 2001).

Objectives

1. To select the highest ethanol producing strain
2. To optimize the medium composition and environmental condition using raw glycerol from biodiesel process
3. To compare the ethanol production in the batch, fed-batch and two-stage fermentations
4. To study the ethanol production in 20 L-fermenter

CHAPTER 2

MATERAILS AND METHODS

1. Materials

1.1 Bacterial strains

Klebsiella pneumoniae SU32, *Klebsiella terrigena* SU3 and *Citrobacter freundii* SU17 were isolated from domestic wastewater. These enterobacteria have the optimum temperature of 37 °C (Sattayasamitsathit *et al*., 2010). They were kept in the Environmental Biotechnology Laboratory, Faculty of Agro-Industry, Department of Industrial Biotechnology, Prince of Songkla University, Thailand.

Enterobacter aerogenes TISTR1468 and *Klebsiella oxytoca* TISTR556 were purchased from Thailand Institute of Scientific and Technological Research (TISTR).

1.2 Glycerol

Raw glycerol was obtained from biodiesel production pilot plant at Faculty of Engineering, Prince of Songkla University, Thailand. Biodiesel was produced from waste cooking oil by conventional transesterification batch process using methanol as a reactant and sodium hydroxide as a catalyst (Tongurai *et al*., 2001). The solid raw glycerol (containing about 50% w/w glycerol) was used as a carbon source in the medium without purification.

1.3 Culture medium

Preculture medium (per liter) contained 20.0 g pure glycerol, 5 g yeast extract, 5 g tryptone, 7.0 g K_2HPO_4, 5.5 g KH_2PO_4, 1.0 g $(NH_4)_2SO_4$, 0.25 g $MgSO_4.7H_2O$, 0.021 g $CaCl_2.2H_2O$, 0.12 g $Na_2MoO_4.2H_2O$, 2.0 mg nicotinic acid, 0.172 mg Na_2SeO_3, 0.02 mg $NiCl_2$, and 10 ml trace element solution (0.5 g $MnCl_2.4H_2O$, 0.1 g H_3BO_4, 0.01g $AlK(SO_4)_2.H_2O$, 0.001 g $CuCl_2.2H_2O$ and 0.5 g Na_2EDTA (per liter)), pH 6.8 (Ito *et al*., 2005).

Fermentation medium contained the same compositions as preculture medium except that raw glycerol was used instead of pure glycerol.

Trypticasein soy agar (TSA) (per liter) contained 15 g casein, 5 g soy peptone, 5 g NaCl and 15 g agar. This medium was used for culture maintenance (Iversen, 2004).

1.4 Instruments

All instruments used in this work were given in Table 5.

Table 5. List of some instruments

Instrument name	Model	Source
Incubator shaker	G 25 - KLG	New Brunswick, USA
Spectrophotometer	U-2000	Hitachi, Ltd., Japan
pH meter	TOLEDO 320	Mettler, China
Hot air oven	ULM	Memmert, Germany
Autoclave	SS 325	Tomy, Japan
Refrigerated centrifuge	5403	Eppendorf, Germany
Laminar flow	V6	Clean, Thailand
GC-FID	HP 6890	Hewlette Packard, USA
Fermenter (3-L)	MDL-300	B.E.Marubishi, Japan
Fermenter (20-L)	BioFlo 415	New Brunswick, USA

2. Analytical methods

2.1 Determination of dry cell weight (DCW)

Dry cell weight (DCW) was determined by centrifuging the sample for 8 min at 8000*g* and 4 °C. The cell precipitated was washed and dried at 105 °C overnight and then weighed (Barbirato *et al*., 1997).

2.2 Determination of ethanol concentration

Ethanol concentration of glycerol fermentation was determined by gas chromatography (GC) using a capillary column (model number J&W 123-3232 DB-FFAP, packed with polyethylene glycol) with flame ionization detector (Hewlette Packard 6890) to separate and determine the amount of volatile components of a very small sample. The column was operated at a temperature of 250 °C, with He (helium) as carrier gas. The sample was loaded at the injection port (via a hypodermic syringe) which was heated in order to volatilize the sample. Once in the gas phase, the sample was carried onto the column by the carrier gas (modified from Jennings, 2001). Ethanol concentration was calculated by standard curve (Appendix Fig. 3-A)

2.3 Determination of glycerol concentration

Glycerol was spectrophotometrically determined by chromotropic acid method (modified from Handel, 1961 by Dr. Ausa Chanumpai). Sample (0.1 mL) was added into capped test tube and 0.1 mL of 0.5% sodium metaperiodate was added, shaked, and standed at room temperature for 10 min. 0.1 mL of 5% sodium metabisulfite was added and mixed and standed for 10 min. Chromotropic acid solution (3.0 mL) was added, shaked, and put in boiling water bath (100 °C, 30 min), and allowed to cool to room temperature, before adding 0.3 mL of thiourea. After that, samples were determined for optical density of 570 nm within 2 h using spectrophotometer. In case of blank reagent, distilled water was used instead of the sample.

3. Methods

3.1 Selection of the highest ethanol producing strain

Five bacterial strains, *Klebsiella pneumoniae* SU32, *Klebsiella Terrigena* SU3 and *Citrobacter freundii* SU17, *Enterobacter aerogenes* TISTR1468 and *Klebsiella oxytoca* TISTR556 were cultivated in 50 mL of the fermentation medium in the 125 mL flask and incubated at 37 °C (the optimum temperature obtained from the preliminary results, data not shown) and 120 rpm for 16-18 h. The cultures were diluted with the fresh fermentation medium to OD_{600} of 0.5 before using as a starter.

The cultivation was performed in 90 mL of the fermentation medium in 250 mL flask with 10 mL starter at 37 °C and 120 rpm. Sample (7 mL) was taken every 4 h up to 24 h cultivation to measure for pH, dry cell weight, glycerol, and ethanol concentrations. The bacterial strain which gave the highest ethanol concentration was selected for the production of ethanol in this work.

3.2 Optimization of ethanol production using raw glycerol from biodiesel process

3.2.1 Optimization of fermentation medium using conventional methodology

Starter preparation

Starter culture was prepared by inoculating the bacteria into a 125 mL flask containing 50 mL of the fermentation medium (20 g/L pure glycerol) and cultivated under aerobic conditions on a shaker (120 rpm) at 37 °C for 16-18 h. The culture was diluted with the fresh medium to obtain OD_{600} of 0.5 before using as the starter culture.

Time course for ethanol production

Cultivation in a 250 mL shake flask containing 90 mL of fermentation medium with the addition of 10 mL starter culture was conducted at 37 °C and 120 rpm. Sample (7 mL) was taken every 4 h up to 24 h cultivation to measure for pH, glycerol and, ethanol concentration. The time giving the highest ethanol concentration was selected for further studies. The influence of the following parameters was investigated and the best result in each experiment was selected for the subsequent studies. The experimental data was represented averages of triplicate treatments and statistics was analyzed using program of Statistical Package for the Social Sciences (SPSS) version 16.0 (SPSS Inc.).

3.1.1 Study the effect of aerate on ethanol production

In this study the effect of oxygen was investigated. Four conditions of cultivation for ethanol production by bacteria were studied. The conditions include 1) in shake flask with 120 rpm of agitation speed 2) in shake flask without agitation 3) in serum bottle and 4) in serum bottle with flushing nitrogen gas. Samples were taken every 4 h in the 24 h cultivation to measure for pH and ethanol concentration

3.2.1.1 Effect of initial raw glycerol concentration

Effect of raw glycerol concentration (50% glycerol w/w) from biodiesel plant on the ethanol production was studied in the range of 10-50 g/L.

3.2.1.2 Effect of nitrogen sources and concentration

Effect of various inorganic nitrogen sources: ammonium chloride (NH_4Cl), ammonium acetate (CH_3COONH_4), ammonium nitrate (NH_4NO_3), ammonium phosphate [$(NH_4)_2HPO_4$] and ammonium sulfate [$(NH_4)_2SO_4$] (base on equal nitrogen concentration (mol) to 1 g/L $(NH_4)_2SO_4$ in complex medium) was studied on ethanol production. Inorganic nitrogen source which gave the highest ethanol concentration was used to study the concentration in the range of 0-3.0 g/L.

3.2.1.3 Effect of organic nitrogen source and concentration

Effect of various organic nitrogen sources (5 g/L): yeast extract, tryptone, peptone and malt extract was studied on ethanol production. Organic nitrogen source which gave the highest ethanol concentration was used to study the effect of its concentration in the range of 0-10 g/L.

3.2.2 Optimization of the medium for ethanol production by mathematical modeling

3.2.2.1 Experimental design

Based on the best results from conventional method, the selected factors were studied for their interactive behaviors using a statistical approach (Response surface methodology). The Centered Composite Design (CCD) was employed to analyze the

experimental design. The levels of three major variables significantly affecting ethanol concentration from the selected strain were selected for this study. The design matrix with three variables set at five levels (-α, -1, 0, +1, +α) were calculated using the statistical software package 'Design-Expert®6.0 Stat-Ease, Inc. (Minneapolis, USA). Code and real values of the three selected factors were given in Table 6.

The experiments were performed in 250 mL shake flask containing 90 mL medium with the addition of 10 mL starter culture, cultivated under 120 rpm shaking speed at 37 °C for 24 h. Samples were taken to measure for pH, glycerol and ethanol concentrations. To examine the combined effect of the medium components on ethanol production, the total of 20 experiments were performed as shown in Table 7.

3.2.2.2 Statistical analysis and modeling

The data on ethanol production was subjected to analysis of variance (ANOVA) appropriate to the design of the experiments. The mathematical relationship of the independent variable and the response (ethanol production) were calculated by the second order polynomial equation;

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{1,1} A^2 + \beta_{2,2} B^2 + \beta_{3,3} C^2 + \beta_{1,2} AB + \beta_{1,3} AC + \beta_{2,3} BC \quad (6)$$

where Y is response variable (ethanol production), β_0 is intercept, β_1 β_2 β_3 are linear coefficients, $\beta_{1,1}$, $\beta_{2,2}$, $\beta_{3,3}$ are squared coefficients, $\beta_{1,2}$, $\beta_{1,3}$, $\beta_{2,3}$ are interaction coefficients, and A, B, C, A^2, B^2, C^2, AB, AC, BC are level of independent variables (Bandaru *et al*., 2006)

3.2.2.3 Model validation and confirmation

The predicted condition obtained by RSM was selected and confirmed. The experiments were operated in shake-flask with optimum condition which is obtained from section 3.2.2.1. The percentage of derivation between the predicted and experimental value of ethanol production was investigated.

Table 6. Real and code values of independent variables in the experimental plan[a]

Factor	Name	Level				
		-α	1	0	+1	+α
A	Crude glycerol	13.18	20.00	30.00	40.00	46.82
B	$(NH_4)_2HPO_4$	-0.85	0.00	1.25	2.50	3.35
C	Yeast extract	0.80	2.50	5.00	7.50	9.20

[a]α = 1.628, Unit = (g/L),

Table 7. Experimental design with the code value

Exp. no	A: Glycerol (g/L)	B: $(NH_4)_2HPO_4$ (g/L)	C: Yeast extract (g/L)
1	0	-α	0
2	0	0	0
3	-1	-1	+1
4	-α	0	0
5	+α	0	0
6	-1	+1	+1
7	0	0	0
8	0	0	0
9	+1	-1	-1
10	0	0	-α
11	+1	+1	-1
12	-1	-1	-1
13	0	0	+α
14	0	+α	0
15	0	0	0
16	-1	+1	-1
17	0	0	0
18	0	0	0
19	+1	-1	+1
20	+1	+1	+1

3.2.3 Effect of environmental conditions

The effect of environmental conditions on the ethanol production was studied in 3 L fermenter with 2 L working volume of the optimized medium obtained from response surface methodology (RSM).

3.2.3.1 Effect of initial pH

The inoculum (10%) was added into a 3 L fermenter containing 2 L the optimal medium (from section 3.2.2), cultivated at 37 °C, 0.5 vvm aeration rate and 120 rpm agitation speed. Effect of initial pH at 6.5, 7.0, 7.5, 8.0 and 8.5 was studied without pH-control.

3.2.3.2 Effect of controlling pH

The inoculum (10%) was added into a 3 L fermenter containing 2 L the optimal medium (from section 3.2.2), cultivated at 37 °C, 0.5 vvm aeration rate and 120 rpm agitation speed. Cultivations were carried out under uncontrolled and controlled-pH at the initial pH values (from section 3.2.3.1).

3.2.3.3 Effect of aeration rate

The inoculum (10%) was added into a 3 L fermenter containing 2 L the optimal medium (from section 3.2.2), cultivated at 37 °C, and 120 rpm agitation speed. Cultivations were carried out under different aeration rates (0, 0.25, 0.5, and 1.0 vvm).

3.2.3.4 Effect of agitation speed

The inoculum (10%) was added into a 3 L fermenter containing 2 L optimal medium (from section 3.2.2), cultivated at 37 °C, and selected aeration rate (section 3.2.3.3). Cultivations were carried out under the different agitation speeds at 0, 60, 120 and 180 rpm.

3.3 Study on the kinetics of the ethanol production in the batch, fed-batch and two-stage process

Starter preparation

The culture of selected bacteria from section 2.1 was inoculated into the preculture medium (200 ml) in a 500 ml flask and cultivated on a rotary shaker (120 rpm) at 37^{o}C for 18 h. The culture was diluted with the preculture medium to obtain OD_{600} of 0.5.

3.3.1 Study on the kinetics of the ethanol production in batch culture

The starter culture (10% v/v) was added into 3 L fermenter with a 2 L working volume of the optimum medium and fermentation conditions (from section 3.2). Samples were taken to measure for dry cell weight, glycerol and ethanol concentrations.

The following kinetics parameters were determined by using equation 7-16. The kinetic values are determined by cultivation in the fermenter with various initial crude glycerol concentrations between 10 and 60 g/L.

Cells growth;

$$\frac{dX}{dt} = \mu X \tag{7}$$

$$InX = InX_0 + \mu t \tag{8}$$

$$\mu = \frac{In(\frac{X}{X_0})}{t} \tag{9}$$

$$\mu = \frac{\mu_{\max} S}{K_s + S + \frac{S^2}{K_I}}[P] \tag{10}$$

Product formation;

$$\frac{dP}{dt} = \rho X \tag{11}$$

$$\rho X = Y_{P/S}\frac{dX}{dt} \tag{12}$$

Substrate utilization;

$$-\frac{dS}{dt} = \frac{1}{Y_{x/s}}\left(\frac{dX}{dt}\right) + \frac{1}{Y_{p/s}}\left(\frac{dP}{dt}\right) \quad (13)$$

$$S_{opt} = \sqrt{K_s K_I} \quad (14)$$

$$Y_{P/S} = \frac{\Delta P}{\Delta S} \quad (15)$$

$$Y_{X/S} = \frac{\Delta X}{\Delta S} \quad (16)$$

Where, substrate concentration (S), cell concentration (X), initial cell concentration (X_0), production concentration (P), the specific growth rate (μ), maximum specific growth rate (μ_{max}), amount of biomass produced (ΔX), amount of product produce (ΔP), amount of substrate utilized (ΔS), cellular yield coefficient ($Y_{X/S}$), conversion yield of substrate to product ($Y_{P/S}$), specific production rate (ρ), optimum substrate concentration (S_{opt}), saturation constant (K_s) and substrate inhibition constant (K_I) (Phisalaphong *et al*., 2006).

3.3.2 Ethanol production in repeated batch fermentation

Repeated batch fermentation was performed in 3 L fermenter with the fixed working volume of 2 L (1.8 L of the optimum medium and 200 mL of starter culture), cultivated at 37 ^{o}C under the optimum condition (section 3.2.3). In these experiments, fermentation was first carried out in the batch mode until 24 h, then the 500 mL fermentation broth was removed and 500 mL fresh fermentation medium was added and cultivated until 48 h. The last batch was started after 48 h by removing 500 mL fermentation broth and 500 mL fresh fermentation medium was fed in and cultivated until 72 h.

The effect of glycerol concentration (40 and 50 g/L) in fermentation medium was studied. The samples were taken every 4 h until 72 h for measurement of pH, dry cell weight, glycerol, and ethanol concentrations.

3.3.3 Ethanol production in variable volume intermittent fed-batch

In this experiment, the fermentation was performed in 3 L fermenter with 1 L working volume (0.9 L of medium and 100 mL of starter culture), cultivated at 37 ^{o}C, under the optimum condition (section 3.2.3). The fermentation was first carried out in batch mode until 24 h fermentation. After 24 and 48 h fermentation, the 500 mL of fresh fermentation medium was added and cultivated until 72 h.

The effect of glycerol concentration (40 and 50 g/L) in fermentation medium was studied. The samples were taken every 4 h until 72 h for measurement of pH, dry cell weight, glycerol, and ethanol concentrations.

Ethanol production by *Saccharomyces cerevisiae* in fed-batch bioreactor, the mathematical models consisting of differential and algebraic equations are given as follow;

Cells growth;

$$\frac{dX}{dt} = \mu X - \frac{X}{V}F \tag{17}$$

Substrate utilization;

$$\frac{dS}{dt} = -\frac{\mu X}{Y_{x/s}} + \frac{(S_0 - S)}{V}F \tag{18}$$

Product formation;

$$\frac{dP}{dt} = \rho X - \frac{P}{V}F \tag{19}$$

$$\frac{dV}{dt} = F \tag{20}$$

Where X, S and P are the concentration of cell mass, substrate, and product (ethanol), respectively, V is the liquid volume within the reactor, μ is the specific growth rate, ρ is the specific productivity, $Y_{x/s}$ is the yield coefficient, S_0 is the feed concentration of substrate, and F is the feed flow rate into the fed-batch reactor which

is the only manipulated input in this process (Arpornwichanop and Shomchoam, 2007).

3.3.4 Two-stages process for ethanol production

In this experiment, fermentation was carried out in 3 L fermenter with 2 L working volume (1.8 L of the optimum medium and 200 mL of starter culture), cultivated at 37 ^{o}C, under the optimum condition (section 3.2.3) until 24 h fermentation. After 24 h, the second stage was started and performed under anaerobic condition. Fermentation was conducted at 37 ^{o}C, under the optimum condition (section 3.2.3) without aeration until 48 h. The samples were taken every 4 h until 48 h for measurement of pH, dry cell weight, glycerol, and ethanol concentrations.

3.3.5 Ethanol production in continuous fermentation

Continuous experiments were performed in 1.5 L fermenter with 1 L working volume (900 mL of the optimum medium and 100 mL of starter culture), cultivated at 37 ^{o}C, under the optimum condition (section 3.2.3). The batch operation was conducted until 24 h and then the continuous operation was started by feeding the fresh fermentation medium with a desired flow rate. The hydraulic retention time (HRT) was varied by changing the feed flow rate at HRT 30, 24, 18 and 12 h, respectively (with the dilution rate of 0.033, 0.042, 0.056 and 0.083 h^{-1}, respectively). The samples were withdrawn from the fermentation broth every 24 h for measurement of pH, dry cell weight, glycerol, and ethanol concentrations. The kinetic parameters of the continuous fermentation were determined on steady state operation. The Monod model was suitable to represent the kinetics as follow;

$$\frac{1}{\mu} = \frac{1}{D} = \frac{K_S}{\mu_m S} + \frac{1}{\mu_m} \qquad (21)$$

Where, substrate concentration (S), the specific growth rate (μ), maximum specific growth rate (μ_{max}), dilution rate (D), saturation constant (K_s) and substrate inhibition constant (K_I)

3.4 Ethanol production in 20 L fermenter

In this study, the optimum aeration rate and agitation speed (results from 3 L fermenter) were used. Starter culture for 20 L fermentation was performed in a 3 L fermenter with 2 L working volume (1.8 L of the optimum medium and 200 mL of starter culture), at 37 ^{o}C and initial pH 8.0 without pH-control. Batch cultivation in 20 L fermenter with 15 L working volume (13.5 L fermentation medium and 1.5 L starter culture). The cultivation was performed under micro-aerobic condition, anaerobic condition and two-stage process. The samples were taken every 6 h until 48 h for measurement of pH, dry cell weight, glycerol, and ethanol concentrations.

3.5 Ethanol production from pure glycerol by immobilized cells

3.5.1 Preparation of carrier materials for immobilization

Sponge, rapeseed straw, activated carbon and UASB granule were used as the material for immobilization.

Sponge was cut into 0.5 x 0.5 x 0.5 cm cubes then washed with distilled water and soaked in distilled water overnight to remove the contaminant particles.

Activated carbon (Filtrasorb 400) (diameter 0.75 mm) was purchased from Chemviron Carbon Aps, Copenhagen, Denmark. The activated carbon was boiled at 100 ºC for 1 h to degas and removes contaminants such as salt, strong acid and base which used during the activation production process then washed with distilled water.

UASB granules were obtained from a full-scale biogas plant (55 °C) in Denmark. UASB granules were boiled at 100 ºC for 2 h to inhibit methanogenic bacteria after that sterilized at 121 ºC for 1 h (repeated three times) to inhibit all of microorganisms in the UASB granules.

Rapeseed straw was cut into 0.1 x 0.3 x 1.0 cm rectangles and then boiled in 1% NaOH for 1 h to remove lignin and fibers which might react with the cell after that washed three times with distilled water and soaked in distilled water overnight. The ratio of rapeseed straw and 1% NaOH was 1:10 (w/v).

Rapeseed straw, sponge and activated carbon were sterilized at 121 ºC for 15 min after that dry at 105 ºC for 30 min to remove water prior usage.

3.5.2 Immobilization of cells

The culture of selected bacteria (from section 3.1) was inoculated into the preculture medium (200 mL) in a 500 mL flask and cultivated on a rotary shaker (120 rpm) at 37 ^{o}C. After 24 h, 1.0 g sterile carriers (section 3.5.1) was added and cultivated at 37 ^{o}C, 120 rpm for 24 h. After 24 h, 100 mL fermentation broth was withdrawn and 100 mL fresh medium was refilled. This step was repeated at 48 and 72 h. After 96 h, the fermentation broth was removed and the immobilized carriers were washed with sterile distilled water.

3.5.3 Selection of the carrier material on the ethanol production

The ethanol production by the immobilized cells on carrier material (from section 3.5.1) was studied compare with the free cells. The fermentation was carried out in 250 mL flask with 100 mL preculture medium and cultivated at 37 ^{o}C, initial pH 8.0 and 120 rpm shaking speed. Fermentation broth of 90 mL was withdrawn and 90 mL fresh preculture medium was added every 24 h until 10 days. The samples were taken every 24 h for measurement ethanol concentrations. The carrier material that gave the highest ethanol production was selected and photographed by scanning electron microscopy (SEM).

3.5.4 Effect of product inhibition on ethanol production

The immobilized cells with the selected carrier (giving the highest ethanol production) (from section 3.5.2) was used to study on the effect of production inhibition and compared with the free cells. The fermentation was carried out in 250 mL flask with 100 mL preculture medium initial pH 8.0, at 37 ^{o}C, and 120 rpm shaking speed for 24 h. Effect of production inhibition was studied by adding 10-50 g/L ethanol in the preculture medium. The samples were taken at 24 h for measurement of pH and ethanol concentration.

CHAPTER 3

RESULTS AND DISCUSSIONS

1. Selection of the highest ethanol producing strain

Fermentation of glycerol for ethanol production occurs in different bacterial groups. Under anaerobic or micro-aerobic conditions, glycerol can be used as a substrate for growth by bacteria belonging to the genera *Klebsiella, Clostridia, Citrobacter* and *Enterobacter* (Deckwer, 1995; Chen *et al*, 2003). During fermentation, glycerol was oxidized to glyceraldehyde-3-phosphate via dihydroxyacetone and via pyruvate for converting to ethanol as well as the other products such as acetate, formate, CO_2, butanol, etc (Barbirato *et al*, 1997).

Five strains of ethanol producing bacteria were cultivated in the fermentation medium containing 20 g/L pure and raw glycerol as a carbon source. All of them could produce ethanol from glycerol (Fig. 5). The highest ethanol production was obtained from *Enterobacter aerogenes* TISTR1468 followed by *Klebsiella oxytoca* TISTR556, *Klebsiella terrigena* SU3, *Klebsiella pneumoniae* SU32, and *Citrobacter freundii* SU17 with the values of 7.54, 5.77, 3.26, 3.24 and 2.58 g/l, respectively using pure glycerol and 7.24, 5.22, 3.06, 3.02, and 2.34 g/L, respectively using raw glycerol. The ethanol concentration using pure glycerol was slightly higher than those using raw glycerol which contained impurities such as methanol, non-glycerol organic matter, potassium and sodium salts, and water obtained from the process of biodiesel production (Mu *et al*, 2006; Sattayasamitsathit *et al*., 2011). The results indicated that the impurities in the raw glycerol had no effect on the ethanol production. This agreed with the results of Ito and co-workers (2005) that the ethanol yield on commercial glycerol was higher than raw glycerol with the yield of 0.43 and 0.34 g/g, respectively.

The ethanol production (ΔP), glycerol consumption (ΔS), productivity (ΔP/t) and ethanol yield ($Y_{P/S}$) of five the strains on pure and raw glycerol are shown in Table 8 and Table 9, respectively. The high glycerol consumption, productivity and yield was also achieved from *E. aerogenes* TISTR1468 (19.23 g/L, 0.38 g/L/h and

0.39 g/g, respectively on pure glycerol), and also gave 17.75 g/L, 0.45 g/L/h and 0.41 g/g on raw glycerol, respectively. The ethanol yield ($Y_{P/S}$) from this strain was 1.2 folds higher than that from *E. aerogenes* HU-101 grown on waste glycerol (Ito *et al*., 2005). However, these results showed ethanol yield lower on raw glycerol lower than ethanol yield of *Aerobacter aerogenes* 1033 of 0.43 g/g (Makasanik *et al*., 1953) and Engineering *E. coli* SY03 of 0.51 g/g (Wu *et al*., 2011). Therefore, *Enterobacter aerogenes* TISTR1468 was selected to optimize for ethanol production using raw glycerol from biodiesel plants as a substrate.

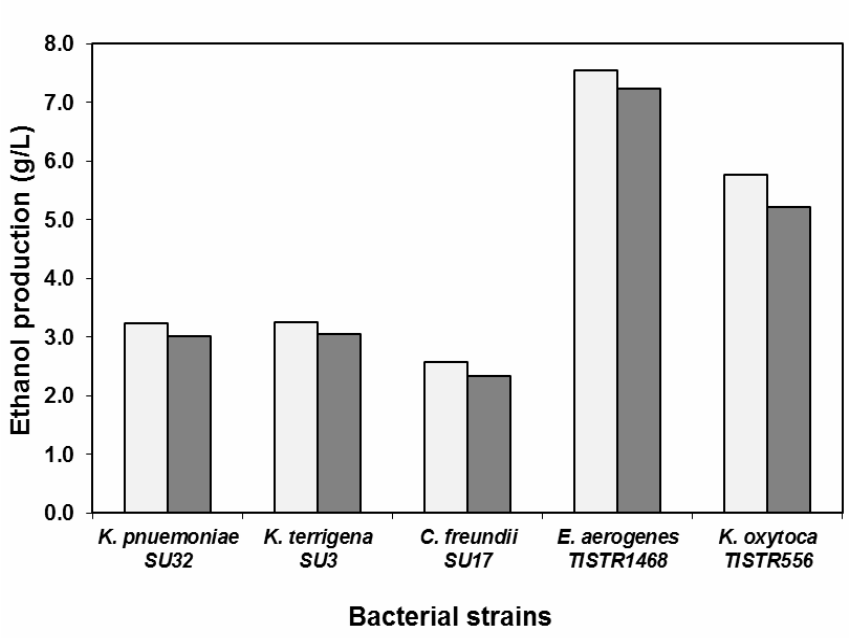


Figure 5. The maximum ethanol production on 20 g/L pure glycerol (□) and raw glycerol (■) by five strains of bacteria in shake-flask culture at 37 ^{o}C, and 120 rpm shaking speed for 24 h

Table 8. Maximum ethanol, glycerol consumption, yield and productivity on pure glycerol by five strains of bacteria (in shake-flask culture at 37 ^{o}C, and 120 rpm shaking speed for 24 h)

Strain	Pure glycerol*				
	Time (h)	ΔP (g/L)	ΔS (g/L)	ΔP/t (g/L/h)	$Y_{P/S}$ (g/g)
K. pnuemoniae SU32	20	3.24	11.13	0.16	0.29
K. terrigena SU3	16	3.26	10.19	0.20	0.32
C. freundii SU17	20	2.58	10.75	0.13	0.24
E. aerogenes TISTR1468	20	7.54	19.23	0.38	0.39
K. oxytoca TISTR556	16	5.77	11.47	0.36	0.50

* Initial glycerol concentration = 20 g/L

Table 9. Maximum ethanol, glycerol consumption, yield and productivity on raw glycerol by five strains of bacteria (in shake-flask culture at 37 ^{o}C, and 120 rpm shaking speed for 24 h)

Strain	Raw glycerol*				
	Time (h)	ΔP (g/L)	ΔS (g/L)	ΔP/t (g/L/h)	$Y_{P/S}$ (g/g)
K. pnuemoniae SU32	20	3.02	9.53	0.15	0.32
K. terrigena SU3	16	3.06	6.68	0.19	0.46
C. freundii SU17	20	2.34	8.01	0.12	0.29
E. aerogenes TISTR1468	20	7.24	17.75	0.45	0.41
K. oxytoca TISTR556	16	5.22	11.68	0.33	0.45

* Initial glycerol concentration = 20 g/L

2. Optimization of ethanol production using raw glycerol from biodiesel plant

2.1 Medium optimization for ethanol production by conventional methodology

The effect of nutrient compositions using raw glycerol as a sole of carbon source on ethanol production by *Enterobacter aerogenes* TISTR1468 was stepwise investigation in the fermentation medium (as described above in Chapter 2).

2.1.1 Study the effect of aerate on ethanol production

Comparison between growth and ethanol production on raw glycerol of *Enterobacter aerogenes* TISTR1468 at 37 ˚C and four conditions was illustrated in Fig. 6 and 7. Cell growth was determined by measured pH values, pH values could be as an indicator for indicating the growth cell. The pH values from Fig. 6 the growth cell on pure glycerol at 120 rpm in shake flask was better than the other conditions. The pH values at 120 rpm of agitation speed gave the maximum decreasing of pH values (≈ pH 5.6). The pH value on four conditions gradually decreased after 12 h of cultivation the growth cell toward to stationary phase the constant pH values. Therefore from this results can conclude that the cultivation in the condition which gave the air or agitate supported the cell growth.

Comparison of ethanol production by *Enterobacter aerogenes* TSTR1468 (Fig. 7) at four conditions, ethanol was produced associate with the growth cell. Ethanol production in the shake flask with 120 rpm of agitation speed was better than the other conditions as well as the cell growth. The highest of ethanol concentration was given at 12 h of cultivation that gave 4.84 g/L, maximum productivity was 0.426 g/L/h that was given at 8 h of cultivation. The maximum ethanol concentration which given from the shake flask without agitation speed, in serum bottle and in serum bottle with nitrogen gas were 2.92, 1.90 and 1.59 g/L, respectively. The ethanol concentration and the productivity were showed in the table 10.

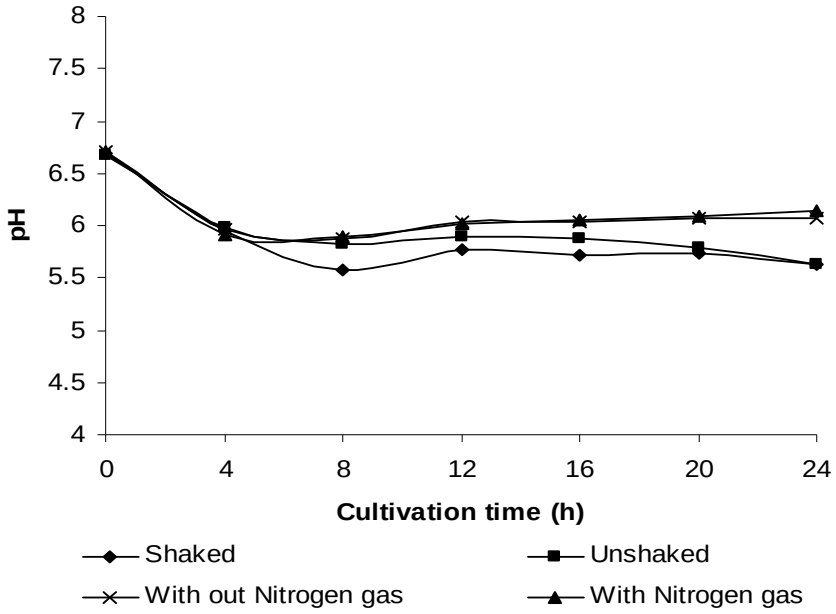


Figure 6. Time profile of pH of *E. aerogenes* TISTR1468 on different culture condition at 37 ºC for 24 h

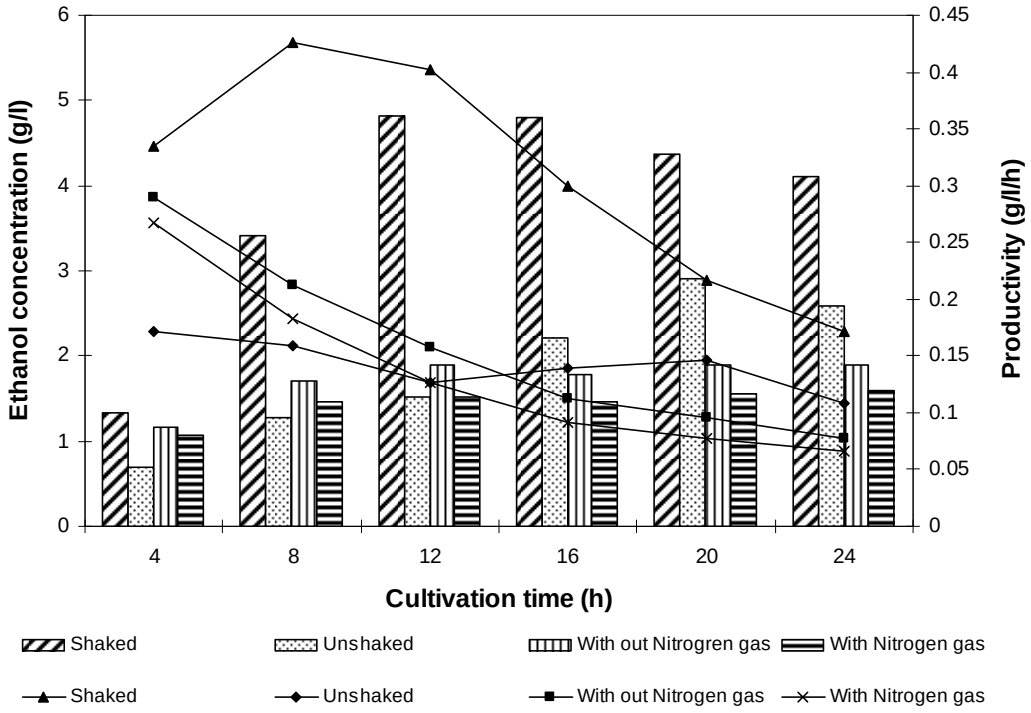


Figure 7. Time profile of ethanol production (bar symbols) and productivity (line symbols) of *E. aerogenes* TISTR1468 on different culture condition at 37 ºC for 24 h

Table 10. Maximum ethanol concentration and productivity of *E. aerogenes* TISTR1468 on different culture condition at 37 ºC for 24 h

Condition	Maximum ethanol concentration (g/L)	Maximum productivity (g/L/h)
Shake flask (120 rpm)	4.84	0.426
Shake flask (0 rpm)	2.92	0.171
Serum bottle	1.90	0.289
Serum bottle (Nitrogen gas)	1.59	0.267

From this experiment could be concluded that the aeration effected for cell growth and ethanol production by *Enterobacter aerogenes* TISTR1468. The maximum growth rate and ethanol concentration were obtained at the condition which gave the air or shaking. Therefore, the condition at 120 rpm of agitation speed in shake flask will be used as a suitable condition for the production of ethanol from glycerol.

2.1.2 Effect of raw glycerol concentration

Time course on the changed of pH, glycerol concentration and ethanol production at various concentration of initial raw glycerol was shown in Fig. 8, respectively. Higher concentration of raw glycerol from 10 up to 20, and 30 g/L resulted in lower values of final pH of 6.17 (at 4 h), 5.69 (at 8 h) and 4.9 (at 16 h), respectively. At concentrations higher than 30 g/L the final pH was 4.9. The decrease of pH in the fermentation was due to the fact that the strain produced acidic by-products such as acetic acid, succinic acid, (etc) via oxidative pathway (Barbirato *et al*., 1997).

Time course of glycerol utilization (Fig. 8B), showed that at low glycerol concentrations (10-20 g/L), glycerol was completely utilized at 8 h which gave the consumption rate of 1.09 and 1.41 g/L/h, respectively. At higher glycerol concentration (30, 40 and 50 g/L), glycerol was not utilized completely within 24 h, 70-85% glycerol was converted to ethanol with the consumption rate of 1.89, 1.87 and 1.75 g/L/h, respectively.

Ethanol concentration increased with the increase of raw glycerol concentration (Fig. 8C). The initial raw glycerol of 10 g/L gave the lowest ethanol concentration (4.10 g/L at 8 h) with the productivity of 0.513 g/L/h, as all glycerol was consumed completely in a short time. Cultivation at the initial raw glycerol concentration of 20, 30, 40 and 50 g/L, the ethanol production was 8.61, 10.08, 9.54 and 7.97 g/L with the productivity of 0.72, 0.84, 0.80 and 0.50 g/L/h, respectively. The decrease of ethanol production and productivity at above 30 g/L was because of the effect of substrate inhibition and impurities that interfere with cell growth and ethanol production, respectively (Ito *et al*., 2005; Choi *et al*., 2011).

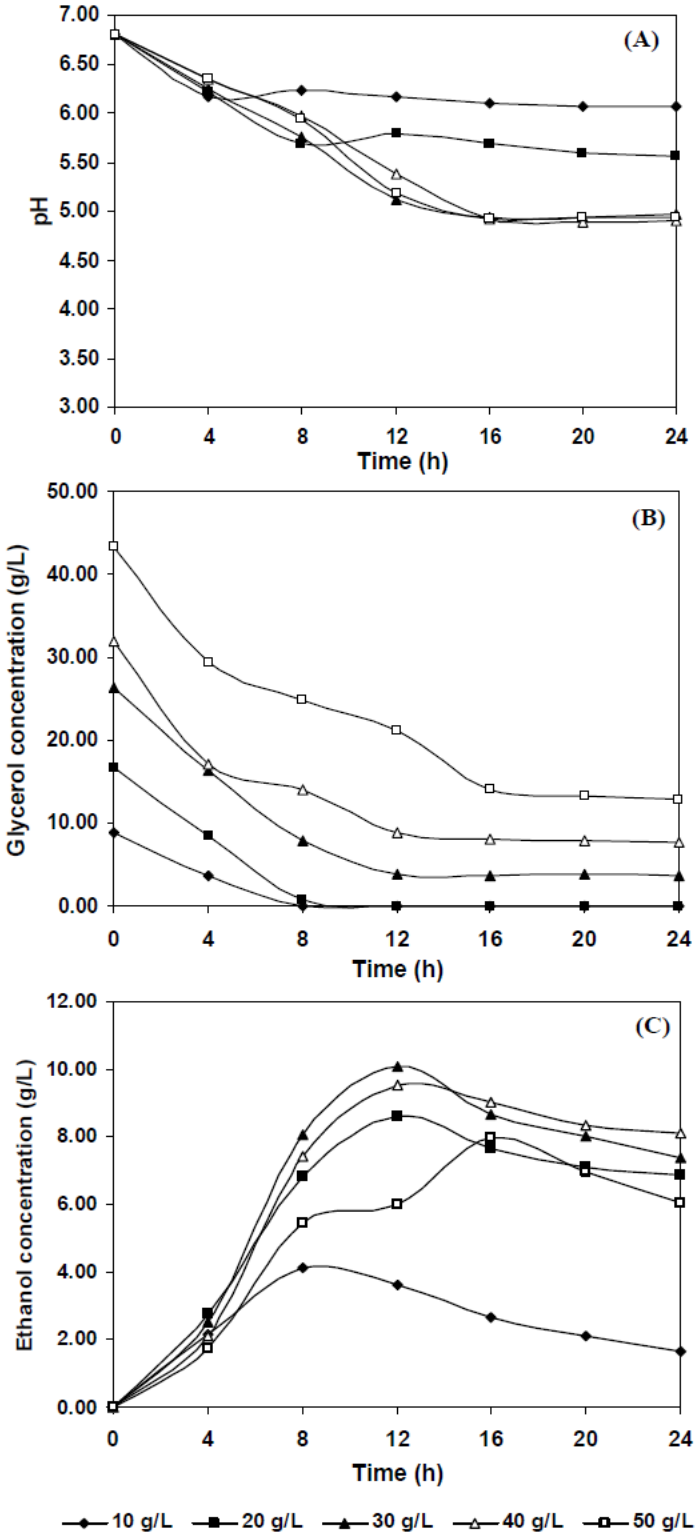


Figure 8. Time profile of pH (A), glycerol concentration (B) and ethanol concentration (C) of *E. aerogenes* TISTR1468 on various initial raw glycerol concentrations in shake-flask culture at 37 ºC, 120 rpm for 24 h

The optimum glycerol concentration for ethanol production at 30 g/L was higher than that (25 g/L) from *Enterobacter aerogenes* HU-101 (Ito *et al*., 2005). On the other hand, the inhibitory level of crude glycerol was about 5% (v/v) for *Pachysolen tannophilus* (Liu *et al*., 2012). Also the inhibition on growth of *Clostridium butyricum* VPI 3266 was evident when raw glycerol (65% w/v) was used and a growth inhibition of 86% was observed when the medium contained 100 g/L of glycerol (Pajuelo *et al*., 2004). In addition, growth of *C. butyricum* DSM 5431 was inhibited by 59% using 100 g/L of commercial glycerol (Petitdemange *et al*., 1995). The effect of initial glycerol concentration on the maximum ethanol, productivity and ethanol yield were shown in Fig. 9. The maximum ethanol production and productivity were 10.08 g/L and 0.84 g/L/h at 30 g/L glycerol.

The effect of initial glycerol concentration on the maximum ethanol production, productivity, glycerol consumption rate and ethanol yield were shown in Fig. 10. The productivity and glycerol consumption rate increased with the increasing glycerol concentration upto 40 g/L. The highest ethanol productivity and glycerol consumption rate (0.84 and 1.89 g/L/h, respectively) were obtained at 30 g/L raw glycerol. Whereas the highest ethanol yield of 0.51 g/g was obtained at 20 g/L raw glycerol. Higher glycerol concentration (50 g/L) gave the lowest productivity and ethanol yield (0.50 g/L/h and 0.28 g/g, respectively) that was the result from the effect of substrate inhibition (as describe above).

To know the best result for next experiment, there is a need to select the specific variable using statistical analysis by SPSS program. The results concluded that the best cultivation of 30 g/L raw glycerol gave the highest ethanol production, productivity and glycerol consumption rate of 10.08 g/L, 0.84 g/L/h, and 1.89 g/L/h, respectively. Therefore, the experiment using initial raw glycerol concentration 30 g/L was selected for further studies.

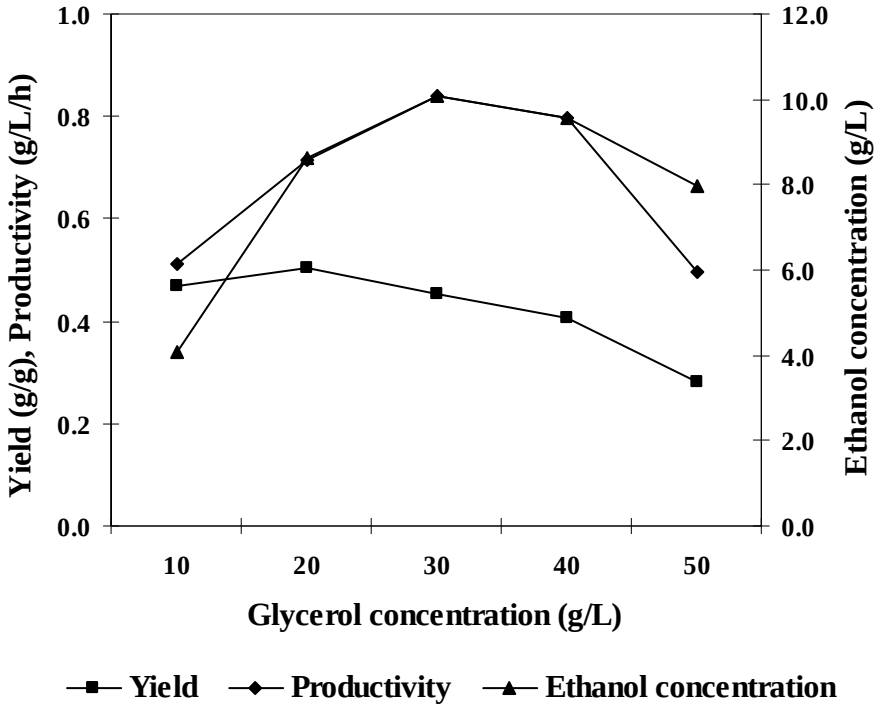


Figure 9. Effect of raw glycerol concentration on ethanol yield, productivity and ethanol production of *E. aerogenes* TISTR1468 in shake-flask culture at 37 ºC, 120 rpm for 24 h

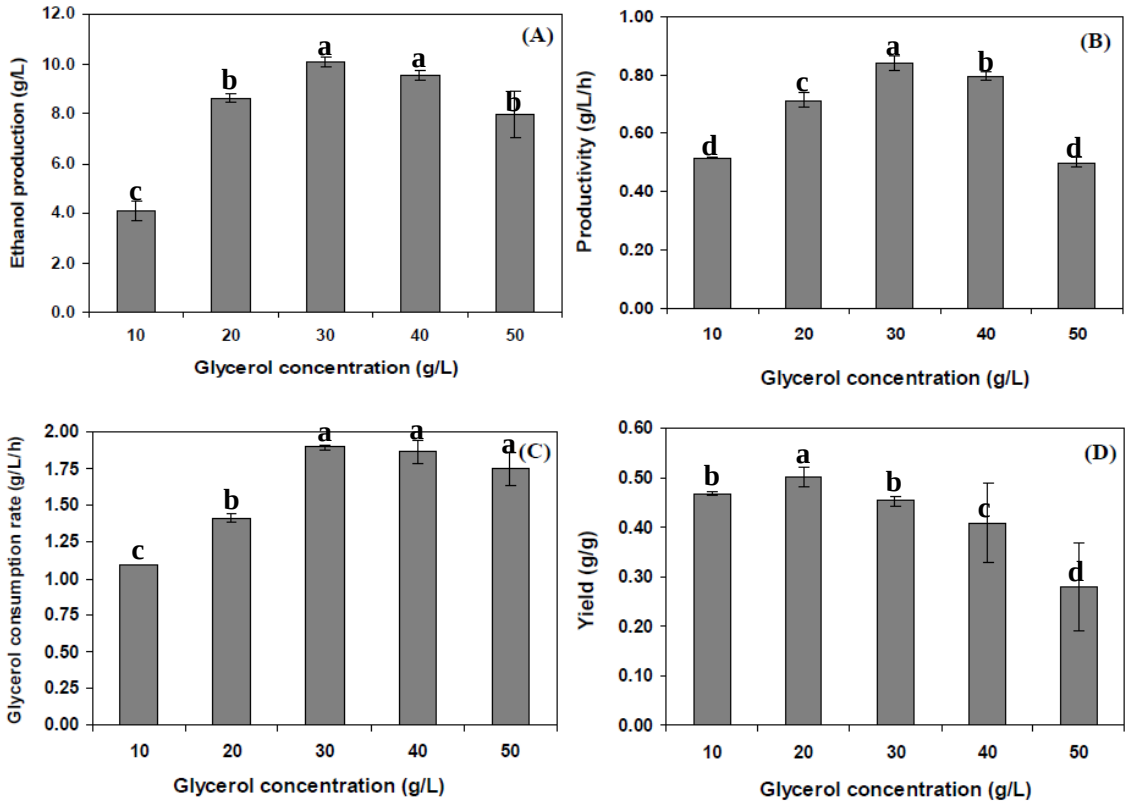


Figure 10. Effect of raw glycerol concentration on ethanol production (A), productivity (B), glycerol consumption rate (C) and ethanol yield (D) of *E. aerogenes* TISTR1468 in shake-flask culture at 37 ºC, 120 rpm for 24 h. The different superscripts in the bar graph denote a significant difference ($p < 0.05$)

2.1.3 Effect of inorganic nitrogen sources and concentration

Cultivation of *E. aerogenes* TISTR1468 in the medium with 30 g/L raw glycerol with five different inorganic nitrogen sources was conducted in shaking flask. Among nitrogen sources tested, $(NH_4)_2HPO_4$ gave the highest ethanol concentration (12.74 g/L), followed by using NH_4NO_3 (12.60 g/L) (Fig. 11A). They were 1.25 and 1.24 folds higher than the control ($(NH_4)_2SO_4$) (10.20 g/L). NH_4Cl gave the highest ethanol productivity and glycerol consumption rate of 0.91 and 1.76 g/L/h, respectively, whereas the highest ethanol yield of 0.57 g/g was obtained using NH_4NO_3 (Fig. 11B-11D).

Statistical analysis using SPSS program showed that the highest ethanol production, productivity and glycerol consumption rate were obtained from using $(NH_4)_2HPO_4$ as inorganic nitrogen source. This result was the same as the previous study on the ethanol production of *E. aerogenes* TISTR1468 on pure glycerol, that $(NH_4)_2HPO_4$ gave higher ethanol production than using $(NH_4)_2SO_4$ and $NH_2C_2H_3O_3$, respectively of 1.00 and 1.44 folds, respectively (Ciptanto *et al*., 2008). In addition, the yield of ethanol from *Pachysolen tannophilus* in the presence of NH_4^+ was 44% higher than using NO_3^- as nitrogen source (Liu *et al*., 2012). $(NH_4)_2HPO_4$ is the important factor for ethanol production because it has a buffering capacity that has an effect on the pH changes during fermentation (Ergun and Multa, 2000)

Therefore, $(NH_4)_2HPO_4$ was selected and its optimum concentration was studied by varying in the range of 0-3.0 g/L (Fig. 10). The highest ethanol concentration and ethanol yield (Fig. 12A and 12C) of 12.98 g/L and 0.44 g/g, respectively, were obtained from 2.5 g/L $(NH_4)_2HPO_4$. In addition, the highest ethanol productivity and glycerol consumption rate of 0.82 and 2.09 g/L/h, respectively, were obtained when using 1.5 g/L $(NH_4)_2HPO_4$ (Fig. 12B and 12D). Statistical analysis using SPSS program showed that (2.0 g/L) $(NH_4)_2HPO_4$ gave the high ethanol production, productivity and glycerol consumption rate. Therefore, this concentration of $(NH_4)_2HPO_4$ was chosen for next study.

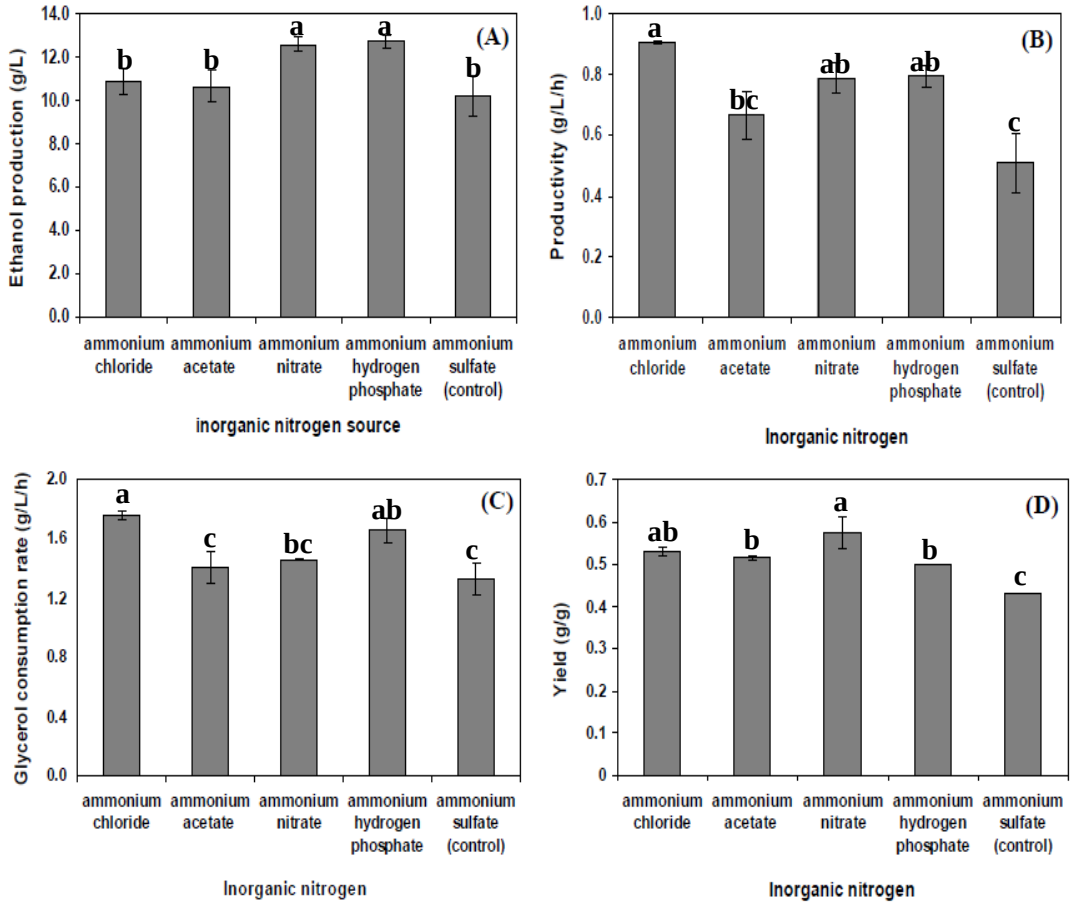


Figure 11. Effect of inorganic nitrogen sources on the ethanol production (A), productivity (B), glycerol consumption rate (C) and ethanol yield (D) of *E. aerogenes* TISTR1468 on 30 g/L raw glycerol concentrations in shake-flask culture at 37 ºC, 120 rpm for 24 h. The different superscripts in the bar graph denote a significant difference ($p < 0.05$)

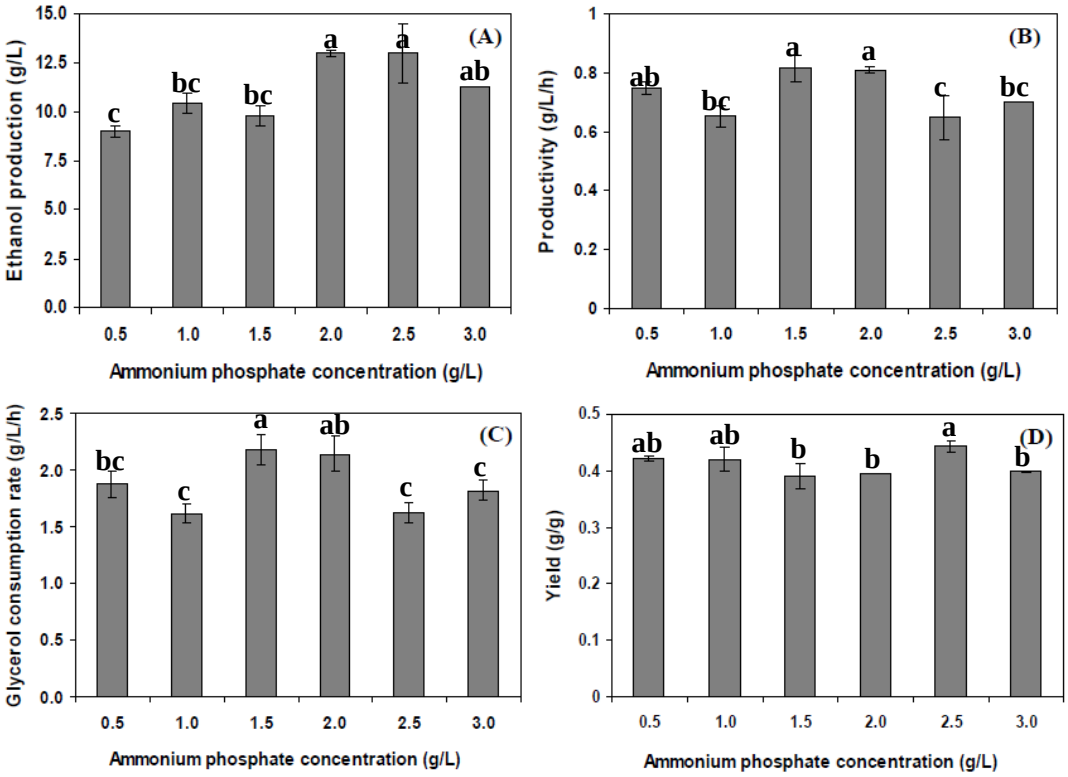


Figure 12. Effect of $(NH_4)_2HPO_4$ concentration on ethanol production (A), productivity (B), glycerol consumption rate (C) and ethanol yield (D) of *E. aerogenes* TISTR1468 on 30 g/L raw glycerol concentrations in shake-flask culture at 37 ºC, 120 rpm for 24 h. The different superscripts in the bar graph denote a significant difference ($p < 0.05$)

2.1.4 Effect of organic nitrogen sources and concentration

The effect of organic nitrogen source on the ethanol production was investigated because of some nutritional components present in the complex nutrient sources can be essential for cell growth and metabolite production (Choi *et al*., 2011). Therefore, the addition of some nutrients is essential for the fermentation especially the utilization of waste as a carbon source. Glycerol from biodiesel waste was diluted with deionized water, glycerol was not completely consumed and no growth of *E. aerogenes* HU-101 was observed. This indicated that some nutrients should be added to ferment glycerol waste (Ito *et al*., 2005). Studies on the effect of organic nitrogen (Fig. 13), revealed that yeast extract and malt extract could enhance the ethanol production, compared with the control (yeast extract and tryptone). Among nitrogen

sources tested, yeast extract exhibited the highest ethanol production, productivity, glycerol consumption rate and yield of 12.86 g/L, 0.64 g/L/h, 1.73 g/L/h and 0.37 g/g, respectively. They were 1.15, 1.38, 1.25 and 1.08 folds compared with the control. This result was similar to the ethanol production using crude glycerol by *Kluyvera cryocrescens* (about 11 g/L) in which yeast extract gave higher ethanol production than polypeptone and tryptone. Since yeast extract is known to contain nitrogen and carbohydrate, it may be utilized as carbon source to synthesized biomass or ethanol (Choi *et al*., 2011). Moreover, yeast extract has a buffering capacity and this might contribute to high productivity (Gaudreau *et al*., 1997).

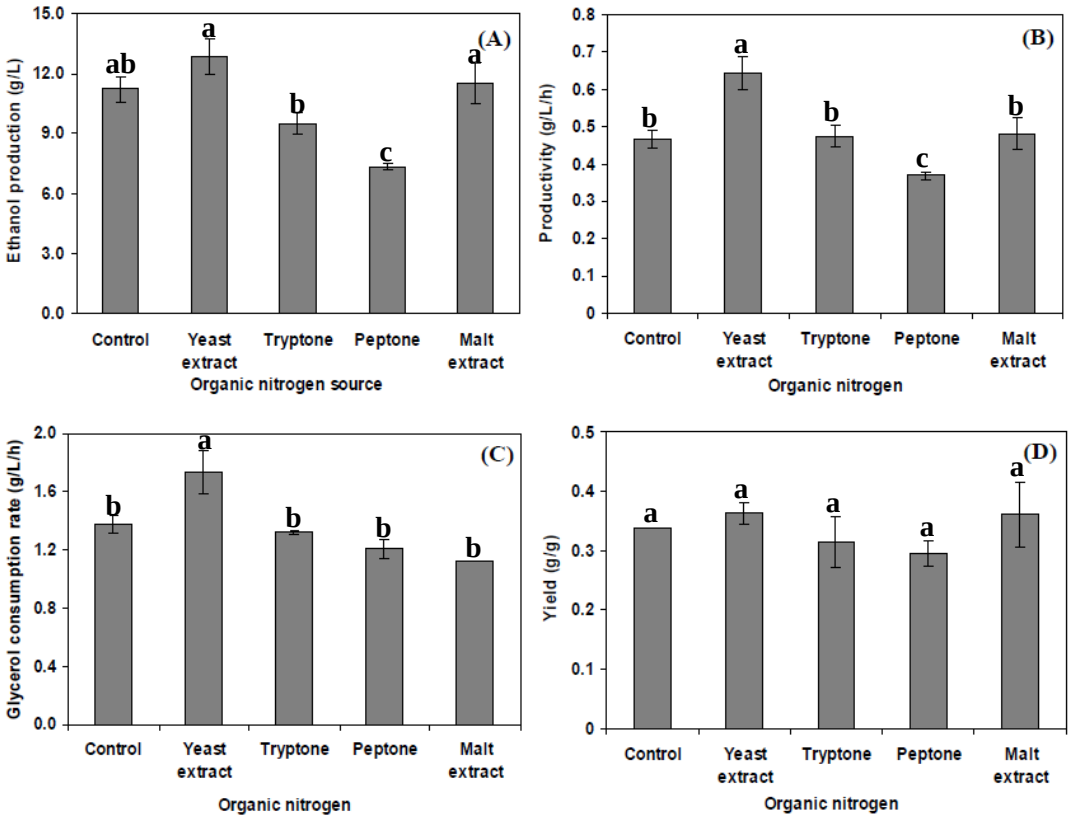


Figure 13. Effect of organic nitrogen sources on ethanol production (A), productivity (B), glycerol consumption rate (C) and ethanol yield (D) of *E. aerogenes* TISTR1468 on 30 g/L raw glycerol concentrations in shake-flask culture at 37 ºC, 120 rpm for 24 h. The different superscripts in the bar graph denote a significant difference ($p < 0.05$)

Therefore, yeast extract was the selected organic nitrogen source and the effect of yeast extract concentration was tested (Fig. 14). The highest ethanol production, productivity and glycerol consumption rate of 15.37 g/L, 0.96 g/L/h and 1.67 g/L/h, respectively, were obtained from 10 g/L yeast extract. However, statistical analysis using SPSS program showed that the result from using 5.0 g/L yeast extract was not significantly different from those of using 7.5 and 10 g/L yeast extract. As yeast extract is costly supplement nutrient therefore 5.0 g/L yeast extract was selected for ethanol production. Under this condition *E. aerogenes* TISTR1468 could produce ethanol 14.53 g/L after 16 h cultivation, with 0.91 g/L/h productivity, 1.44 g/L/h glycerol consumption rate and 0.62 g/g ethanol yield.

By conventional methodology, the optimum medium composition was consisted of 30 g/L glycerol, 2.0 g/L $(NH_4)_2HPO_4$ and 5.0 g/L yeast extract. Comparison to the original cultivation medium, under the optimum condition, the ethanol production, productivity and ethanol yield increased 2.01, 2.02 and 1.54 folds, respectively (Table 11). This meant that the optimization by 'one factor at the time' in the batch fermentation process could increase the ethanol production by 1.5-2.0 folds.

Table 11. Ethanol production from raw glycerol by *E. aerogenes* TISTR1468 before and after medium optimization

	Before optimization	After optimization
Ethanol production (g/L)	7.24	14.53
Productivity (g/L/h)	0.45	0.91
Ethanol yield (g/g)	0.41	0.63

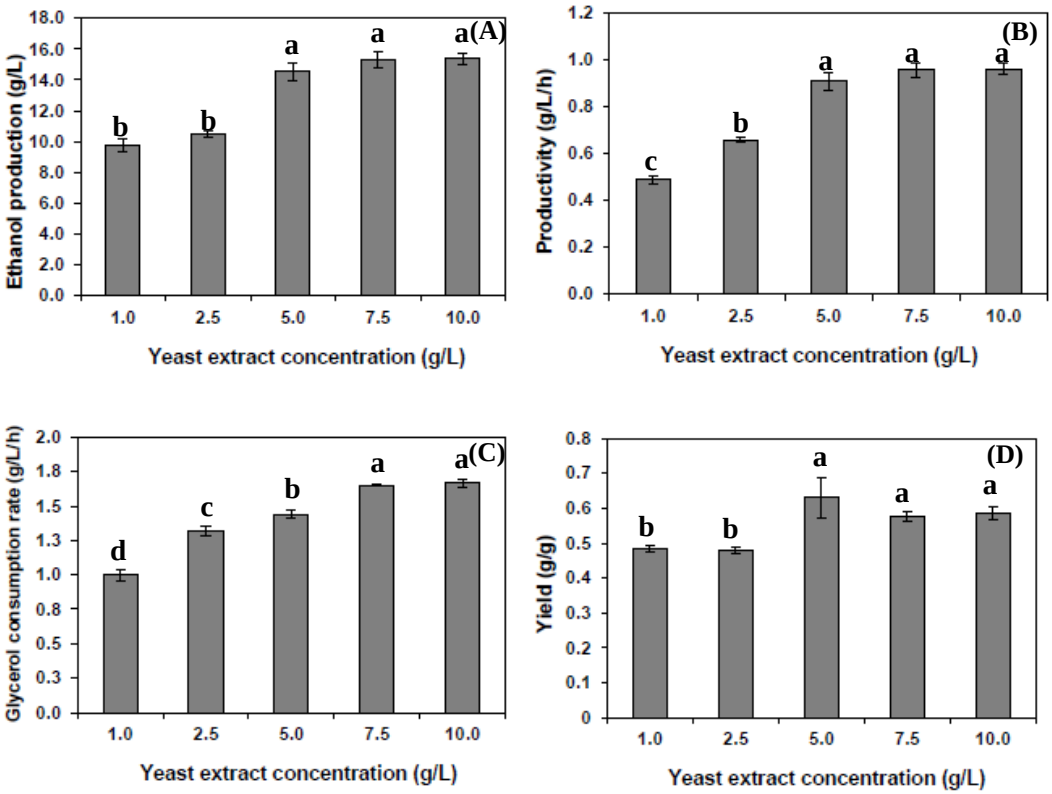


Figure 14. Effect of yeast extract concentration on ethanol production (A), productivity (B), glycerol consumption rate (C) and ethanol yield (D) of *E. aerogenes* TISTR1468 on 30 g/L raw glycerol concentrations in shake-flask culture at 37 ºC, 120 rpm for 24 h. The different superscripts in the bar graph denote a significant difference ($p < 0.05$)

2.2 Medium optimization for ethanol production by response surface methodology

The results from the conventional method revealed that glycerol, yeast extract and $(NH_4)_2HPO_4$ are the important factors in fermentation, so they were selected as the variables in experimental design. Central composite experimental design matrix and the observed response under various factors are summarized in Table 12 and the response surface constructed using equation (22) is illustrated in Fig. 15. The regression model of equation was considered to represent the experimental data accuracy as value of R^2 was 0.95. The optimum condition was found to be at 40 g/L

crude glycerol, 2.50 g/L $(NH_4)_2HPO_4$ and 7.5 g/L yeast extract and 16.09 g/L ethanol was produced.

The effect for model was calculated and statistics such as *F*-value, lack of fit and R^2-value were used for comparing the model, and consequently, quadratic model was selected. The model was test through lack of fit *F*-test. Lack of fit is not desirable, so a low *F*-value and probability greater than 0.1 are desired (non-significant), that indicated in Table 13. Lack of fit showed non-significant. The model is highly significant with very low probability values ($P<0.0001$). The R^2-value provides a measure of how much variability in observed response value can be explained by the experimental variables and their interactions. The data showed the R^2-value was 0.95, which was closer to 1.0 and was the better the model predicts the response. The low value of coefficient of variation (CV) was 8.5 indicated that good precision and reliability of experiment.

Final Equation in Terms of Coded Factors:

$$Y = 13.72 + 2.88(X_1) + 0.031(X_2) + 0.24(X_3) - 1.64(X_1)^2 - 0.43(X_2)^2 + 0.15(X_3)^2 + 1.23(X_1X_2) + 1.50(X_1X_3) - 1.00(X_2X_3) \quad (22)$$

Where Y is the response values as the ethanol (g/L), X_1 is glycerol (g/L), X_2 is yeast extract (g/L) and X_3 is $(NH_4)_2HPO_4$ (g/L).

The results showed that the model term of X_1 and $(X_1)^2$, were significant with 95% probability and interaction of all factors (X_1X_2, X_1X_3 and X_2X_3) were significantly influence on ethanol production (Table 14). The isoresponse contour and surface plots for optimization condition of ethanol production is given in Fig. 15. The plot illustrated the interactions between two nutrients and also to locate the optimum levels. The 3D response surface showed the effect of glycerol and yeast extract interaction (Fig. 15A), glycerol and $(NH_4)_2HPO_4$ (Fig. 15B) and yeast extract and $(NH_4)_2HPO_4$ (Fig. 15C) on ethanol production. It showed that all factors significantly interactive influence on ethanol production.

Table 12. Experimental design and comparison of observed and predicted ethanol production

Exp. No	A: Glycerol (g/L)	B: $(NH_4)_2HPO_4$ (g/L)	C: Yeast extract (g/L)	Response (Ethanol) (g/L)	
				Predicted	Observed
1	0	-α	0	12.45	13.61
2	0	0	0	13.72	14.83
3	-1	-1	+1	9.85	9.17
4	-α	0	0	4.23	4.92
5	+α	0	0	13.91	14.95
6	-1	+1	+1	5.45	5.07
7	0	0	0	13.72	14.15
8	0	0	0	13.72	12.52
9	+1	-1	-1	10.67	9.82
10	0	0	-α	13.72	14.62
11	+1	+1	-1	15.20	14.66
12	-1	-1	-1	10.37	9.73
13	0	0	+α	16.15	15.38
14	0	+α	0	12.55	13.12
15	0	0	0	13.72	13.83
16	-1	+1	-1	9.97	9.52
17	0	0	0	13.72	13.15
18	0	0	0	13.72	13.52
19	+1	-1	+1	16.15	15.38
20	+1	+1	+1	16.67	16.09

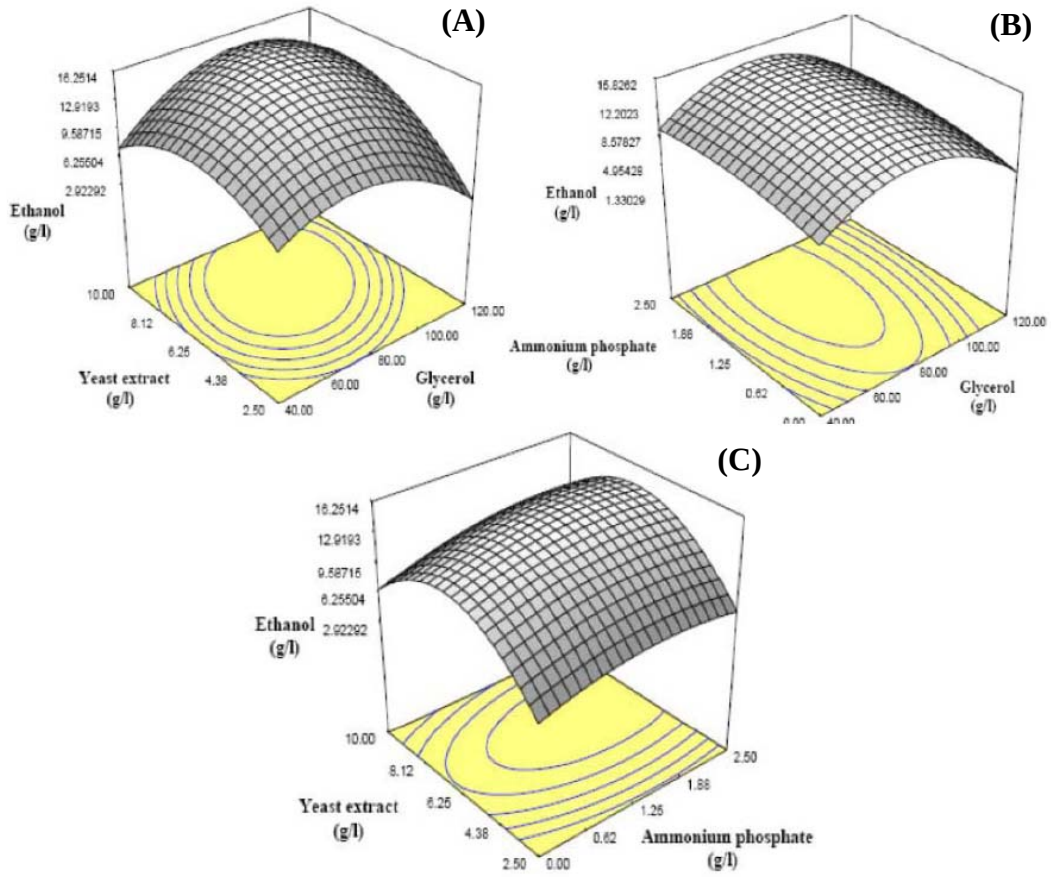


Figure 15. Response surface of ethanol production: fixed $(NH_4)_2HPO_4$ concentration at 1.25 g/L (A), fixed glycerol concentration at 30 g/L (B), and fixed yeast extract concentration at 5.00 g/L (C)

The predicted conditions obtained by RSM were selected and confirmed. The experiments were operated in shake-flask with the optimum condition obtained above (Table 15). The percentage of deviation between the predicted and experimental values of ethanol production was investigated. The study using RSM was based on CCD established and efficient model to describe the process. The high similarly between the observed value (16.19 g/L ethanol) and the predicted value (16.31 g/L ethanol) confirmed that the RSM was an accurate and applicable tool to optimize the ethanol production from raw glycerol. By utilizing the statistical methodology, the maximum ethanol production from raw glycerol (16.19 g/L) was obtained under the

optimum medium composition of 38.28 g/L raw glycerol, 2.10 g/L $(NH_4)_2HPO_4$ and 7.20 g/L yeast extract.

Table 13. Summary of the analysis of variance result for the response models for ethanol production*

Source	Sum of square	Degree of freedom	Mean square	*F*-value	*P*-value
Model	193.81	9	21.53	19.37	<0.0001
Residual	11.12	10	1.11		
Lack of fit	7.90	5	1.58	2.46	0.1732
Pure error	3.22	5	0.64		
Total	204.93	19			

*Coefficient of determination R^2= 0.95, R^2 adjusted= 0.90, CV = 8.5

Table 14. Quadratic model coefficient estimated by multiples linear regression (significance of regression coefficients)

Independent variables [a]	Coefficient estimate	Standard error	Degrees of freedom	Prob > F [b]
Intercept	13.72	0.43	1	
X_1	2.88	0.29	1	<0.0001
X_2	0.031	0.29	1	0.9169
X_3	0.24	0.29	1	0.4226
X_1^2	-1.64	0.28	1	0.0001
X_2^2	-0.43	0.28	1	0.1519
X_3^2	0.15	0.28	1	0.6072
X_1X_2	1.23	0.37	1	0.0079
X_1X_3	1.50	0.37	1	0.0024
X_2X_3	-1.00	0.37	1	0.0227

[a]X_1 = glycerol concentration (g/L), X_2 = $(NH_4)_2HPO_4$ (g/L), and X_3 = Yeast extract (g/L), [b]$p < 0.05$ are significant, R^2 = 0.95

Table 15. Experimental design and comparison of observed and predicted ethanol production

Exp. no	Glycerol (g/L)	$(NH_4)_2HPO_4$ (g/L)	Yeast extract (g/L)	Response (Ethanol) (g/L)		%Deviation
				Predicted	Observed	
1	38.77	0.93	7.24	16.41	15.41	6.09
2	39.87	2.45	6.60	16.30	14.85	8.90
3	38.28	2.10	7.20	16.31	16.19	0.74

By utilizing the statistical methodology (RSM), the maximum ethanol production from raw glycerol (16.19 g/L) was obtained under the optimum medium composition of 38.28 g/L raw glycerol, 2.10 g/L $(NH_4)_2HPO_4$ and 7.20 g/L yeast extract, with the productivity of 0.67 g/L/h. Comparison to the original medium, under the optimum condition, the ethanol production and productivity increased 2.24 and 1.50 folds, respectively. This meant that the optimization by 'response surface methodology' in the batch fermentation process could increase the ethanol production.

2.3 Effect of environmental conditions for ethanol production

The effect of environmental condition on the ethanol production by *Enterobacter aerogenes* TISTR1468 was stepwise investigation on optimum medium from section 2.2. The effect of each factor was shown as follow.

2.3.1 Effect of initial pH and pH-control

The effect of initial pH (6.5, 6.8, 7.0, 7.5, 8.0 and 8.5) on ethanol production, productivity, glycerol consumption rate and ethanol yield was studied in 3 L fermenter (Fig. 16). The ethanol production (Fig. 16A) increased with the increase of initial pH up to 8.0 and decreased thereafter. The optimum initial pH on raw glycerol for ethanol production was weakly alkaline pH of 7.5 and 8.0. The lowest of ethanol concentration and productivity (9.45 g/L and 0.39 g/L/h, respectively) were obtained at the initial pH of 6.5. Initial pH at 8.0 gave the high ethanol production, productivity and glycerol consumption rate were 12.74 g/L, 0.53 g/L/h and 1.23 g/L/h, respectively

and under this condition gave 0.43 g/g ethanol yield. Fig 17 showed the optimum initial pH at 8.0. This result indicated that the amount of glycerol consumed and ethanol production was dependent on the culture pH. Similar result was obtained from the ethanol production from *Enterobacter agglomerans* on 720 mM glycerol at varying pH (6-8) that glycerol consumption increased with the increased pH and was complete at pH 8 (Barbirato *et al*., 1996). Moreover, *Clostridium pasteurianum* could produce the maximum ethanol yield at the initial pH at 7.5 with 0.44 g/g ethanol yield (Biebl, 2001). The optimum pH at 8.0 was the same as that optimum for mixed culture from a distillery wastewater treatment plant (Temudo *et al*., 2007). The acidic condition was unsuitable for cell growth that consequently affects the ethanol production. Therefore, the optimum initial pH 8.0 was chosen to study on the effect of pH-control.

Results of pH-control (Fig. 18) showed that the highest ethanol production, productivity, glycerol consumption rate and yield were 12.75 g/L, 0.53 g/L/h, 1.34 g/L/h, and 0.40 g/g, respectively. Under uncontrolled-pH condition, these values were only slightly lower (12.11 g/L, 0.51 g/L/h, 1.34 g/L/h, and 0.38 g/g, respectively). The results revealed that the ethanol production under controlled-pH and uncontrolled-pH was not significantly different. So, the optimum condition for ethanol production was the initial pH 8.0 without pH-control.

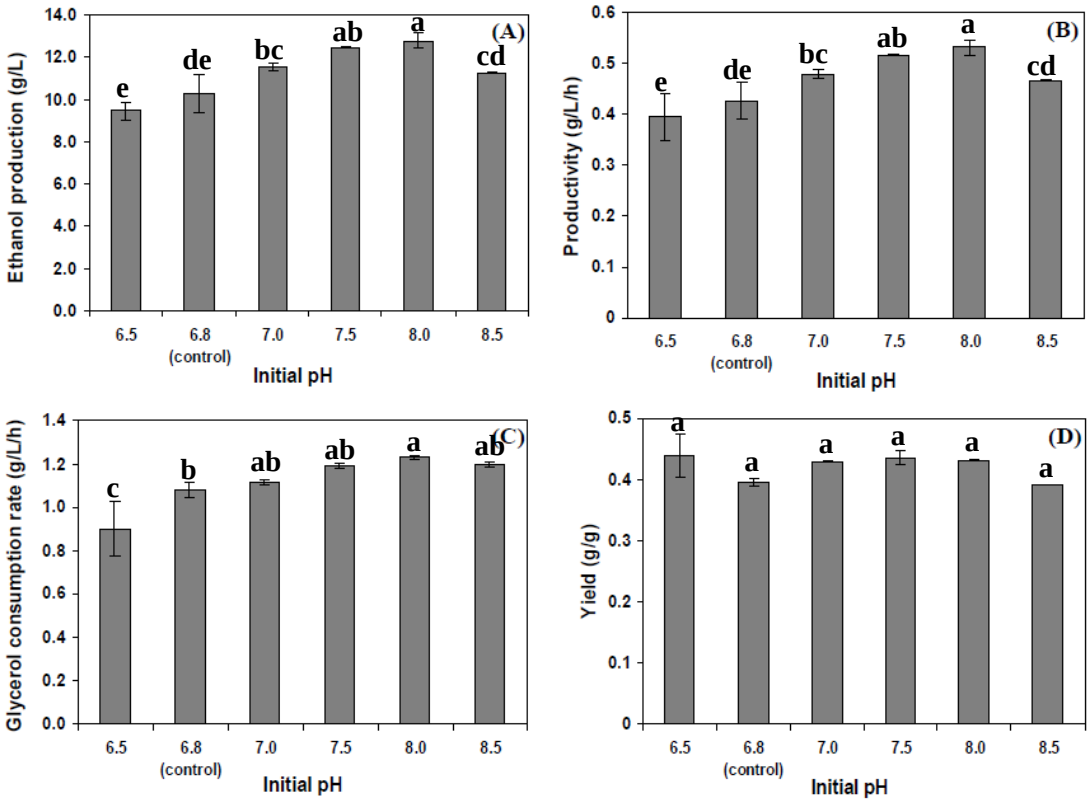


Figure 16. Effect of initial pH on ethanol production (A), productivity (B), glycerol consumption rate (C) and ethanol yield (D) of *E. aerogenes* TISTR1468 on optimum medium in 3 L fermenter culture at 37 ºC, 0.5 vvm aeration rate and 120 rpm agitation rate for 24 h. The different superscripts in the bar graph denote a significant difference ($p < 0.05$)

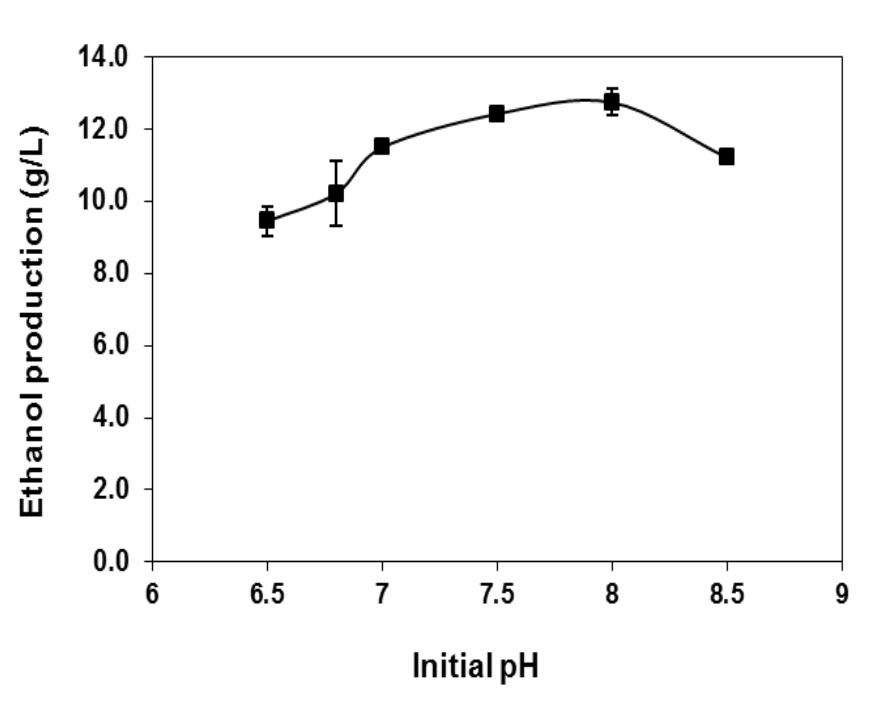


Figure 17. Effect of initial pH on ethanol production of *E. aerogenes* TISTR1468 on optimum medium in 3 L fermenter culture at 37 ºC, 0.5 vvm aeration rate, and 120 rpm agitation rate for 24 h.

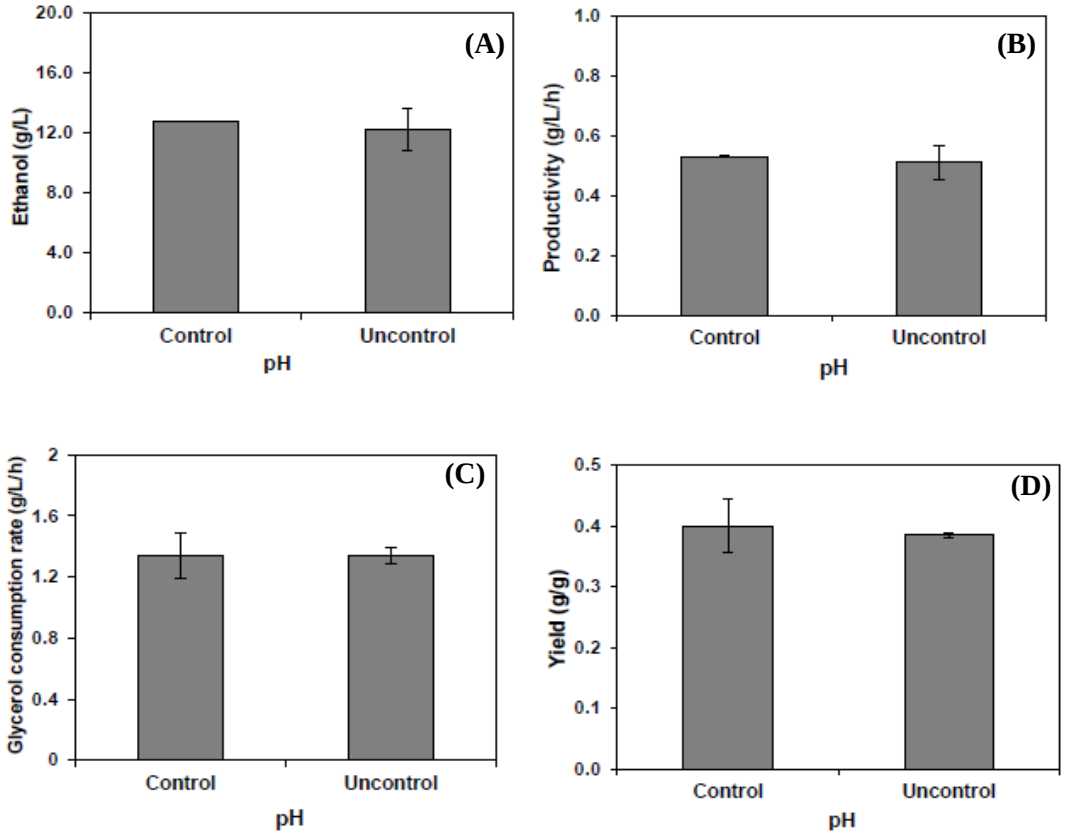


Figure 18. Effect of pH-control at pH 8.0 on ethanol production (A), productivity (B), glycerol consumption rate (C) and ethanol yield (D) of *E. aerogenes* TISTR1468 cultivated in the optimum medium in 3 L fermenter culture at 37 ºC, 0.5 vvm aeration rate and 120 rpm agitation rate for 24 h.

2.3.2 Effect of aeration rate and agitation rate

Aeration rate had an influence on ethanol production, productivity, glycerol consumption rate and ethanol yield (Fig. 19). The ethanol production (Fig. 19A) increased with the increasing aeration rate up to 0.50 vvm and decreased thereafter. The results revealed that micro-aerobic condition (0.25 and 0.50 vvm) was more favorable for ethanol production than anaerobic (0.0 vvm) and aerobic (1.00 vvm) condition. The highest ethanol production, productivity and ethanol yield of 11.66 g/L, 0.73 g/L/h and 0.58 g/g, respectively, were achieved under 0.50 vvm. The highest glycerol consumption rate of 1.49 g/L/h was obtained under aerobic condition (1.00 vvm). This was due to the fact that the dissolved oxygen activates faster cell growth of the gene expression system for enzymes of the citric acid cycle, which provides

energy for cell growth. In addition, oxygen supply at high level can enhance the generation of ATP by reducing NADH, which is then used for biomass synthesis (Chen *et al*., 2003). Therefore, under 1.00 vvm, glycerol might be used for synthesize the cells rather than ethanol production, resulting in high glycerol consumption rate and low ethanol production. Under anaerobic condition, small amount of glycerol was consumed and converted to ethanol production, giving ethanol production only 4.95 g/L with the ethanol yield of 0.41 g/g. Therefore, micro-aerobic condition could enhance ethanol production and 0.50 vvm was chosen for the next study.

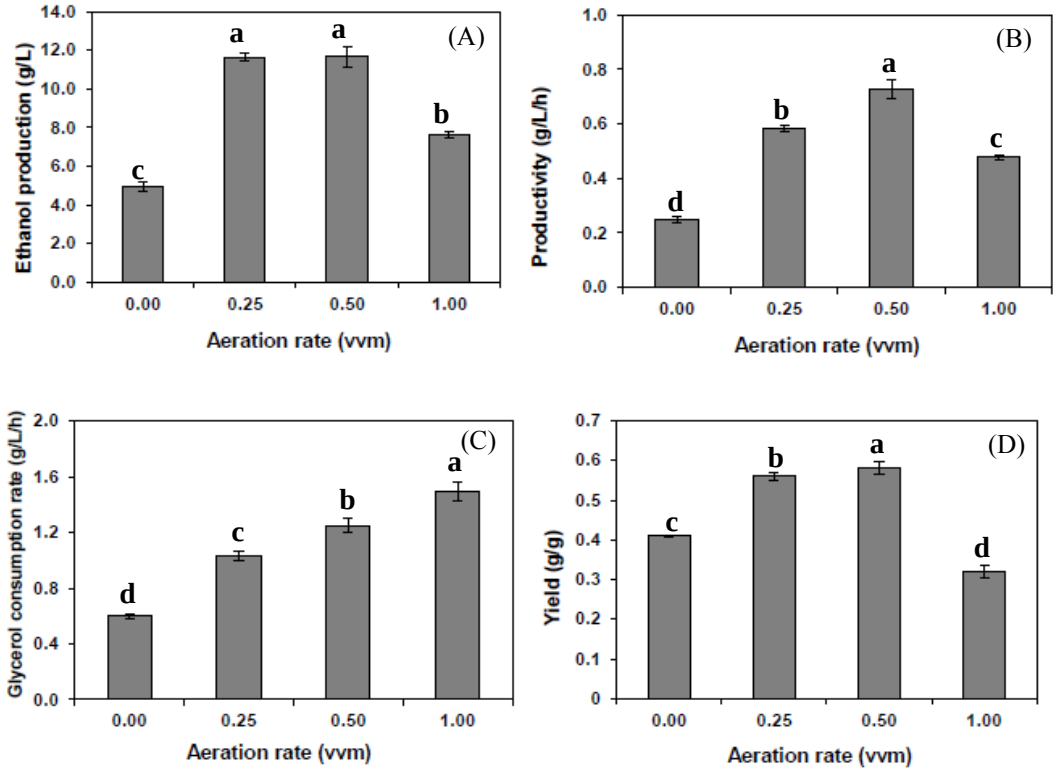


Figure 19. Effect of aeration rate on ethanol production (A), productivity (B), glycerol consumption rate (C) and ethanol yield (D) of *E. aerogenes* TISTR1468 on optimum medium in 3 L fermenter culture at 37 ºC, initial pH 8.0 without pH-control and 120 rpm agitation rate for 24 h. The different superscripts in the bar graph denote a significant difference ($p < 0.05$)

The effect of agitation speed on ethanol production was studied in 3 L fermentor with 0.5 vvm aeration rate (Fig. 20). The highest ethanol production and ethanol yield were 14.03 g/L and 0.58 g/g, respectively using 60 rpm agitation speed. However, the highest productivity and glycerol consumption rate of 1.05 g/L/h and 1.89 g/L/h, respectively, were obtained from 180 rpm agitation speed. The rate of stirring on glycerol fermentation using *Klebsiella pneuminiae* IC 15 showed a significant effect on fermentation process, and the rate of stirring conferred more influence of dissolved oxygen (Zheng *et al*., 2008). The high stirring rate enhances oxygen transfer rate to a culture and facilitates diffusion (Kirkpatrick *et al*., 2001). This study, agitation speed of 60 rpm was selected for further study.

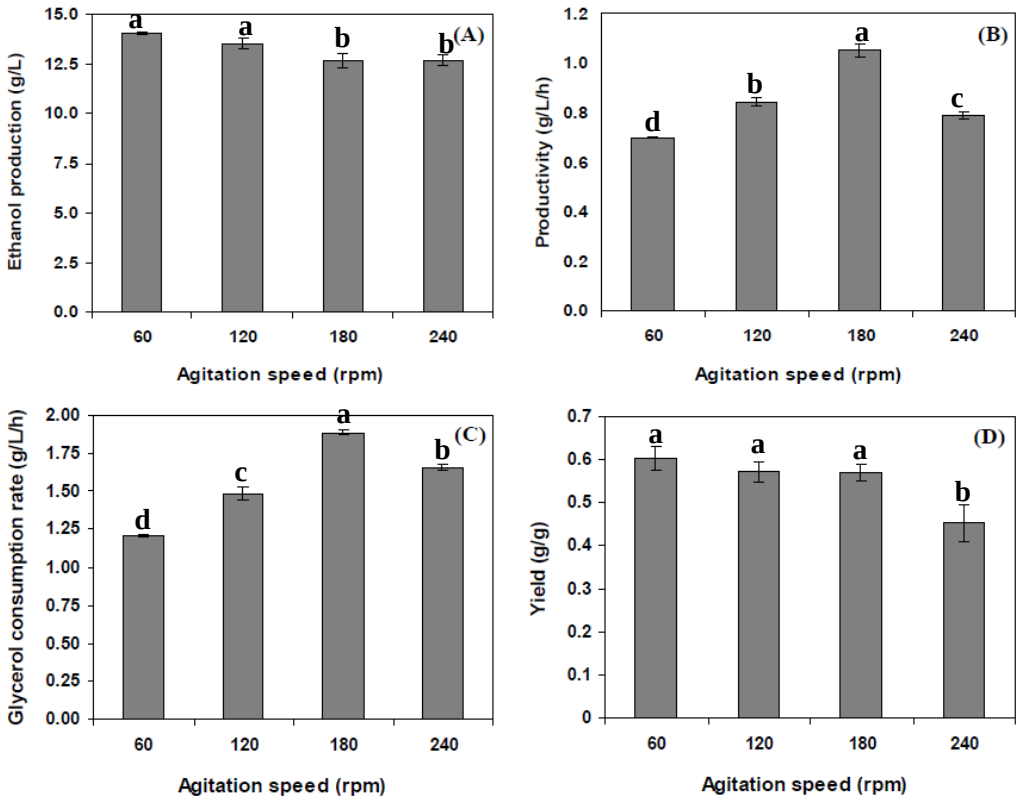


Figure 20. Effect of agitation speed on ethanol production (A), productivity (B), glycerol consumption rate (C) and ethanol yield (D) of *E. aerogenes* TISTR1468 cultivated in the optimum medium in 3 L fermenter at 37 ºC, initial pH 8.0 without pH-control and 0.5 vvm aeration rate for 24 h. The different superscripts in the bar graph denote a significant difference ($p < 0.05$)

The optimum environmental condition for ethanol production from raw glycerol in a 3 L fermenter was initial pH 8.0 without pH-control, 0.5 vvm aeration rate and 60 rpm agitation speed. Under the optimum condition, *E. aerogenes* TISTR1468 was able to produce 14.0 g/L ethanol, 0.60 g/g yield, 0.70 g/L/h productivity and 1.21 g/L/h glycerol consumption rate. Therefore, the ethanol production was 1.94 times higher than the original condition.

3. Kinetics of the ethanol production in the batch, fed-batch and two-stage process

3.1 Kinetics of the ethanol production in batch culture

3.1.1 Cell growth rate

Effect of the initial crude glycerol concentrations between 10-60 g/L with the fixed initial biomass concentration of 0.05 g/L was studied (Fig. 21). The time profile illustrated the influence of glycerol concentration as the growth increased with the increase of raw glycerol concentration upto 40 g/L then decreased thereafter (Fig 21A). The lag phase was 4 h while the log phase was during 4-16 h except at 60 g/L where the lag phase prolonged to 8 h. At 10, 20, 30 and 40 g/L glycerol concentration, the maximum cell concentrations were 1.35, 1.95, 2.15 and 5.75 g/L, respectively with the cell growth rate of 0.11, 0.14, 0.17 and 0.43 g/L/h, respectively. Above the optimum value (50 and 60 g/L glycerol), the cell concentration dropped sharply to 3.95 and 1.75 g/L, respectively with the cell growth rate of 0.26 and 0.08 g/L/h, respectively. The inhibitory effect was pronounced at over 40 g/L glycerol and leads to significantly suppress the cell growth. Besides glycerol itself (i.e., high level of non-refined raw glycerol), the inhibitory effect on cell growth was also derived from impurities like sodium or potassium salts (Choi *et al*., 2011). The highest cell yield of 0.15 g/g (from 40 g/L glycerol) was 3 times higher than the value of 0.05 g/g (from 60 g/L glycerol) (Table 16).

Table 16. Effect of initial glycerol concentration on maximum biomass, cell growth rate, cell yield and specific growth rate

Glycerol (S_0, g/L)	Maximum biomass (X_m, g/L)	Cell growth rate (g/L/h)	Yield of cell ($Y_{X/S}$, g /g)	Specific growth rate (μ, h^{-1})
10	1.35 ± 0.04	0.11 ± 0.01	0.09 ± 0.01	0.660 ± 0.02
20	1.95 ± 0.16	0.14 ± 0.02	0.11 ± 0.01	0.708 ± 0.01
30	2.15 ± 0.21	0.17 ± 0.01	0.12 ± 0.01	0.611 ± 0.02
40	5.75 ± 0.18	0.43 ± 0.03	0.15 ± 0.004	0.613 ± 0.01
50	3.95 ± 0.25	0.26 ± 0.02	0.09 ± 0.003	0.507 ± 0.01
60	1.75 ± 0.15	0.08 ± 0.01	0.05 ± 0.001	0.297 ± 0.03

The specific growth rate increased gradually with the increase of glycerol concentration and decreased sharply at the concentration higher than 20 g/L. The relationship between specific growth rates and the initial glycerol concentrations (Fig 22) revealed that the maximum specific growth rate (0.708 h^{-1}) was at 20 g/L glycerol and substrate saturation constant (K_s) of 6 g/L. Over 20 g/L glycerol concentration, inhibition effect was observed and gave substrate inhibition constant (K_I) of 57 g/L with gave optimum glycerol concentration ($S_{opt} = \sqrt{(K_S K_I)}$) of 18.5 g/L.

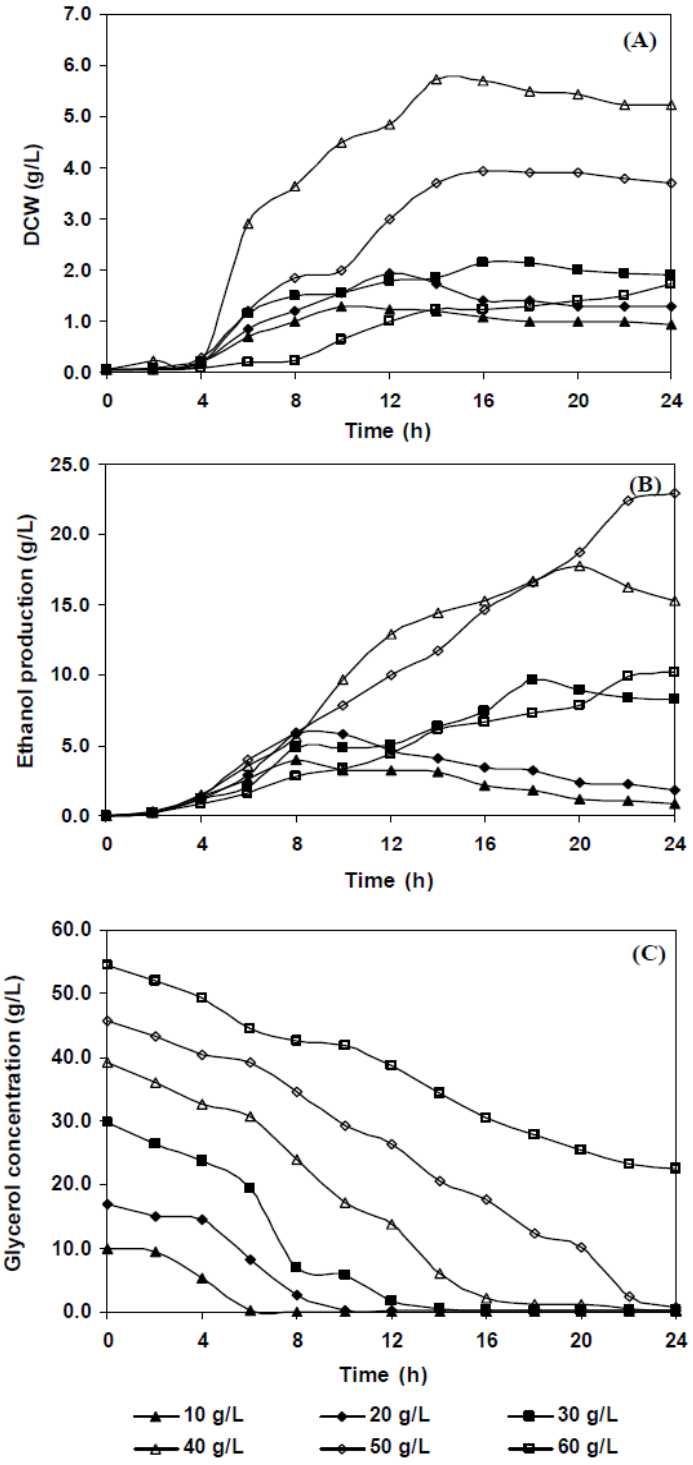


Figure 21. Time course of ethanol production from raw glycerol by *Enterobacter aerogenes* TISTR1468, in 3 L fermenter culture at 37 ºC, initial pH 8.0 without pH-control, 0.5 vvm aeration rate and 60 rpm agitation rate for 24 h, biomass (A), ethanol (B) and glycerol (C) concentrations

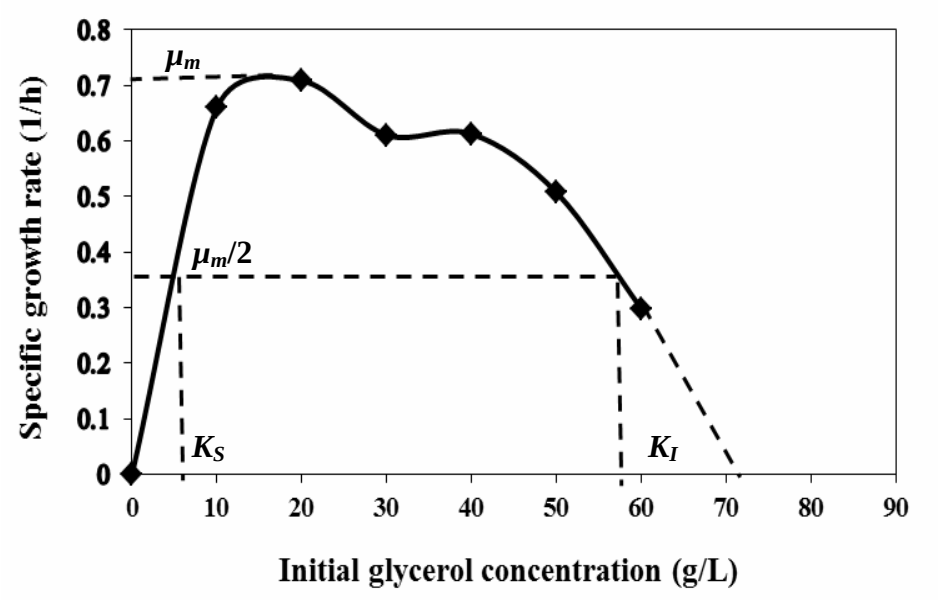


Figure 22. Monod plot between specific growth rate and initial glycerol concentration of *Enterobacter aerogenes* TISTR1468 in 3 L fermenter culture at 37 ºC, initial pH 8.0 without pH-control, 0.5 vvm aeration rate and 60 rpm agitation rate for 24 h

3.1.2 Ethanol production rate

The ethanol concentration increased proportionally to the increase of biomass concentration in the exponential growth phase and its production decreased during the stationary phases (Fig. 21A and 21B). Hence, ethanol was growth-associated product. As the glycerol concentration increased 3 times (from 10 to 30 g/L), the maximum ethanol concentrations increased 2.4 times at 8 h cultivation (from 3.99 to 9.67 g/L, respectively). However, the production rates were about the same (0.51, 0.56 and 0.53 g/L/h, respectively). The maximum ethanol concentration at 40 g/L glycerol (17.78 g/L) obtained at 20 h cultivation was nearly 2 times higher than that at 30 g/L glycerol. The highest ethanol production of 22.97 g/L was achieved at 50 g/L glycerol at 22 h cultivation, giving also the highest production rate (1.00 g/L/h) and the ethanol yield (0.52 $g_{ethanol}/g_{glycerol}$). Therefore, ethanol production, production rate and ethanol yield increased with the increased of glycerol concentration up to 50 g/L and decreased thereafter (Table 17). The specific production rate (ρ) was related to the cell concentration and ethanol production as described in equation (3). Specific production

rate decreased with the increase of glycerol concentration with the highest value of 0.42 h^{-1} at 10 g/L glycerol. Fig 23 show the effect of ethanol concentration on specific production rate, the results shown that the specific production rate decreased with the increasing of ethanol production.

Table 17. Effect of initial glycerol concentration on maximum ethanol, production rate, ethanol yield and specific production rate

Glycerol (S_0, g/L)	Maximum ethanol (P_m, g/L)	Production rate (g/L/h)	Yield of ethanol ($Y_{P/S}$, g /g)	Specific production rate (ρ, h^{-1})
10	3.99 ± 0.01	0.51 ± 0.01	0.33 ± 0.06	0.42 ± 0.12
20	5.82 ± 0.06	0.56 ± 0.04	0.36 ± 0.02	0.36 ± 0.03
30	9.67 ± 0.06	0.53 ± 0.01	0.33 ± 0.01	0.20 ± 0.06
40	17.78 ± 0.03	0.96 ± 0.09	0.47 ± 0.02	0.15 ± 0.01
50	22.97 ± 0.17	1.00 ± 0.06	0.52 ± 0.01	0.22 ± 0.01
60	10.23 ± 0.07	0.44 ± 0.01	0.33 ± 0.01	0.19 ± 0.07

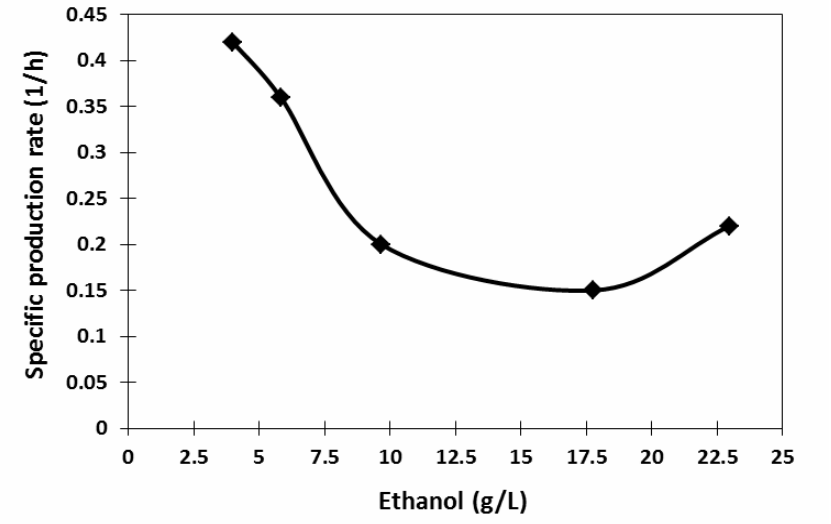


Figure 23. Effect of ethanol concentration on specific production rate by *Enterobacter aerogenes* TISTR1468, in 3 L fermenter culture at 37 ºC, initial pH 8.0 without pH-control, 0.5 vvm aeration rate and 60 rpm agitation rate for 24 h.

3.1.3 Glycerol consumption rate

Time course of glycerol utilization was shown in Fig. 21C. Glycerol was utilized completely at 6, 10 and 14 h at low glycerol concentrations (10-30 g/L) which gave the consumption rate of 1.67, 2.00 and 2.14 g/L/h, respectively. At 40 and 50 g/L glycerol concentration, glycerol was utilized completely at about 18 and 24 h, respectively and gave the consumption rate of 2.22 and 2.08 g/L/h, respectively. At 60 g/L glycerol concentration, 60% of glycerol was consumed and less effectively converted to biomass and ethanol (with the yields of 0.05 g/g and 0.33 g/g). Similar results *E. aerogenes* HU-101 on batch with varying waste glycerol concentration (1.7, 3.3, 10 and 25 g/L), found that the culture times for complete utilization of glycerol. At 25 g/L needed time higher than 48 h for glycerol utilization, because of the substrate inhibition and impurities that interfere with cell growth and ethanol production, respectively (Ito *et al*., 2005). The specific substrate consumption rate decreased with the increase of glycerol concentration because the glycerol was utilized completely and rapidly within a short time at low glycerol concentration. The highest specific consumption rate (0.89 h^{-1}) was obtained from using 10 g/L glycerol (Table 18). Fig 24 show the effect of glycerol concentration on specific substrate consumption rate, the results shown that the specific consumption rate decreased with the increasing of glycerol concentration.

Table 18. Effect of initial glycerol concentration on % substrate consumption, consumption rate and specific consumption rate

Glycerol (S_0, g/L)	Substrate consumption (%)	Substrate consumption rate (g/L/h)	Specific consumption rate (q_s, h^{-1})
10	100	1.67 ± 0.03	0.89 ± 0.09
20	100	2.00 ± 0.01	0.83 ± 0.10
30	100	2.14 ± 0.10	0.49 ± 0.03
40	100	2.22 ± 0.09	0.11 ± 0.02
50	100	2.08 ± 0.07	0.18 ± 0.05
60	60	1.37 ± 0.05	0.04 ± 0.08

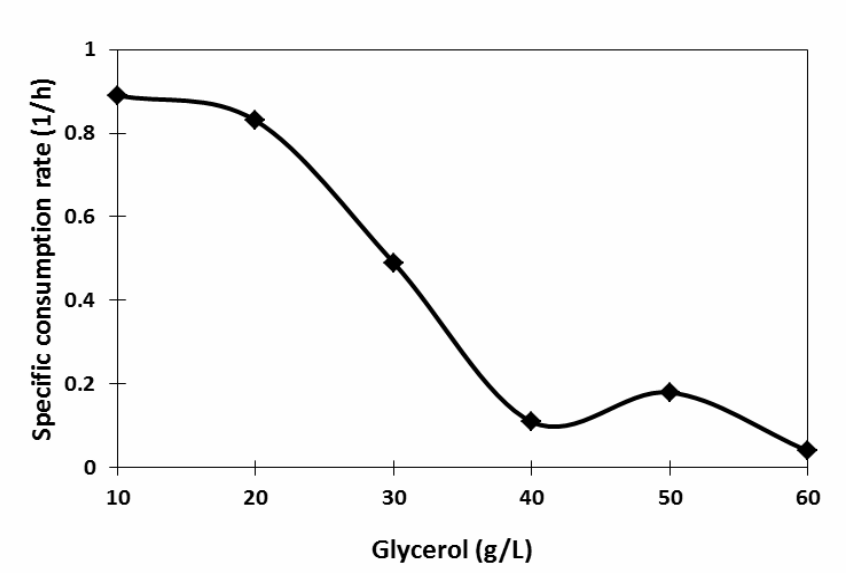


Figure 24. Effect of glycerol concentration on specific consumption rate by *Enterobacter aerogenes* TISTR1468, in 3 L fermenter culture at 37 ºC, initial pH 8.0 without pH-control, 0.5 vvm aeration rate and 60 rpm agitation rate for 24 h

In this study, the kinetic model (equation 7-16 in Chapter 2) for the batch production of ethanol from *Enterobacter aerogenes* TISTR1468 was studied. The results of the model simulations showed good agreement with the experimental data obtained at varying initial glycerol concentrations.

3.2 Ethanol production in repeated batch fermentation

Repeated batch fermentation means that the culture broth is harvested at the end of ordinary batch fermentation and fresh medium is refilled at the same volume for next batch fermentation (Meneil and Harvey, 2008). This kind of fermentation process can reduces inhibition of by-product occurred as the results of oxidative pathway of glycerol, including acetate, lactate succinate metabolism (Narendranath *et al* 2001; Vasseur *et al* 1999).

Effect of glycerol concentration (40 and 50 g/L) on ethanol production by repeated batch fermentation was studied and cultivated in 3 L fermenter culture at 37 ºC, initial pH 8.0 without pH-control, 0.5 vvm aeration rate and 60 rpm agitation rate for 24 h,. In this study the culture broth (25%) was harvested and fresh glycerol

solution was refilled at the 24 and 48 h fermentation. Time course of fixed volume intermittent fed-batch with 40 and 50 g/L raw glycerol is illustrated in Fig. 25A-B, respectively. Results (Fig. 25A) indicated that during first cycle (24 h), the cells grow rapidly and reached the highest cell growth (3.70 g/L), cell growth rate (0.15 g/L/h), yield of cell (0.11 g/g), specific growth rate (0.23 h^{-1}), ethanol production (15.51 g/L), productivity (0.65 g/L/h), ethanol yield (0.47 g/g). During the first cycle, glycerol decreased and almost completely consumed with the glycerol consumption rate of 1.38 g/L/h. In the second cycle (24-48 h), the fresh glycerol solution was added and gave the glycerol concentration about 34.04 g/L. This cycle the cell increased and reached the cell of 3.30 g/L at 48 h, cell growth rate (0.06 g/L/h), yield of cell (0.048 g/g) and specific growth rate (0.02 h^{-1}). The ethanol production in this cycle, the highest ethanol production of 18.64 g/L at 36 h and gave productivity of 0.53 g/L/h with yield of 0.45 g/g and 1.176 g/L/h of glycerol consumption rate. In last cycle (48-72 h), only 16% glycerol was utilized with small amount of increased cells and ethanol. Biomass concentration increased gradually and decreased thereafter because of cell growth in stationary or death phase. Also with the ethanol production, the ethanol production associated with the reduction of glycerol concentration. At 50 g/L glycerol concentration (Fig. 25B), the similar time profile of cell, ethanol and glycerol concentration with 40 g/L glycerol. The first cycle gave high rate of cell with specific growth rate of 0.18 h^{-1}, ethanol production and glycerol consumption and decreased in second and last cycle respectively. The highest ethanol production on 50 g/L glycerol was 19.97 g/L at 32 h (second cycle) with 1.17 g/L/h productivity. In the last cycle, glycerol accumulation in high level (higher than 50 g/L) might inhibit cell growth and ethanol production.

Results in this study indicated that the first and second cycle gave higher ethanol production rate and glycerol consumption rate than the third cycle. However, by comparing with batch fermentation at 40 and 50 g/L glycerol concentration, the repeated batch fermentation gave lower ethanol production than the batch fermentation. Therefore fed-batch fermentation by variable volume intermittent was tested for comparison.

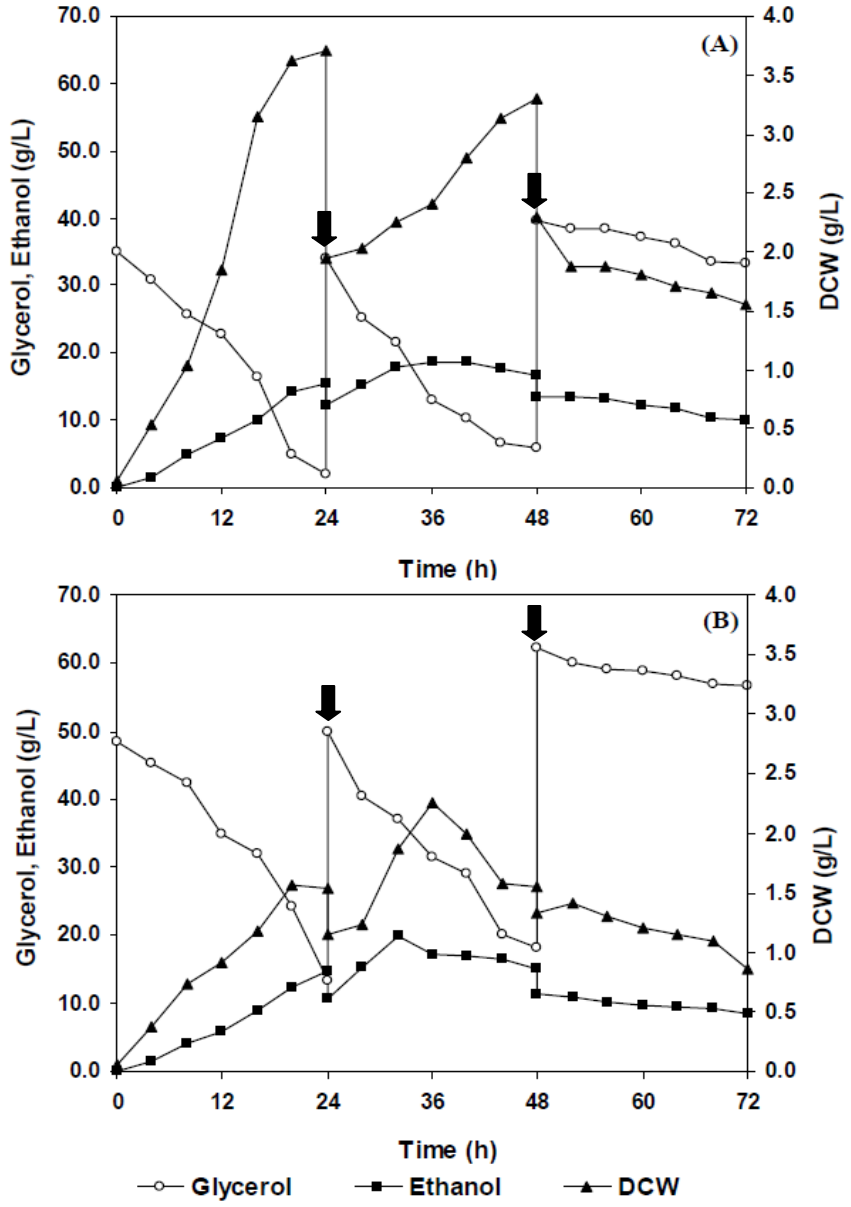


Figure 25. Time course on glycerol, ethanol and cell concentrations during cultivation of *E. aerogenes* TISTR1468 in 3 L fermenter culture at 37 ºC, initial pH 8.0 without pH-control, 0.5 vvm aeration rate and 60 rpm agitation, under repeated batch feeding with 40 g/L (A) and 50 g/L (B) glycerol at 24 and 48 h

3.3 Ethanol production in variable volume intermittent fed-batch

This experiment *Enterobacter aerogenes* TISTR1468 was cultivated in 3 L fermenter and culture at 37 ºC, initial pH 8.0 without pH-control, 0.5 vvm aeration rate and 60 rpm agitation rate. Initially, the 1 L fermentation medium containing 40 and 50 g/L glycerol and the intermittent feeding at 500 mL was added at 24 and 48 h, respectively. The final working volume was 2 L with 40 and 50 g/L glycerol. Cell growth, ethanol production and glycerol consumption in the first and second cycle exhibited similar pattern (Fig. 26). Fig. 26A, during the first cycle (24 h), the growth was rapid and gave the values of cell concentration (2.17 g/L DCW), growth rate (0.09 g/L/h), cell yield (0.055 g/g), specific growth rate (0.13 h^{-1}), ethanol production (14.73 g/L), productivity (0.61 g/L/h) and ethanol yield (0.41 g/g). During the first cycle, glycerol decreased and almost completely consumed with the rate of 1.51 g/L/h. In the second cycle (24-48 h), the fresh glycerol solution was added and gave the glycerol concentration of 43.05 g/L. This cycle exhibited lower cell concentration (2.02 g/L at 40 h), growth rate (0.023 g/L/h) and cell yield (0.013 g/g). However, the ethanol production was higher (19.21 g/L at 44 h) with lower productivity (0.46 g/L/h), ethanol yield of 0.27 g/g and glycerol consumption rate (1.48 g/L/h). In the last cycle (48-72 h), only 25% glycerol was utilized, hence giving small amount of cell and ethanol (1.7 g/L and 12 g/L, respectively). Biomass concentration increased gradually (48-60 h) and decreased thereafter because of cell growth in stationary or death phase (60-72 h). Also with the ethanol production, the ethanol production associated with the reduction of glycerol concentration. At 50 g/L glycerol concentration (Fig. 26B), time course on growth, ethanol and glycerol concentration were similar to those obtained at 40 g/L glycerol. The first cycle gave higher rates of growth (specific growth rate of 0.12 h^{-1}), ethanol production and glycerol consumption than the second and third cycle respectively. At 50 g/L glycerol, the highest ethanol production was 16.85 g/L at 44 h (second cycle) with 0.39 g/L/h productivity. In the last cycle, the accumulation of glycerol from the second cycle (78 g/L) that might affect to inhibit cell growth and ethanol production which giving the cell of 1.65 g/L at 52 h with also produced ethanol 15.70 g/L at 52 h.

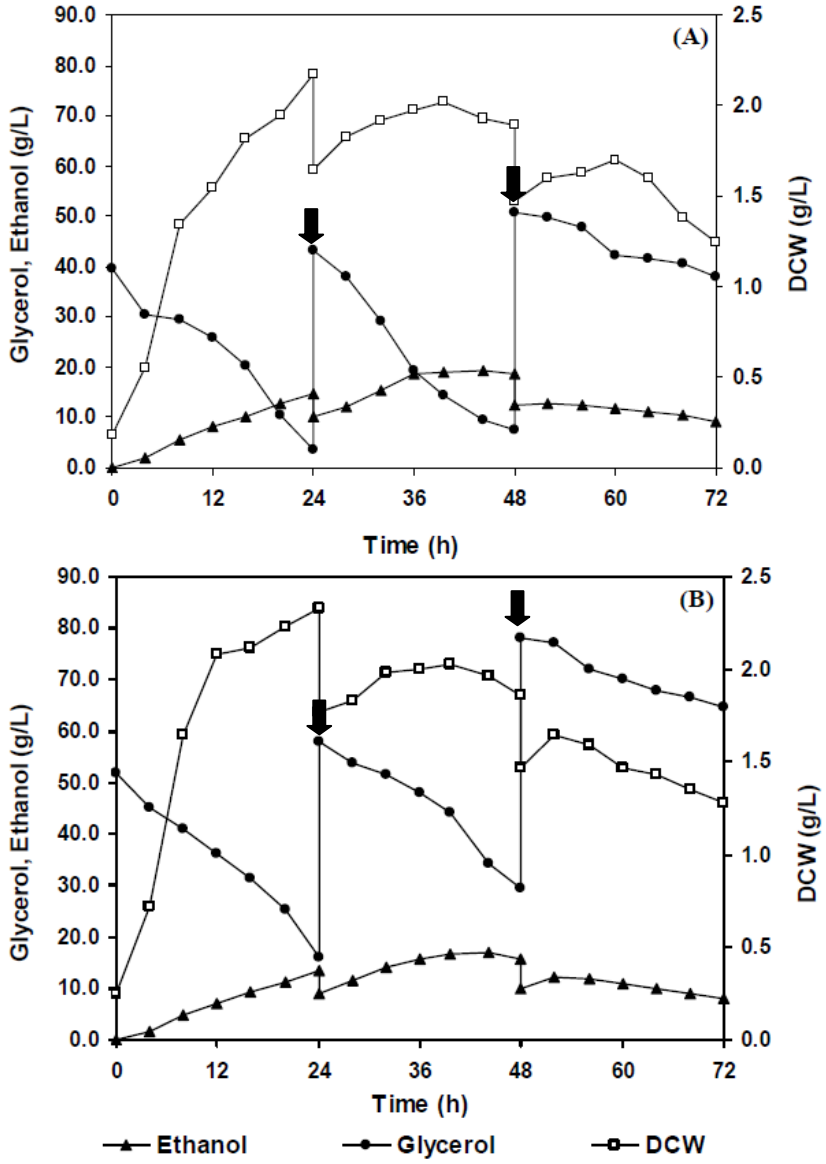


Figure 26. Time course on glycerol, ethanol and cell concentrations during cultivation of *E. aerogenes* TISTR1468 in 3 L fermenter culture at 37 ºC, initial pH 8.0 without pH-control, 0.5 vvm aeration rate and 60 rpm agitation, under variable volume intermittent fed-batch feeding with 40 g/L (A) and 50 g/L (B) glycerol at 24 and 48 h

The above studies indicated that, fed-batch fermentation was lower ethanol production than the batch fermentation, due to the cell loss activity for long time (Tomás-Pejó *et al*., 2009). Therefore, the next strategy was to use two-stage process for comparison.

3.4 Two-stage process for ethanol production

The previous results (section 2.3.2 in chapter 3) showed that cultivation under micro-aerobic condition was more favorable for ethanol production and glycerol consumption than under anaerobic condition. However, the ethanol was generally produced under anaerobic condition (Yazdani and Gonzalez, 2007). Therefore, this strategy was used by using micro-aerobic condition for growth (first stage) and anaerobic condition for ethanol production (second stage) to enhance the ethanol production.

Time course on biomass, ethanol and glycerol concentration are depicted in Fig. 27. Under micro-aerobic condition, the cell concentration increased rapidly during 4-12 h giving a growth rate of 0.34 g/L/h, which was almost stable until the end of cultivation (48 h). The highest biomass production under micro-aerobic condition (4.0 g/L) was 3.3 folds higher than that under anaerobic condition (1.2 g/L) (Fig. 27A). This gave the specific growth rate of 0.38 and 0.07 h^{-1}, respectively.

Ethanol was growth-associated product (Fig. 27B) as it increased with the increase of biomass. The production of ethanol under micro-aerobic condition (20.7 g/L) was also 3.3 times higher than that under anaerobic condition (6.3 g/L). This gave the ethanol productivity of 1.18 and 0.240 g/L/h, respectively. The glycerol consumption (Fig. 25C) was rapid under micro-aerobic condition and was depleted completely in the stationary phase (24 h cultivation). The glycerol consumption rate was 2.61 g/L/h and gave high cell yield (0.094 g/g) and ethanol yield (0.47 g/g). Under anaerobic condition, only 19% of glycerol was consumed and converted to small amount of biomass and ethanol with the yield of 0.09 g biomass/g glycerol consumed and 0.72 g ethanol/ g glycerol consumed.

The above results indicated that oxygen could activate the growth of *E. aerogenes* TISTR1468 (4.0 g/L), substrate consumption (2.61 g/L/h), maximum ethanol production (20.7 g/L) with ethanol productivity (1.18 g/L/h). The values were

similar to the mutant strain of *Klebsiella pneumoniae* (21.5 g/L and 0.93 g/L/h, respectively) (Oh *et al*., 2011) and *Kluyvera cryocrescens* S26 (18.33 g/L and 0.92 g/L/h, respectively) (Choi *et al*., 2011). Besides ethanol, the production of 1,3-propanediol from *Klebsiella pneumoniae* was also higher under micro-aerobic condition than anaerobic condition, because the dissolved oxygen activates faster cell growth and the gene expression system for enzymes of the citric acid cycle, which provides energy for cell growth (Chen *et al*., 2003). In addition, oxygen supply at high level can enhance the generation of ATP by reducing NADH, which is then used for biomass synthesis. Under micro-aerobic condition, limited amount of oxygen managed to convert NADH generated during cell growth into NAD while maintaining carbon flux into ethanol synthesis.

The concentration of biomass, ethanol, and glycerol (Fig. 28) under two-stage process, were similar to those obtained under micro-aerobic condition. In the first stage (micro-aerobic condition), the cell concentration increased rapidly (4-12 h) with the growth rate of 0.29 g/L/h and specific growth rate (0.40 h^{-1}), which was 19 times higher than that under anaerobic condition. The highest biomass production was 4.4 g/L at 32 h. Ethanol production increased rapidly in first stage (8-16 h) with 1.17 g/L/h productivity and decreased in second stage with 0.188 g/L/h productivity. The highest ethanol concentration was 24.5 g/L (at 36 h). The glycerol consumption in first stage was rapidly utilized (4-20 h) with the rate of 2.29 g/L/h and depleted in anaerobic condition.

The kinetic parameters are presented in Table 19. The ethanol productivity, biomass productivity and glycerol consumption rate under micro-aerobic condition were higher than those two-stage process. However, specific growth rate, maximum ethanol, ethanol yield and biomass yield under the two-stage condition (0.40 h^{-1}, 24.5 g/L, 0.56 g/g, and 0.10 g/g, respectively) were higher than those obtained from micro-aerobic condition.

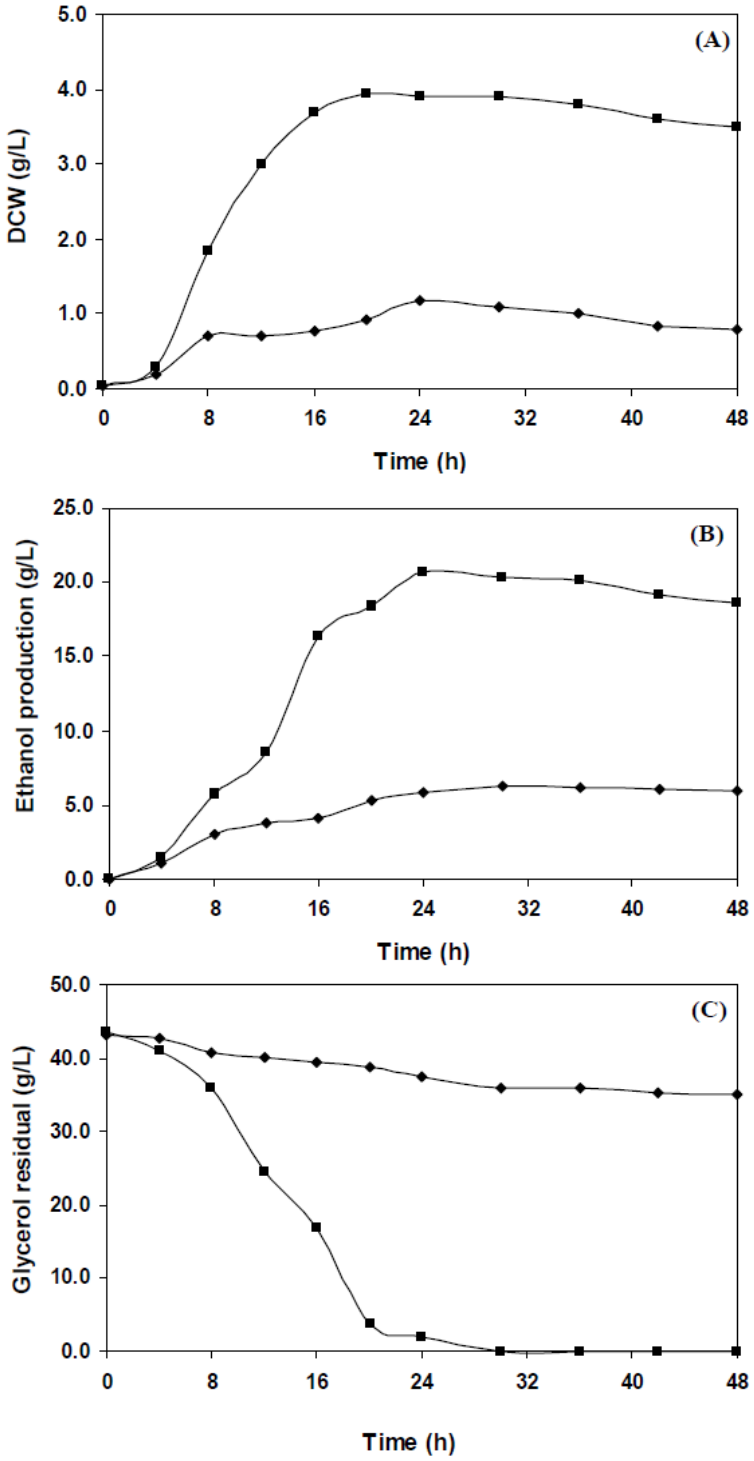


Figure 27. Time course of concentrations of biomass (A), ethanol (B), and residual glycerol (C) during batch culture of *Enterobacter aerogenes* TISTR1468 under micro-aerobic (■) and anaerobic (◆) conditions in 3 L fermenter culture at 37 ºC, initial pH 8.0 without pH-control, and 60 rpm agitation for 48 h

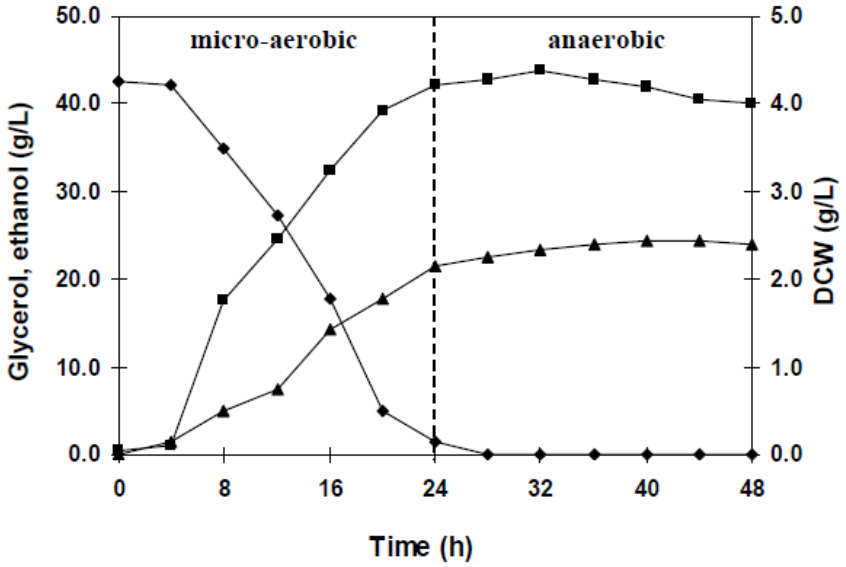


Figure 28. Time course of concentrations of ethanol (▲), biomass (■), and residual glycerol (◆) during batch cultures of *Enterobacter aerogenes* TISTR1468 under two-stage condition in 3 L fermenter culture at 37 ºC, initial pH 8.0 without pH-control, and 60 rpm agitation for 48 h

Table 19. Comparison of kinetic values in micro-aerobic and two-stage in batch fermentation by *Enterobacter aerogenes* TISTR1468

	Condition	
	micro-aerobic	two-stage
Specific growth rate (1/h)	0.38 ± 0.02	0.40 ± 0.003
Ethanol production (g/L)	20.70 ± 0.08	24.50 ± 0.01
Ethanol productivity (g/L/h)	1.18 ± 0.08	1.17 ± 0.01
Biomass productivity (g/L/h)	0.34 ± 0.02	0.29 ± 0.01
Glycerol consumption rate (g/L/h)	2.61 ± 0.13	2.29 ± 0.11
Ethanol yield (g/g)	0.47 ± 0.01	0.56 ± 0.01
Biomass yield (g/g)	0.09 ± 0.01	0.10 ± 0.003

The ethanol production by *Enterobacter aerogenes* TISTR1468 using crude glycerol under the two-stage process was higher than those using micro-aerobic or anaerobic conditions. The highest ethanol concentration of 24.5 g/L and productivity of 1.17 g/L/h were achieved.

3.5 Continuous culture for ethanol production from crude glycerol: effect of hydraulic retention time (HRT)

Continuous experiments were performed at four different HRT levels (30, 24, 18 and 12 h with the dilution rate of 0.033, 0.042, 0.056 and 0.083 h^{-1}, respectively), which were established by changing the feed flow rate while keeping the fermentation volume constant at 1 L. Fig. 29A shows the variation of effluent total glycerol concentration and percent glycerol utilization with the HRT for constant feed glycerol content of initial glycerol (40 g/L). The effluent glycerol content decreased and percent utilization increased with the increasing HRT. The percent glycerol utilization increased from 45 to 85 % with the HRT increased from 12 to 30 h.

Variation of ethanol concentration and productivity with HRT are shown in Fig. 29B. Ethanol concentration and productivity increased with the increase HRT and reached a maximum of 14.72 g/L and 0.49 g/L/h, respectively. The ethanol production and productivity increased with HRT due to higher percent glycerol utilization at high level. Similar to the results of Ozmihci and Kargi (2007b), the ethanol production from cheese whey powder solution by *Kluyvermyces marxianus* DSMZ7239. The percent of sugar utilization and ethanol production increased with the increase of HRT, giving the increasing of sugar utilization from 15 to 86 % with the HRT increased from 12.5 to 60 h. Low HRT or high dilution rate the ethanol production was reduced, probably due to the cells are washed-out from the system (Ozmihci and Kargi, 2007b).

The result of this study indicated that ethanol production in continuous operation is affected by the HRT employed. At high HRT (30 h) or low dilution rate gave the high ethanol production as well as glycerol consumption.

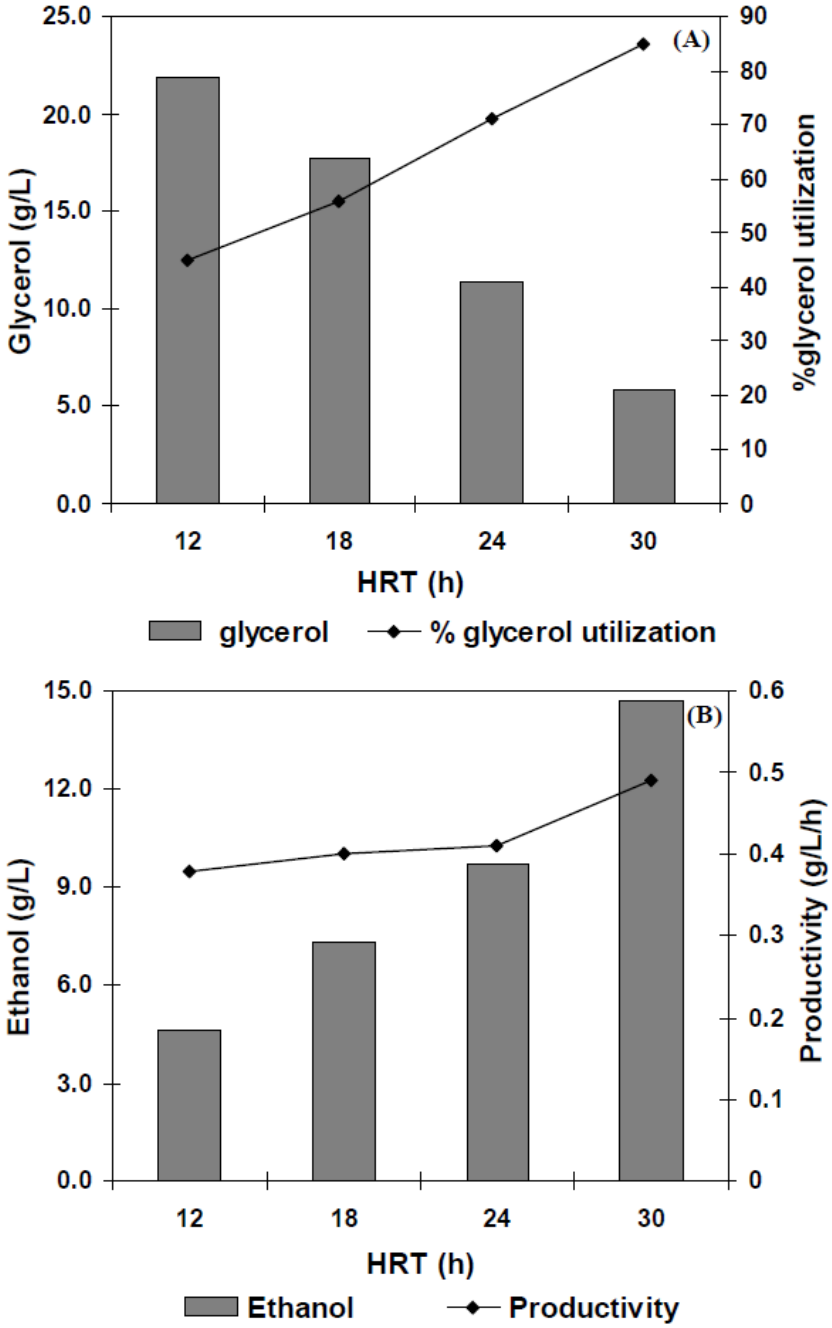


Figure 29. Variation of glycerol and percent glycerol utilization (A), ethanol and productivity (B) with hydraulic retention time (HRT) (in 1.5 L fermenter culture at 37 ºC, initial pH 8.0 without pH-control, 0.5 vvm aeration rate and 60 rpm agitation)

To compare the ethanol under different type of fermentation found that, the ethanol production under two-stage was higher than batch, fed-batch and continuous operation, respectively. When compared to the ethanol production from glycerol by different microorganisms, it was found that *E. aerogenes* TISTR1468 using two-stage process exhibited the high ethanol concentration (Table 20). Therefore, the ethanol production under two-stage process was chosen for the ethanol production in semipilot scale.

Table 20. Comparison of ethanol production from glycerol by other studies with this study

Organism	Fermentation Type	Ethanol Production (g/L)	Productivity (g/L/h)	References
Enterobacter aerogenes HU-101	Batch	10.0	0.83	Ito *et al*., 2005
Klebsiella oxytoca M5a1	Fed-batch	19.5	0.56	Yang *et al*., 2007
Escherichia coli EH05	Batch	20.7	0.22	Durmin *et al*., 2009
Klebsiella pneumonia GEM167	Fed-Batch	21.5	0.93	Oh *et al*., 2011
Klebsiella pneumonia GEM167	Batch	8.6	0.72	Oh *et al*., 2011
Enterobacter aerogenes TISTR1468	Batch	22.97	1.00	This study
Enterobacter aerogenes TISTR1468	Fed-batch	19.97	1.17	This study
Enterobacter aerogenes TISTR1468	Continuous	14.72	0.49	This study
Enterobacter aerogenes TISTR1468	Two-stage	24.47	1.17	This study

4. Ethanol production in 20 L fermenter

Fermentation for ethanol production was carried out in 3 L and 20 L reactors (Fig. 30). Cell growth exhibited similar trend under anaerobic, microaerobic and two-stage fermentation as studied above. Anaerobic condition gave the lowest cell growth (1.0 g/L DCW) in both fermenters. Under microaerobic condition in 3 L and 20 L fermenters, cell growth reached stationary phase around 16 h and 12 h, respectively with the maximum values of 3.79 g/L and 2.76 g/L, respectively. For two-stage fermentation, cell growth reached stationary was reached around 20 h (3.93 g/L), and around 18 h (2.86 g/L), respectively. In 20 L fermenter, two-stage condition gave the highest biomass production (3.14 g/L at 30 h) whereas microaerobic and anaerobic condition gave the maximum cell mass of 2.82 g/L at 24 h and 1.17 g/L at 42 h, respectively. The difference observed between cell growth when fermentation was carried out in 3 L and 20 L reactors, may be due to difference in the particular geometry of each system (Roza *et al*., 2003).

Time course on ethanol production in 3 L and 20 L reactors under the three conditions is given in Fig. 31. Under anaerobic condition, the maximum ethanol production in 3 L reactor was 6.27 g/L at 30 h with 0.21 g/L/h productivity which were higher than those obtained from 20 L reactor (4.00 g/L at 36 h with 0.11 g/L/h productivity). Under microaerobic condition, the maximum ethanol productions were 20.69 g/L and 18.36 g/L, respectively. The two-stage condition gave the highest ethanol production of 24.47 and 18.84 g/L. These results led to the selection of the two-stage condition for the ethanol production.

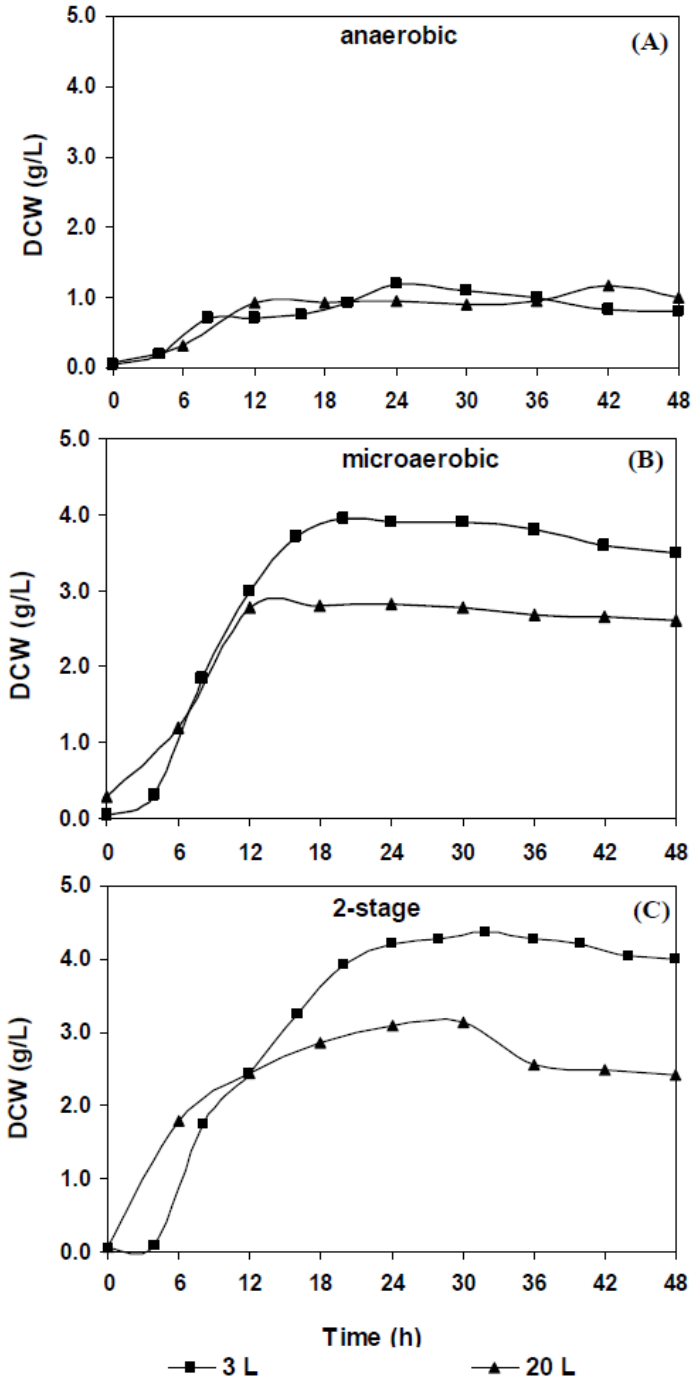


Figure 30. Time course of growth by *Enterobacter aerogenes* TISTR1468 under anaerobic (A), microaerobic (B) and two-stage (C) in 3 L and 20 L reactors (culture at 37 ºC, initial pH 8.0 without pH-control, and 60 rpm agitation for 48 h)

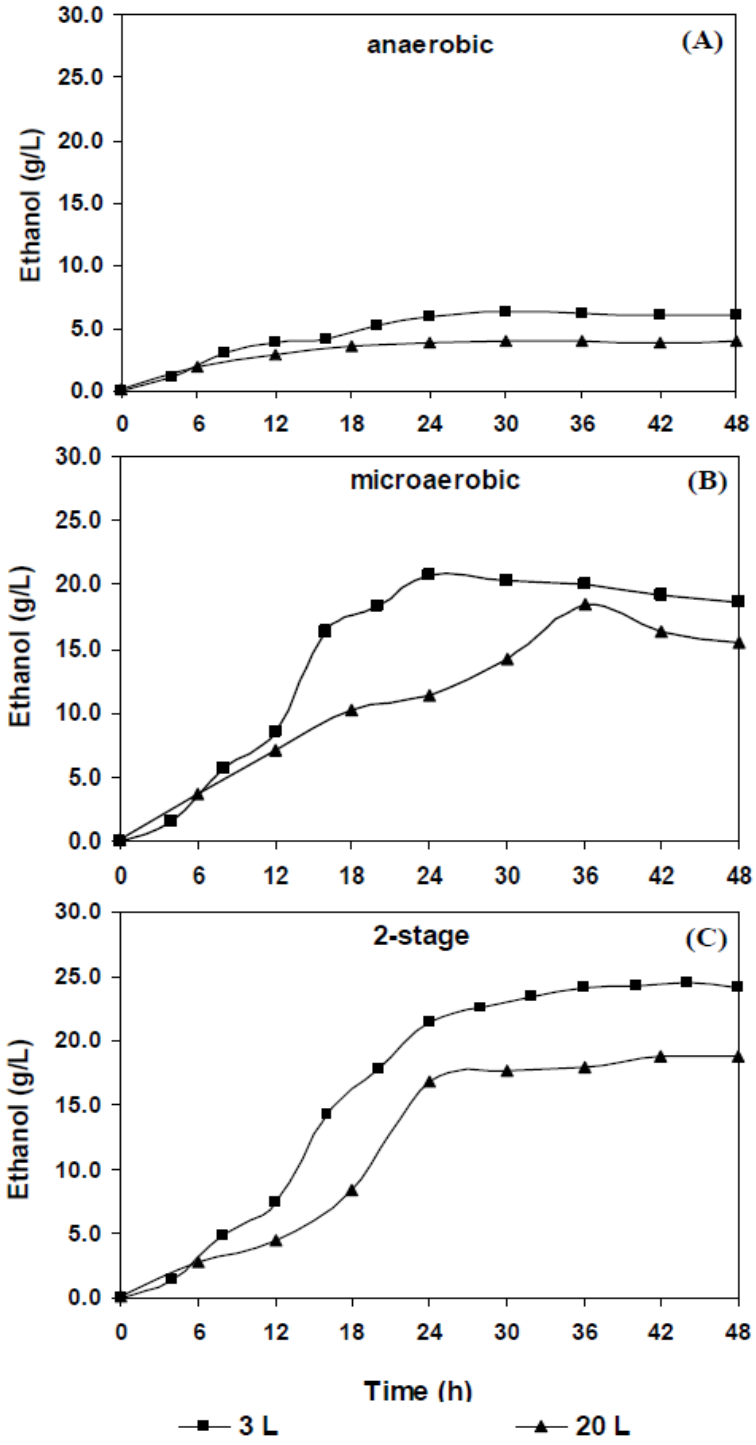


Figure 31. Time course of ethanol production by *Enterobacter aerogenes* TISTR1468 under anaerobic (A), microaerobic (B) and two-stage (C) in 3 L and 20 L reactors (culture at 37 ºC, initial pH 8.0 without pH-control, and 60 rpm agitation for 48 h)

The glycerol consumption in 3 L and 20 L reactors are illustrated in Fig. 32. In Under anaerobic condition in the two reactors, glycerol was consumed only 19% and 16%, respectively therefore, giving low amount of biomass and ethanol. In 3 Lreactor the cell yield was 0.09 g/g and yield ethanol was 0.72 g/g. In 20 L reactor the cell yield (0.13) was slightly higher which the ethanol yield (0.54 g/g) was lower than those obtained at 3 L. Under microaerobic and two-stage condition, the glycerol was completely consumed at around 30 h in 3 L reactor and 48 h in 20 L reactor. In 3 L reactor under microaerobic condition, glycerol was converted to cell and ethanol with the yield of 0.094 g/g and 0.47 g/g, respectively. In 20 L reactor, the yields of cell and ethanol were 0.083 g/g and 0.45 g/g, respectively. For two-stage fermentation in 3 L reactor, the highest yields of cell and ethanol were 0.101 g/g glycerol consumed and 0.56 g/g glycerol, respectively, whereas in 20 L reactor, these values were lower (0.83 g biomass/g glycerol consumed and 0.45 g ethanol/ g glycerol, respectively).

The results revealed that micro-aerobic and two-stage condition were more favorable for cellular growth, ethanol production and glycerol consumption than anaerobic condition. However, the cell and ethanol production in 20 L were lower than those obtained from the 3 L reactor, as also found earlier may be due to difference in the particular geometry of each system and conditions for the production in 3 L was unsuitable condition for 20 L reactor (Roza *et al*., 2003).

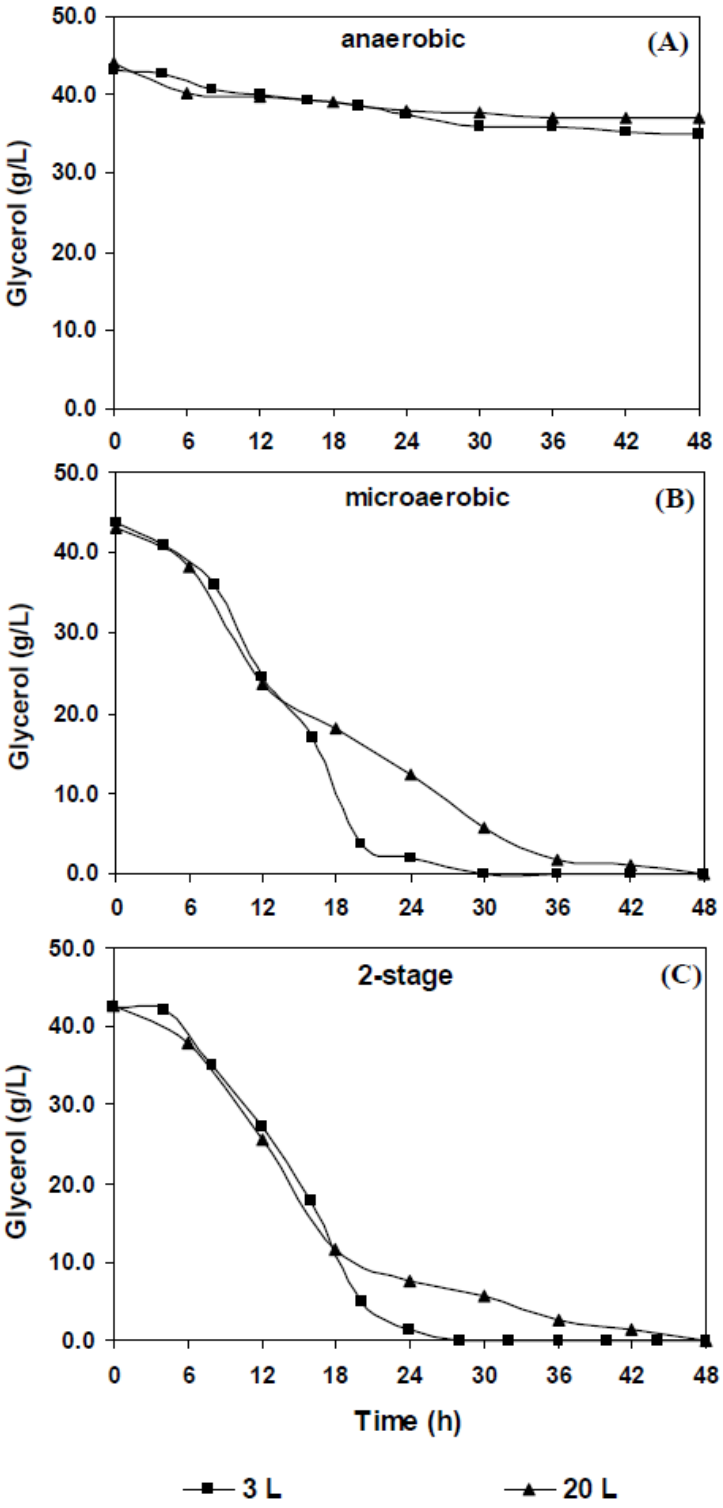


Figure 32. Time course of glycerol consumption by *Enterobacter aerogenes* TISTR1468 under anaerobic (A), microaerobic (B) and two-stage (C) in 3 L and 20 L reactors (culture at 37 ºC, initial pH 8.0 without pH-control, and 60 rpm agitation for 48 h)

5. Ethanol production from pure glycerol by immobilized cells

Enterobacter aerogenes TISTR1468 was immobilized on carrier materials. The carriers for cell immobilization were selected and examined a possibility of reusing for the ethanol production on pure glycerol medium. Ethanol production by repeated-batch using immobilized cells compared with free cells was depicted in Fig. 33. Results demonstrated that the immobilized cells could be reused more than ten times and produced ethanol higher than free cells. The first batch gave similar trend of the ethanol production (5-6 g/L) by immobilized and free cells. The maximum ethanol production using free cells was 5.05 g/L at first batch and decreased thereafter. After ten times repeated-batch, free cells could produce 50% of ethanol compared to the first batch. The maximum ethanol using immobilized cells were 5.88, 5.87, 5.18 and 4.72 g/L on dish sponge, rapeseed straw, activated carbon, and UASB granule, respectively. Moreover, immobilized cell on dish sponge also gave high stability for ethanol production because of the cell could also attach on surface and porous and moreover the porous could protect cell. After ten times repeated-batch, ethanol production by dish sponge immobilized cells decreased only 14%. Therefore, dish sponge was photographed by scanning electron microscopy (SEM) (Fig. 34). The SEM showed that the bacterial cells grow and attached on surface of dish sponge.

The effect of product inhibition was shown in Fig. 35 by varied the initial ethanol concentration (0-50 g/L) and compared between using free cells and immobilized cells. In this study the final pH at 24 h was used as the indicator for cell growth because of during the fermentation the intermediates were produced that caused the drop of pH. The reduction of pH show that the reduction decrease with increasing of ethanol concentration compared with control (ethanol 0 g/L), indicated that cell growth decreased with the increase of initial ethanol concentration (Fig. 35B). Moreover, the slope from linear equation of immobilized cells (0.0264) lower than free cell (0.0285) indicated that the immobilized cells could tolerate higher ethanol concentration than free cells.

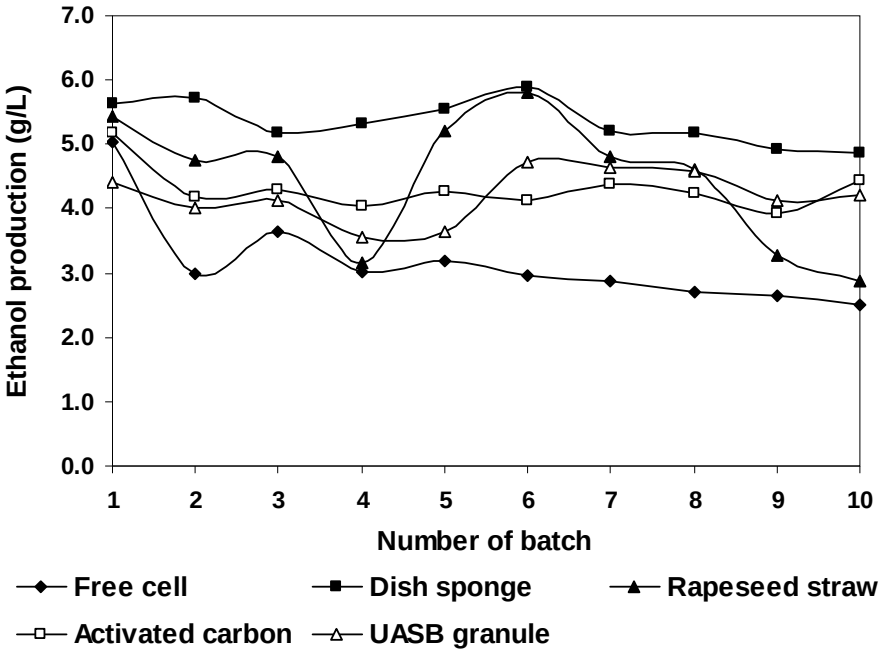


Figure 33. Ethanol production in repeated-batch culture using immobilized *Enterobacter aerogenes* TISTR1468 on different carriers

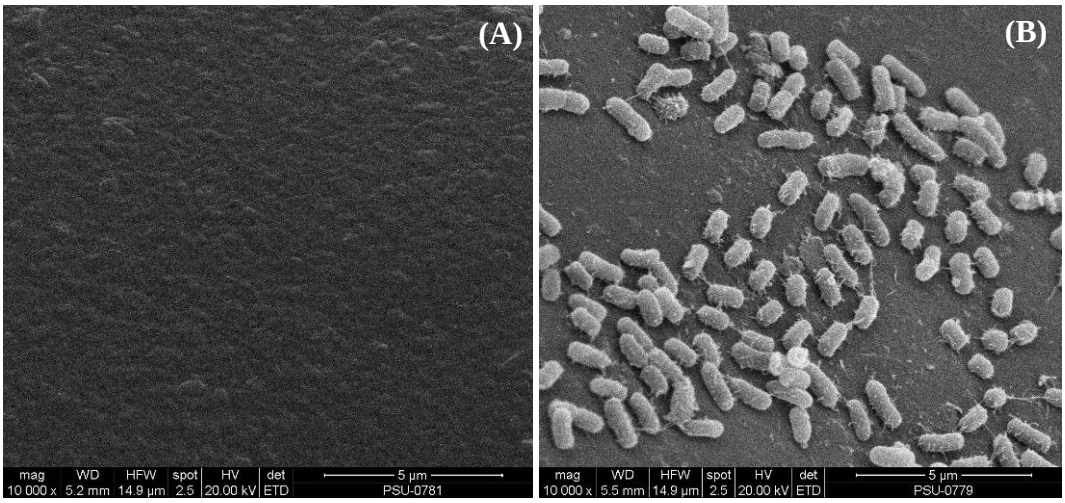


Figure 34. Scanning electron micrograph on dish sponge: (A) before immobilization, (B) immobilization with *Enterobacter aerogenes* TISTR1468

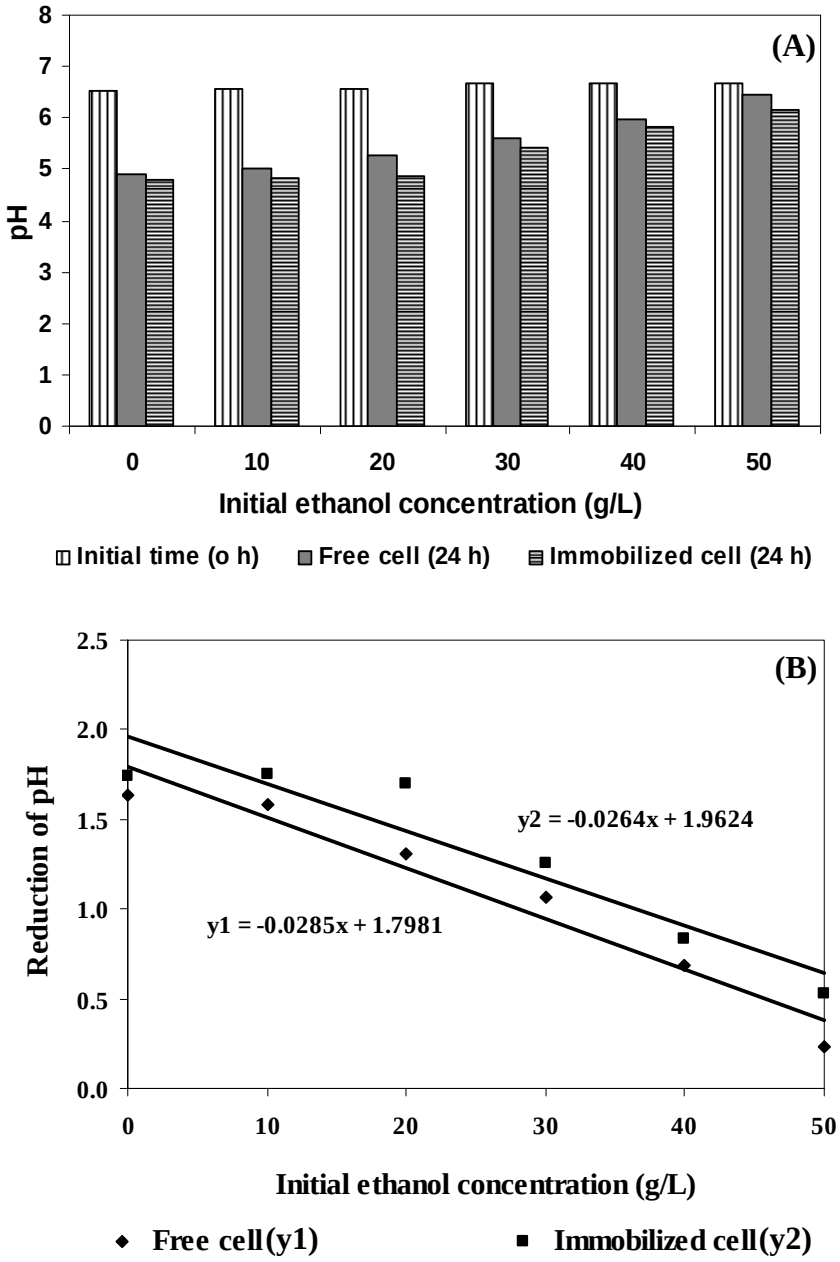


Figure 35. The effect of production inhibition on pH value of free cells and immobilized cells of *Enterobacter aerogenes* TISTR1468 at 0 and 24 h (A), reduction of pH of free cells and immobilized cells at 24 h (B)

The results showed that the immobilized cells on dish sponge were the most suitable for immobilization of *Enterobacter aerogenes* TISTR1468 for ethanol production. The immobilized cells could be reused more than ten times and tolerate higher ethanol concentration than the free cells.

CHAPTER 4

CONCLUSIONS AND SUGGESTIONS

CONCLUSIONS

1. Among five bacterial strains tested, *Enterobacter aerogenes* TISTR1468 was selected as the strain produced higher ethanol from pure and crude glycerol than *Klebsiella oxytoca* TISTR556, *Klebsiella terrigena* SU3, *Klebsiella pneumoniae* SU32 and *Citrobacter freundii* SU17, respectively. The highest ethanol concentration was 7.54 g/L using pure glycerol and 7.24 g/L using raw glycerol.

2. The optimum medium for ethanol production from raw glycerol by *E. aerogenes* TISTR1468 using conventional method was 30 g/L raw glycerol, 2.0 g/L $(NH_4)_2HPO_4$ and 5.0 g/L yeast extract. Under this condition, the highest ethanol production, yield and productivity increased to 14.53 g/L, 0.63 g/g and 0.91 g/L/h, respectively.

3. Using the statistical methodology, the maximum ethanol production from raw glycerol (16.19 g/L) was obtained under the optimum medium composition of 38.28 g/L raw glycerol, 2.10 g/L $(NH_4)_2HPO_4$ and 7.20 g/L yeast extract. Therefore, the ethanol production and productivity increased by 2.24 and 1.50 folds, respectively, compared to the original medium.

4. The optimum environmental condition was initial pH 8.0 without pH-control, 0.5 vvm aeration rate and 60 rpm agitation speed. Under this condition, 14.0 g/L ethanol, 0.60 g/g yield, 0.70 g/L/h of productivity and 1.21 g/L/h glycerol consumption rate were obtained.

5. Simple kinetic model for the batch production of ethanol was developed. Highest ethanol production of 22.97 g/L was achieved at 50 g/L crude glycerol. The results of the model simulations showed good agreement with the experimental data obtained at varying initial glycerol concentrations.

6. The ethanol production under two-stage process was higher than those using micro-aerobic or anaerobic conditions. The highest ethanol concentration of 24.5 g/L

and productivity of 0.68 g/L/h were achieved. This strategy was more suitable for ethanol production than batch, fed-batch and continuous fermentation.

7. The ethanol production in 20 L reactor under two-stage was 18.84 g/L, cell yield 0.083 g/g and ethanol yield 0.45 g/g, which decreased by 1.30, 1.22 and 1.24 folds compared to 3 L reactor.

8. Immobilized cells of *Enterobacter aerogenes* TISTR1468 on dish sponge was the most suitable for the ethanol production. This immobilized cells could be reused more than ten times and tolerate higher ethanol concentration than free cells.

SUGGESTIONS

1. Study the effect of nitrogen source using low cost nitrogen source such as urea to reduce the production cost.

2. Use the results of substrate inhibition from batch fermentation to design substrate feeding strategies such as glycerol concentration and feeding time.

3. Increase the hydraulic retention time (HRT) higher than 30 h to improve the ethanol production.

4. Improve the ethanol production using the combination method of two-stage and fed-batch fermentation by feeding the fresh substrate in second-stage.

5. Optimization of condition for ethanol production in 20 L fermeter, use the criteria from 3 L fermenter such as k_La and OLR.

6. Apply the immobilized cell for ethanol production on crude glycerol.

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APPENDICES

APPENDIX A

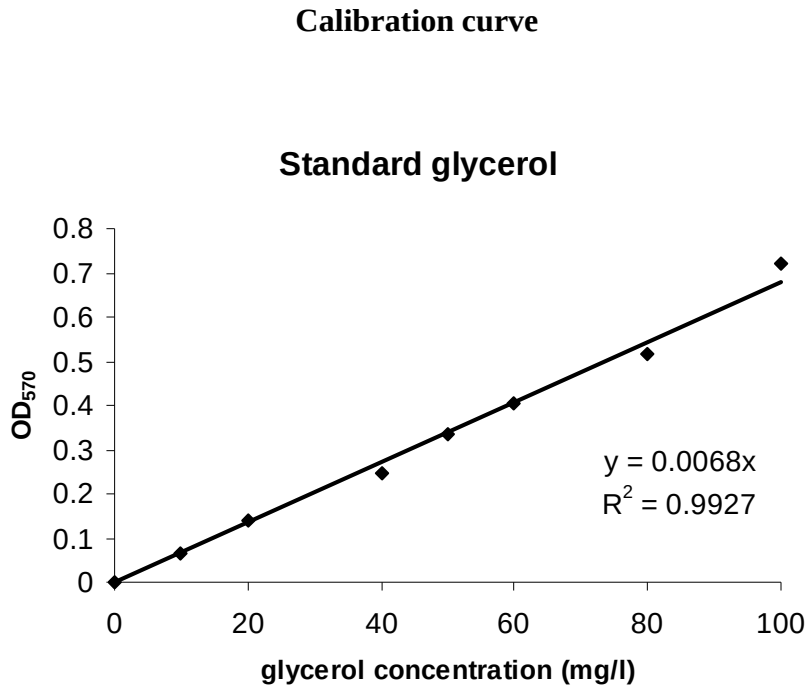

Figure 1-A. Calibration curve of glycerol concentration by absorbance measurements at 570 nm

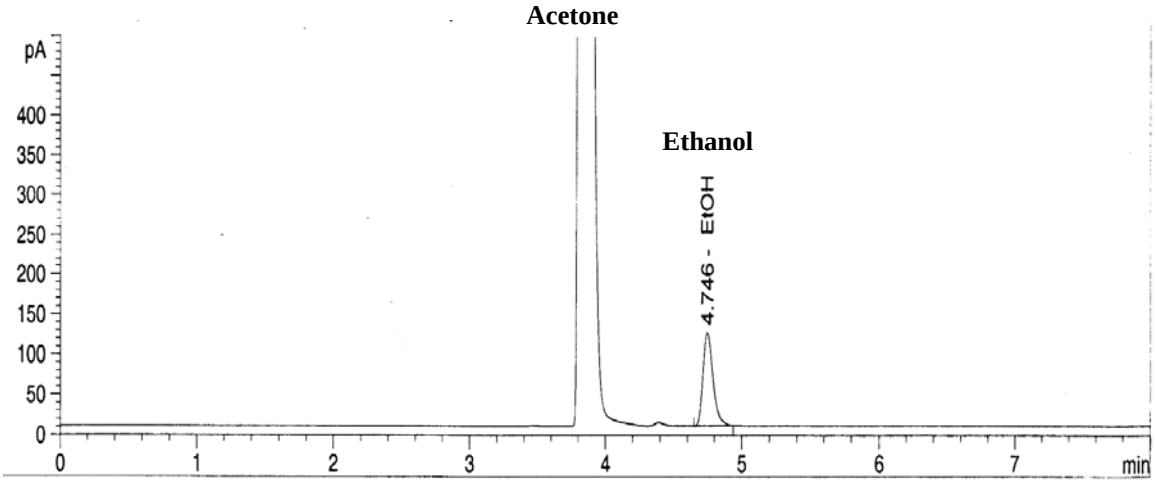

Figure 2-A. Chromatogram of ethanol using gas chromatography

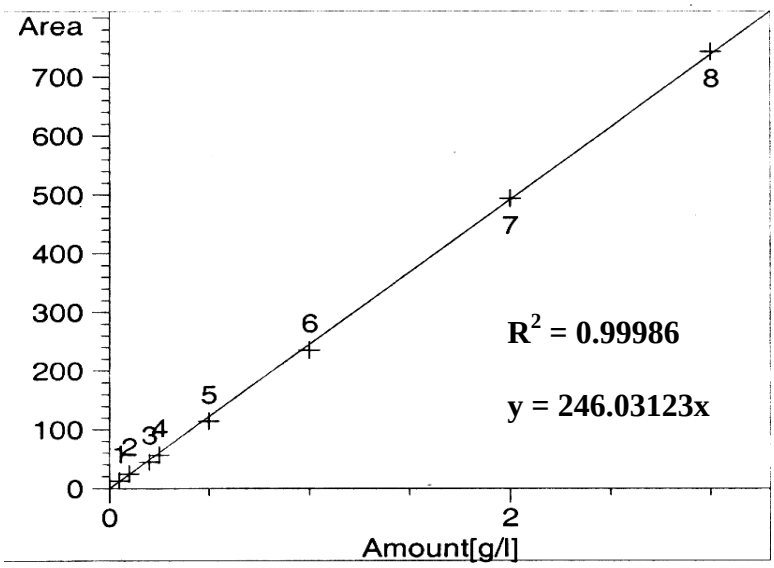

Figure 3-A. Calibration curve of ethanol concentration by GC-FID

APPENDIX B

Experimental data on optimization

Table 1-B. Effect of initial glycerol concentration on ethanol production, glycerol consumption rate, productivity and yield

Glycerol (g/L)	t (h)	ΔP (g/L)	ΔS (g/L)	ΔS/t (g/L)	ΔP/t (g/L/h)	$Y_{P/S}$ (g/g)
10	8	4.10±1.41	8.74±0	1.09±0	0.51±0.030	0.47±0.003
20	12	8.61±2.16	16.96±0.04	1.41±0.04	0.72±0.026	0.51±0.020
30	12	10.08±2.18	22.72±0.02	1.89±0.02	0.84±0.024	0.44±0.010
40	12	9.54±2.21	22.38±0.08	1.87±0.08	0.80±0.013	0.42±0.080
50	16	7.97±1.94	27.97±0.11	1.75±0.11	0.50±0.016	0.28±0.090

Table 2-B. Effect of on inorganic nitrogen on ethanol production, glycerol consumption rate, productivity and yield (*Control = $(NH_4)_2SO_4$)

Inorganic nitrogen source	t (h)	ΔP (g/L)	ΔS (g/L)	ΔS/t (g/L)	ΔP/t (g/L/h)	$Y_{P/S}$ (g/g)
NH_4Cl	12	10.88±0.59	21.12±0.03	1.76±0.03	0.91±0.00	0.52±0.01
CH_3COONH_4	16	10.65±0.75	20.48±0.11	1.28±0.11	0.67±0.08	0.52±0.01
NH_4NO_3	16	12.60±0.34	22.10±0.01	1.38±0.01	0.79±0.05	0.57±0.04
$(NH_4)_2HPO_4$	16	12.74±0.31	25.48±0.08	1.60±0.08	0.80±0.06	0.50±0.00
*$(NH_4)_2SO_4$	20	10.20±0.96	23.72±0.10	1.19±0.10	0.51±0.1	0.43±0.00

Table 3-B. Effect of ammonium hydrogen phosphate $(NH_4)_2HPO_4$ concentration on ethanol production, glycerol consumption rate, productivity and yield

$(NH_4)_2HPO_4$ (g/L)	t (h)	ΔP (g/L)	ΔS (g/L)	ΔS/t (g/L)	ΔP/t (g/L/h)	$Y_{P/S}$ (g/g)
0.5	12	9.00±0.27	21.43±0.11	1.80±0.11	0.75±0.019	0.42±0.004
1.0	16	10.43±0.52	24.83±0.09	1.56±0.09	0.65±0.033	0.42±0.021
1.5	12	9.79±0.52	25.10±0.14	2.09±0.14	0.82±0.044	0.39±0.023
2.0	16	12.96±0.17	32.40±0.15	2.03±0.15	0.81±0.011	0.40±0.000
2.5	20	12.98±1.50	29.40±0.09	1.47±0.09	0.65±0.075	0.44±0.010
3.0	16	11.23±0.01	28.08±0.09	1.76±0.09	0.70±0.001	0.40±0.001

Table 4-B. Effect of organic nitrogen source on ethanol production, glycerol consumption rate, productivity and yield

Organic nitrogen source	t (h)	ΔP (g/L)	ΔS (g/L)	ΔS/t (g/L)	ΔP/t (g/L/h)	$Y_{P/S}$ (g/g)
Control*	24	11.20±0.62	33.12±0.06	1.38±0.06	0.47±0.03	0.34±0.00
Yeast extract	20	12.86±0.88	34.60±0.15	1.73±0.15	0.64±0.04	0.37±0.02
Tryptone	20	9.47±0.55	31.57±0.02	1.58±0.02	0.47±0.03	0.30±0.04
Peptone	20	7.36±0.17	24.20±0.06	1.21±0.06	0.37±0.01	0.30±0.02
Malt extract	24	11.49±1.03	31.92±0.00	1.33±0.00	0.48±0.04	0.36±0.01

*Control = Yeast extract + Tryptone

Table 5-B. Effect of yeast extract concentration on ethanol production, glycerol consumption rate, productivity and yield

Yeast extract (g/L)	t (h)	ΔP (g/L)	ΔS (g/L)	ΔS/t (g/L)	ΔP/t (g/L/h)	$Y_{P/S}$ (g/g)
1.0	20	9.77±0.39	19.94±0.04	1.00±0.04	0.49±0.02	0.49±0.01
2.5	16	10.52±0.20	22.00±0.04	1.38±0.04	0.66±0.01	0.48±0.01
5.0	16	14.53±0.61	23.04±0.03	1.44±0.03	0.91±0.04	0.63±0.06
7.5	16	15.30±0.51	26.40±0.01	1.65±0.01	0.96±0.03	0.58±0.01
10.0	16	15.37±0.35	26.72±0.03	1.67±0.03	0.96±0.02	0.58±0.02

Table 6-B. Effect of initial pH on ethanol production, glycerol consumption rate, productivity and yield

Initial pH	t (h)	ΔP (g/L)	ΔS (g/L)	ΔS/t (g/L)	ΔP/t (g/L/h)	$Y_{P/S}$ (g/g)
6.5	24	9.45±0.42	21.60±0.12	0.90±0.12	0.39±0.05	0.44±0.058
6.8*	24	10.22±0.90	25.92±0.04	1.08±0.04	0.43±0.04	0.40±0.035
7.0	24	11.49±0.18	26.88±0.01	1.12±0.01	0.48±0.01	0.43±0.007
7.5	24	12.42±0.05	28.56±0.01	1.19±0.01	0.52±0.00	0.44±0.002
8.0	24	12.74±0.37	29.52±0.01	1.23±0.01	0.53±0.02	0.43±0.012
8.5	24	11.21±0.03	28.80±0.01	1.20±0.01	0.47±0.00	0.39±0.001

* Control = pH 6.8

Table 7-B. Effect of pH-control on ethanol production, glycerol consumption rate, productivity and yield

pH-control	t (h)	ΔP (g/L)	ΔS (g/L)	ΔS/t (g/L)	ΔP/t (g/L/h)	$Y_{P/S}$ (g/g)
Control	24	12.75±0.04	32.21±0.15	1.34±0.15	0.53±0.01	0.40±0.04
Uncontrol	24	12.19±1.39	32.10.066±	1.34±0.06	0.51±0.08	0.38±0.00

Table 8-B. Effect of aeration rate on ethanol production, glycerol consumption rate, productivity and yield

Aeration rate (vvm)	t (h)	ΔP (g/L)	ΔS (g/L)	ΔS/t (g/L)	ΔP/t (g/L/h)	$Y_{P/S}$ (g/g)
0.0	20	4.95±0.26	12.00±0.02	0.60±0.02	0.25±0.01	0.41±0.00
0.25	20	11.64±0.21	20.60±0.03	1.03±0.03	0.58±0.01	0.56±0.01
0.50	16	11.66±0.55	20.00±0.05	1.25±0.05	0.73±0.03	0.58±0.02
1.00	16	7.63±0.14	23.84±0.07	1.49±0.07	0.48±0.01	0.32±0.01

Table 9-B. Effect of agitation speed on ethanol production, glycerol consumption rate, productivity and yield

Agitation rate (rpm)	t (h)	ΔP (g/L)	ΔS (g/L)	ΔS/t (g/L)	ΔP/t (g/L/h)	$Y_{P/S}$ (g/g)
60	20	14.03±0.09	24.20±0.01	1.21±0.01	0.70±0.00	0.58±0.03
120	16	13.50±0.27	23.84±0.05	1.49±0.05	0.84±0.02	0.57±0.02
180	12	12.64±0.34	22.68±0.01	1.89±0.01	1.05±0.03	0.56±0.02
240	16	12.64±0.26	26.56±0.02	1.66±0.02	0.79±0.02	0.48±0.04

APPENDIX C

Kinetics on batch fermentation

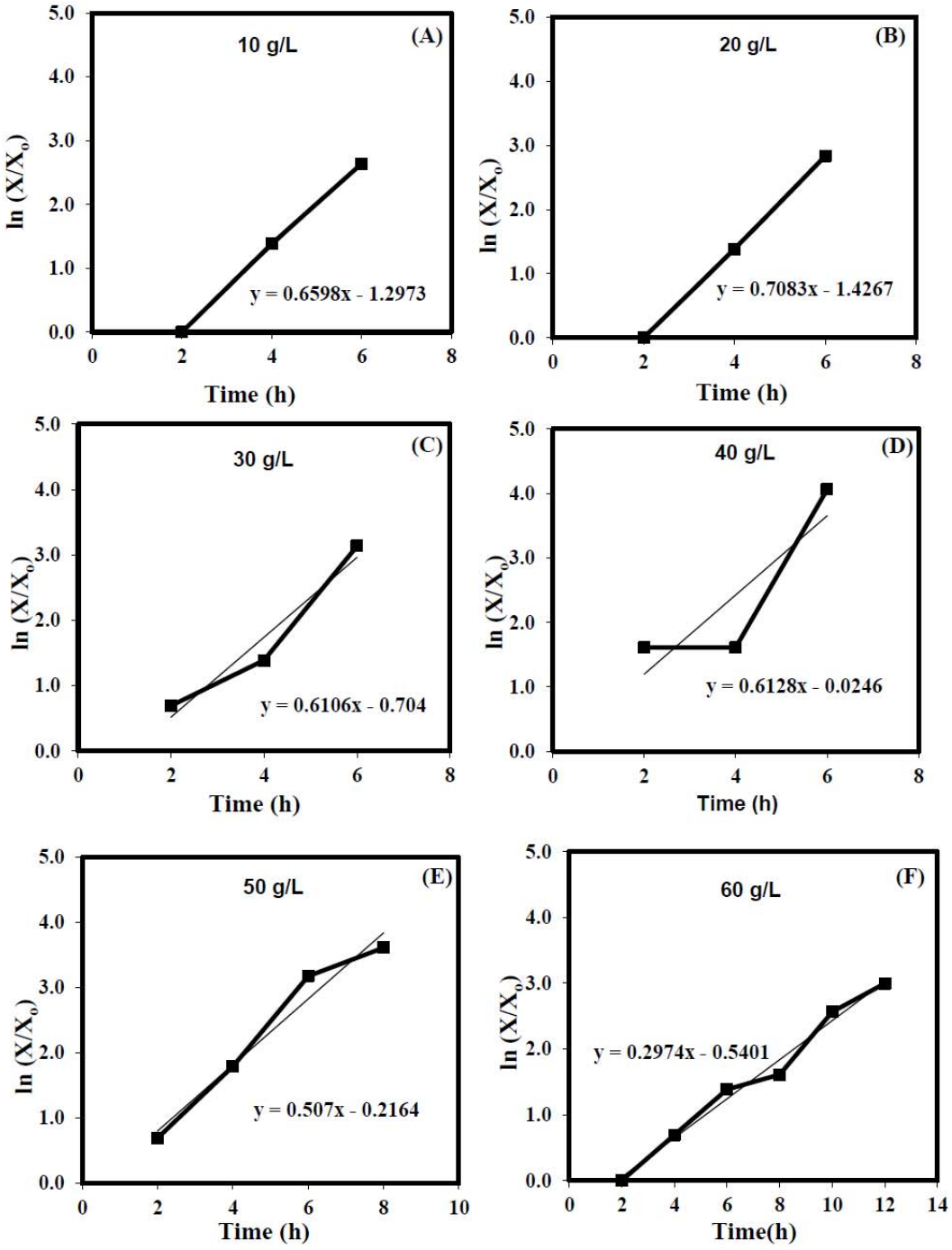

Figure 1-C. Effect of initial glycerol concentration (10-60 g/L) on specific growth by *Enterobacter aerogenes* TISTR1468 in 3 L fermenter, at 37 ºC, 0.5 vvm aeration rate, and 60 rpm agitation speed

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List of Publications and Proceeding

Publications

Saisa-ard, K. and Prasertsan, P. Ethanol Production using Crude Glycerol from Biodiesel Production Plant by *Enterobacter aerogenes* on Batch Fermentation. (Accepted in Wulfenia)

Saisa-ard, K. and Prasertsan, P. Strain selection and optimization for ethanol production using crude glycerol from biodiesel plant. (Manuscript preparation)

Proceeding

Saisa-ard, K., Angelidaki, I. and Prasertsan, P. 2011. Micro-aerobic, anaerobic and two-stage condition for ethanol production by *Enterobacter aerogenes* from biodiesel-derived crude glycerol. World Academic of Science, Engineering and Technology. 77:795-798.

Presentations

Saisa-ard, K. and Prasertsan, P. 2009. Medium optimization for ethanol production using raw glycerol from biodiesel production plant. International Congress of

Malaysian Society for Microbiology (ICMSM), December 1-4'2009, Penang, Malaysia. (Poster presentation)

Saisa-ard, K., Angelidaki, I. and Prasertsan, P. 2011. Micro-aerobic, anaerobic and two-stage condition for ethanol production by *Enterobacter aerogenes* from biodiesel-derived crude glycerol. The International Conference on Agricultural, Biosystems, Biotechnology and Biological Engineering (ICABBBE2011). June 26-28' 2011, Paris, France. (Oral presentation)

Saisa-ard, K. and Prasertsan, P. 2011. Kinetics of batch ethanol production using crude glycerol from biodiesel production plant by *Enterobacter aerogenes*. 11th International Conference on Clean Energy (ICCE) 2011, 2-5 November 2011, Feng Chia University, Taiwan. (Poster presentation)

Saisa-ard, K. and Prasertsan, P. 2011. Optimization of fermentation conditions for ethanol production from biodiesel-derived crude glycerol using *Enterobacter aerogenes*. Thailand Chemical Engineering and Applied Chemistry Conference (TIChE) International Conference 2011, November 10-11'2011, Prince of Songkla University, Thailand. (Oral presentation)