Optimization on Ethanol Production from Glycerol using *Enterobacter aerogenes* TISTR 1468

Akbar Ciptanto

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

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Thesis Title	Optimization on Ethanol Production from Glycerol using *Enterobacter aerogenes* TISTR 1468
Author	Mr. Akbar Ciptanto
Major Program	Biotechnology

Major Advisor	**Examining Committee:**
………………………………………	…………………………Chairperson
(Assoc. Prof. Dr. Poonsuk Prasertsan)	(Assist. Prof. Dr. Benjamas Cheirsilp)
	………………………………………
Co-advisor	(Assoc. Prof. Dr. Poonsuk Prasertsan)
………………………………………	………………………………………
(Assoc. Prof. Dr. Aran H-Kittikun)	(Assoc. Prof. Dr. Aran H-Kittikun)
	………………………………………
	(Assoc. Prof. Dr. Vichien Kitpreechavanich)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Master of Science Degree in Biotechnology.

…………………………………………

(Assoc. Prof. Dr. Krerkchai Thongnoo)

Dean of Graduate School

Thesis Title	Optimization on Ethanol Production from Glycerol Using *Enterobacter aerogenes* TISTR 1468
Author	Akbar Ciptanto
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ABSTRACT

Optimization of ethanol production from glycerol by *Enterobacter aerogenes* TISTR 1468 was investigated using 'one-factor-at-a-time method'. The optimum condition for ethanol production was studied using response surface methodology (RSM) combined with Box–Behnken Design (BBD) to describe the relationship among the selected parameters. Finally, the optimum condition was used to produce ethanol under batch and semi continuous fermentation.

Eight parameters affecting ethanol production from glycerol by *Enterobacter aerogenes* TISTR 1468 were investigated using 'one-factor-at-a-time method'. Time course study gave the optimum cultivation time at 8 hour and shaking speed of 150 rpm. The optimum glycerol concentration giving the highest ethanol concentration (12.14 g/l) was 40 g/l but not significant different with 20 g/l at 11.17 g/l, the highest ethanol yield (0.98 g ethanol/g glycerol) and ethanol productivity (1.36 g/l/h) were achieved at 20 g/l glycerol. However, 40 g/l glycerol concentration was chosen for the further study. Yeast extract at 10 g/l was the optimum organic nitrogen source and concentration that giving the highest ethanol concentration and productivity (11.16 g/l and 1.39 g/l/h) whereas the highest ethanol yield (0.49 g ethanol/g glycerol) was obtained at 1 g/l yeast extract. For inorganic nitrogen source and concentration, 0.4 mmol nitrogen as $(NH_4)_2HPO_4$ gave no significant results with 0.8 mmol nitrogen in $(NH_4)_2HPO_4$, whereas the highest ethanol concentration (11.34 g/l), ethanol yield (0.47 g ethanol/g glycerol) and ethanol productivity (1.42 g/l/h). Trace element solution and $NiCl_2$ had no effect on ethanol production. The optimum initial pH was 7.5, giving the ethanol concentration of 16.31 g/l, the yield of 0.52 g ethanol/g glycerol and ethanol productivity of 1.57 g/l/h.

Optimization studies of the three selected parameters using response surface methodology (RSM) combined with Box–Behnken Design (BBD) was conducted under facultative condition in shake flask (250 ml) culture of *E. aerogenes*

TISTR 1468 with 150 rpm agitation speed at 37ºC and initial pH of 7.5. The RSM results illustrated that the optimal medium composed of 30 g/l glycerol, 8.73 g/l yeast extract and 0.30 mmol nitrogen in $(NH_4)_2HPO_4$. Glycerol concentration had an effect on ethanol production, while yeast extract and $(NH_4)_2HPO_4$ had no significant effect on ethanol concentration, ethanol yield and productivity ($P<0.05$). The predicted maximum ethanol production, ethanol yield and productivity were 14.32 g/l, 0.64 g/g and 1.79 g/l/h, respectively. In the confirmation experiment under the optimized conditions, highly reproducible results were obtained, giving ethanol concentration, ethanol yield and productivity of 16.38 g/l, 0.63 g/g and 2.05 g/l/h, respectively.

The optimum condition from RSM results was employed in a 3 L fermentor with 2 L working volume of the optimum medium with 150 rpm agitation speed, the initial pH 7.5 and cultivated at 37^0C, for 8 h. For semi-continuous cultivation, batch culture was carried out for the period that reached the highest ethanol production during 8 h. Then, 1/3 of the culture broth was withdrawn and the same quantity of the fresh medium was added. This process was conducted 3 times during cultivation. Samples were taken every 8 hour cultivation. Batch and semi-continuous fermentation exhibited no significant difference on the product formation. Ethanol (15 g/l) was the main product with much lower values of butanol (0.3-0.4 g/l), acetic acid (0.02-0.03 g/l) and butyric acid (0.02-0.03 g/l).

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LIST OF ABBREVIATION AND SYMBOLS

ANOVA	Analysis of variance
$AlK(SO_4)_2 \cdot H_2O$	Aluminum potassium sulfate
BBD	Box–Behnken Design
CV	Coefficient variations
$C_3H_5[OH]_3$	Glycerol
C_3H_5	Glyceryl
CH_3CH_2OH	Ethanol
$CaCl_2 \cdot 2H_2O$	Calcium chloride dehydrate
$CuCl_2 \cdot 2H_2O$	Copper (II) chloride dihydride
FFA	Free fatty acid
GC-FID	Gas Chromatography – Flame Ionized Detector
H_2	Hydrogen
H_3BO_4	Boric Acid
He	Helium
HPLC	High Performance Liquid Chromatography
H_2SO_4	Sulfuric Acid
K_2HPO_4	Dipotassium phosphate
KH_2PO_4	Potassium dihydrogen phosphate
KOH	Alkali
KCl	Salt
$MnCl_2 \cdot 4H_2O$	Manganese dichloride tetrahydrate; Manganous chloride tetrahydrate
$MgSO_4 \cdot 7H_2O$	Magnesium sulphate
$Na_2MoO_4 \cdot 2H_2O$	Sodium molybdate
Na_2SeO_3	Sodium selenite
Na_2EDTA	Disodium ethylenediaminetetraacetate
NaOH	Sodium hydroxide
NH_4	Ammonium

LIST OF ABBREVIATION AND SYMBOLS (CONTINUED)

$(NH_4)_2SO_4$	Ammonium sulphate
$(NH_4)_2HPO_4$	Diamonium hydrophosphate
$NH_4C_2H_3O_2$	Ammonium acetate
NOx	Nitrogen oxides
$NiCl_2$	Nickel chloride
nm	Nanometer
OD	Optical Density
PDH	Pyruvate dehydrogenase
RSM	Response Surface Methodology
rpm	Revolution per minute
SPSS	Statistical Package for the Social Sciences
TSA	Tryptone Soy Agar
TISTR	Thailand Institute of Scientific and Technological Research
vvm	Volume of air per volume of medium per minute
w/v	Weight / Volume

CHAPTER 1
INTRODUCTION

Introduction

Biodiesel is a renewable fuel manufactured from vegetable oils, animal fats, and recycled cooking oils. Although biodiesel are produced chemically and enzymatically, glycerol is inevitably generated as the by-product (Vicente *et al*. 2004; Du *et al*. 2003). This waste or raw glycerol could be purified and used in many industries such as in food, pharmaceutical, cosmetics, and many others. Besides utilization as glycerol itself, glycerol could be used as an alternative carbon source for production of many other value-added products particularly ethanol.

Fermentative metabolism of glycerol has been studied in several species of the Enterobacteriaceae family that belong to the genera *Klebsiella, Citrobacter, Enterobacter, Clostridium,* and *E. coli*. From all of the species, only *Enterobacter aerogenes* could convert glycerol to ethanol as a main product. Glycerol fermentation was studied and yielded hydrogen gas and ethanol as main product in bioelectrochemical cells with thionine using wild type of *Enterobacter aerogenes* NBRC 12010. Both of yields are more than 80% even in the wild type (Sakai and Yagishita, 2007). *Enterobacter aerogenes* HU-101 could convert both purified glycerol to produce hydrogen and ethanol as main products. In continuous culture with a packed-bed reactor using self-immobilized cells, the maximum hydrogen production rate from pure glycerol was 80 mmol/l/h with the yield of 0.8 mol ethanol/mol glycerol. When using porous ceramics as a support material to fix cells in the reactor, the maximum hydrogen production from biodiesel wastes was 63 mmol/l/h and 0.85 mol ethanol/mol glycerol (Ito *et al*. 2005). On the other hand, many species belonging to Enterobactericeae only could convert glycerol to ethanol as by-product, such as *Enterobacter aglomerans* (Barbirato *et al*. 1997), *Klebsiella oxytoca* (Yang *et al.* 2007), *Klebsiella pneumoniae* (Zheng *et al*. 2008; Zhanga *et al*. 2006; Zhao *et al*. 2006; Lin *et al*. 2005), *Clostridium butyricum* (Colin *et al*. 2001), *Clostridium acetobutylicum* (Gonzalez-Pajuelo *et al*. 2005).

Objectives

The main objective of this research is to optimize the nutrient and environmental factors for production of ethanol from purified glycerol of biodiesel plant by *Enterobacter*

aerogenes TISTR 1468 using 'one-factor-at-a-time' method. Optimization of the selected parameters culture conditions will be studied using Response Surface Methodology (RSM). Finally, comparison on ethanol production and the other products using batch and semi continuous fermentation will be investigated.

Scope of Research

Optimization on nutrient and environmental factors for production of ethanol from purified glycerol of biodiesel plant by *Enterobacter aerogenes* TISTR 1468 using 'one-factor-at-a-time' method and response surface methodology (RSM) method, then comparison between batch and semi continuous cultivation.

Literature review

1. Biodiesel

Biodiesel is a renewable fuel manufactured from vegetable oils, animal fats, and recycled cooking oils. Biodiesel offers many advantages such us, renewable, energy efficient, displaces petroleum derived diesel fuel, can be used in most diesel equipment with no or only minor modifications, can reduce global warming gas emissions, can reduce tailpipe emissions including air toxics, nontoxic, biodegradable, suitable for sensitive environments, and made from either agricultural or recycled resources.

There are three basic methods of biodiesel (methyl ester) production from oils and fats with glycerol or glycerin as a by-product. They are, base catalyst transesterification of the oil with methanol, directed acid catalyzed esterification of the oil with methanol, conversion of the oil to fatty acids, and then to methyl esters with acid catalysis. Today, biodiesel production usually use base catalyzed reaction which is the most economic process as it uses a low temperature (15^{0}F), pressure (20 psi), and gives a high conversion of 98% with no intermediate steps (Biodiesel Production Technology, 2000).

The transesterification process involves mixing at room temperature methanol (50% excess) with NaOH (100% excess), then mixing vigorously with vegetable oil and letting the glycerol settle (about 15% of the biodiesel mix). The supernatant is biodiesel and contains a mixture of methylated fatty acids and methanol, the catalyst remaining dissolved in the glycerol fraction. Industrially, the esters are sent to the clean-up or purification process which consists of water washing, vacuum drying, and filtration. An *in situ* alkaline transesterification was efficient in preparing fatty acid esters, the simple and direct process eliminating the expense associated with solvent extraction and oil clean-up (Haas *et al*. 2004). Transesterification may be processed using methanol, ethanol, isopropyl alcohol, or butanol, the catalyst being either sodium or potassium hydroxide.

2. Glycerol

Glycerine or glycerol is a a clear, odorless, viscous liquid. It is an alcohol with three hydroxyl (OH) groups, giving the synonyms as 1,2,3-trihydroxypropane or 1,2,3-propanetriol. Glycerol preferably called to indicate its alcohol character (trihydric alcohol $C_3H_5[OH]_3$), containing the trivalent radical "glyceryl" (C_3H_5). It has the chemical formula as $C_3H_8O_3$ (Fig. 1).

Glycerol is soluble in water and alcohol but insoluble in ether, benzene, or oils. Refined glycerin is mostly pure glycerol, with the salt, methanol and free fatty acids

removed. Glycerol is chemically stable under normal storage and handling conditions. It has no self-reactivity, spontaneous combustibility, or explosive properties. However, it may become explosive when in contact with strong oxidizing agents such as potassium chlorate or potassium permanganate. At low temperatures, glycerol sometimes forms crystals which tend to melt at 17.9° C. Liquid glycerol boils at 290° C under normal atmospheric pressure. Its specific gravity is 1.26 and its molecular weight is 92.09.

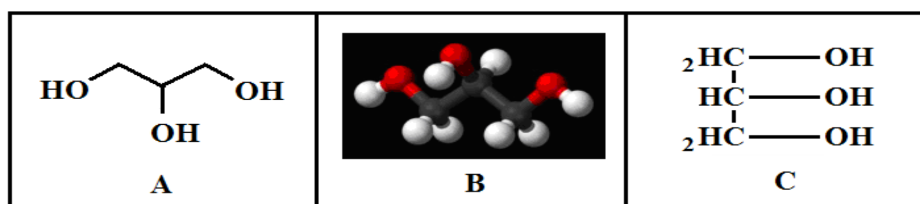


Figure 1. Structure model glycerol (A), 3D model (B), and structure formula (C)
Source: Wikipedia Foundation Inc (2006)

Crude glycerin is a low-value byproduct of the transesterification reaction conducted during production of biodiesel. It is mixed with varying amounts of soap, alcohol, catalyst, and water. This mixture can be in a liquid or solid form, and range in color from transparent and light yellow to opaque and dark brown. From a single stage base catalyzed reaction using waste frying oil feedstock, the amount of actual glycerol in the mixture is typically around 40% with the majority of the rest of it being soap. For every 9 kg of biodiesel produced about 1 kg of a crude glycerol by-product is formed (Dasari *et al*. 2005). There are techniques to reduce the water and FFA content, the most common of which that is applicable at small scales is a two stage, acid-base method that uses sulfuric acid during the pretreatment stage.

Glycerol is competitive with sugar used in the production of chemicals and fuels such as butanol and ethanol via microbial fermentation (Yazdani and Gonzalez, 2007). Additionally, glycerol has yield advantages over sugar due to the highly reduced nature of carbon atoms. A native, nonpathogenic strain of *E. coli* was able to ferment glycerol to ethanol under anaerobic condition without need to do much genetic engineering to have ethanol as the main product. The key factor is not the type of strain, but rather on the appropriate environment including an acidic pH, avoiding accumulation of fermentation gas hydrogen and appropriate medium composition. With the right environment, ethanol is the primary product from glycerol fermentation with *E. coli*.

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. An effective usage or conversion of crude glycerol to specific products will cut down the biodiesel production costs. Glycerol can cover possible conversion into useful products such as 1,3-propanediol, 1,2-propanediol, dihydroxyacetones, hydrogen, polyglycerols, succinic acid, polyesters, and ethanol.

3. Ethanol

Ethanol or ethyl alcohol (CH_3CH_2OH) (Fig. 2) is the most widely used liquid biofuel (as gasohol). Most commercial production of ethanol is from sugar cane or sugar beet as starches and cellulosic biomass. It using as well as for manufacture of cosmetics, pharmaceuticals, and also for the production of alcoholic beverages.

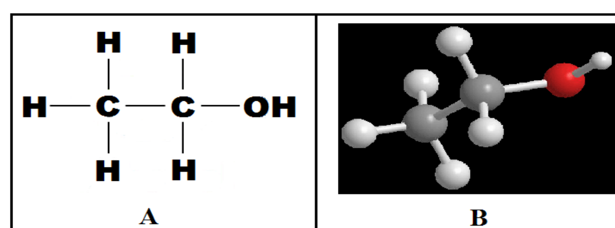


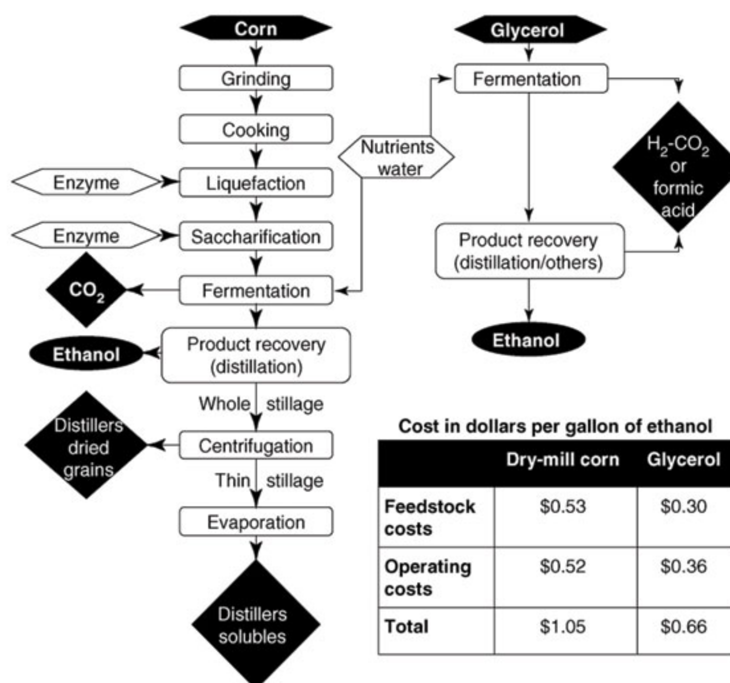
Figure 2. Structure formula ethanol (A) and 3D model (B)
Source: Wikipedia Foundation Inc (2006)

Ethanol, unlike gasoline, is an oxygenated fuel that contains 35% oxygen, which reduces particulate and NOx emissions from combustion. When burned, ethanol derived from fermentation produces no net increase in carbon dioxide in the atmosphere. It is an octane enhancing additive and removes free water which can plug fuel lines in cold climates.

To make ethanol, glycerol is known as competitive raw material comparing the other raw material such as simple sugar, starch, and cellulosic biomass. Glycerol can be used in the production of chemicals and fuels such as butanol and ethanol via microbial fermentation (Yazdani and Gonzalez, 2007).

Additionally, glycerol has yield advantages over sugar due to the highly reduced nature of carbon atoms. A native, nonpathogenic strain of *E. coli* was able to ferment glycerol to ethanol under anaerobic condition without need to do much genetic engineering to have ethanol as the main product. The key factors are not the type of strain, but rather on

the appropriate environment include an acidic pH, avoiding accumulation of fermentation gas hydrogen and appropriate medium composition. With the right environment, ethanol is the primary product from glycerol fermentation with *E. coli*. Comparison of ethanol production cost from glycerol and corn revealed that glycerol exhibited a cheaper total production cost than corn (Fig. 3).



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

Figure 3. Comparing pathway on ethanol production cost from corn and glycerol Source: Yazdani and Gonzalez (2007).

4. Microbial conversion of glycerol to ethanol

Fermentative metabolism of glycerol has been studied in several species of the Enterobacteriaceae family that belong to the genera *Klebsiella, Citrobacter, Enterobacter, Clostridium,* and *E. coli*. The family Enterobacteriaceae is characterized by the following general features; rod shaped, Gram-negative bacteria, which are oxidase negative and posse's variable motility (Prescott *et al*. 2002). The other characteristic is facultative anaerobes that lack cytochrome oxidase activity allows the use of the oxidase test as a quick way to differentiate these organisms from other gram-negative bacilli (Murray *et al*. 2002) and include their ability to ferment glucose, the presence of the enzyme catalase, and their ability to reduce nitrate.

The metabolic pathway in glycerol assimilation to ethanol that shows the metabolic pathways for glycerol fermentation using continuous cultures of *Enterobacter*

agglomerans CNCM 1210 that were performed under regulated pH conditions (pH 7.0) and glycerol or glucose (20 g/l) as carbon source (Fig. 4). Cultures grown on glucose produced mainly acetate, ethanol and formate. In contrast, 1,3-propanediol (PPD) was the main product with glycerol.

The carbon flow distribution at branching metabolic points was investigated. Higher PPD yields with increased dilution rate were correlated with an important increase in the relative ratio of glycerol dehydratase to glycerol dehydrogenase. Determination of intracellular triose-phosphate and fructose 1,6-biphosphate concentrations demonstrated that glyceraldehyde-3-phosphate dehydrogenase is the limiting step in glycerol dissimilation. At the pyruvate branching point, pyruvate dehydrogenase (PDH) activity was systematically detected. The pyruvate flow shifted to PDH is suspected to represent up to 22% of the acetyl-CoA formed. In addition, this enzyme pattern combined with the enhanced in vivo lactate dehydrogenase activity at high growth rates and was correlated with a decrease in the pyruvate formate-lyase activity. A regulation of this latter enzyme by the accumulation of triose-phosphate is suspected.

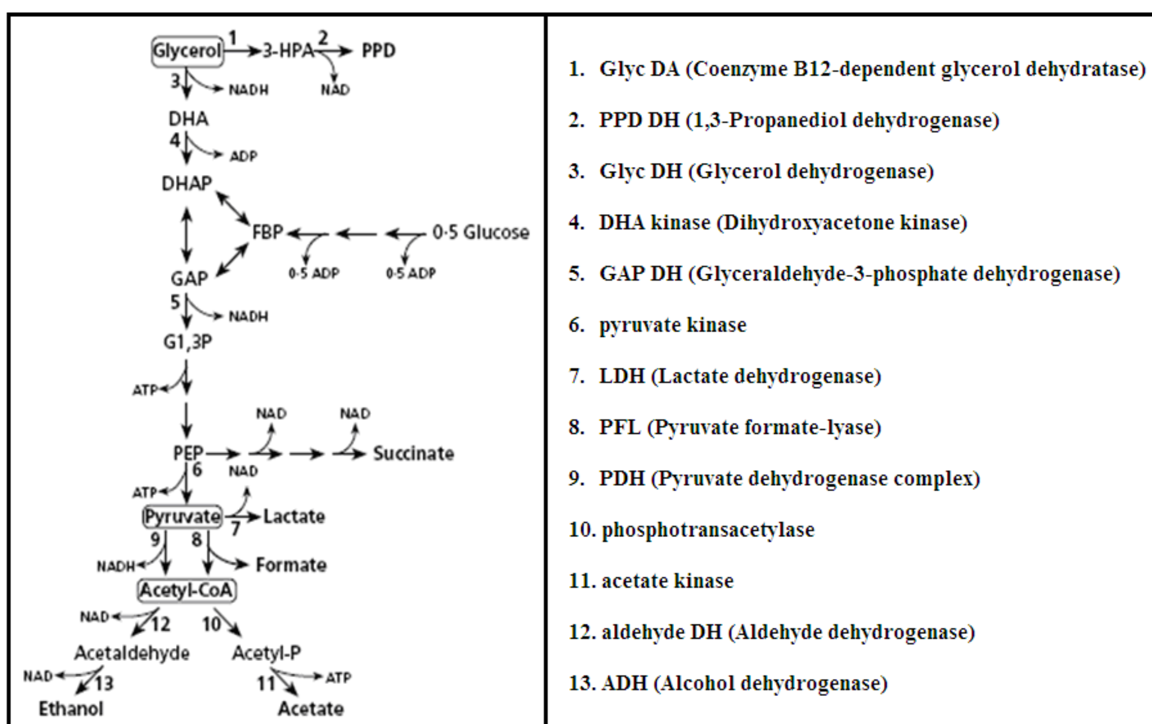


Figure 4. Metabolic pathways involved in glycerol assimilation by *E. agglomerans*. Abbreviations: acetyl-P (acetyl phosphate); DHAP (*dihydroxyacetone phosphate*); PEP (*phosphoenolpyruvate*); G1, 3P, (glyceraldehyde-1,3-biphosphate)

Source: Barbirato *et al* (1997).

4.1 *Aerobacter aerogenes* strain 1033 and 1041

A. aerogenes Strains 1033 and 1041 were isolated from patients at the Boston City Hospital. They found that only strain 1033 is capable to degraded glycerol in the absence of oxygen, though both strains can oxidize this substrate. The result of this study was compared the adaptive patterns and the end products of glycerol oxidation in the two strains suggested that glycerol is 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).

4.2 *Enterobacter aerogenes* HU-101

E. aerogenes HU-101 was studied to converts 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 -80^0C 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. The results of this study is 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).

4.3 *Enterobacter agglomerans* CNCM 1210 and *Clostridium butyricum* CNCM 1211

The strains isolated from a distillery waste-water anaerobic digestor were deposited at the Collection nationale de Cultures de Microorganismes (CNCM, Institut Pasteur, France). *C. butyricurn* CNCM 1211 belongs to a genus often cited in the literature for the production of 1,3-propanediol and *Enterobacter agglomerans* has ability to convert glycerol to 1,3-propanediol (PPD) as the main product.

Barbirato *et al* (1995) found that *E. agglomerans* is described for the first time for its ability to produce 1, 3-propanediol on a medium containing glycerol as the sole carbon source. Compare with the other Enterobacteriaceae. *E. agglomerans* is similar to the genus *Klebsiella* and *Citrobacter* with regard to reducing-equivalent regeneration (1,3-propanediol pathway) and energy production (acetate pathway). But the composition of the end products and 1,3-propanediol yield differ markedly depending on the enterobacterial species. *E. agglomerans* produce s large amounts of formate, which is not a metabolite for the other Enterobacteriaceae. Ethanol and lactate accumulate as they do with the other microorganisms but with different yields. The other hand, *Citrobacter freundii* produces mainly acetate and yields more 1,3-propanediol. By contrast, glycerol conversion by *KIebsiella oxytoca* leads mainly to 1,3- propanediol and ethanol, implying a lower 1,3-propanediol production.

4.4 *Klebsiella planticola* DR3

Kl. planticola DR3 obtained from the rumen contents of red deer (*Cervus elaphus*) from Invermay Agricultural Research Station, Dunedin, New Zealand. *Kl. planticola* DR3 was grown in liquid culture using glycerol as the carbon source (glycerol-based medium with no yeast extract or acetate added). The cultivation of the bacteria was carried out using a modified version of a phosphate-buffer basal medium (pH 7.2–7.4) with $NaHCO_3$ (4.2 g/l) replacing NaCl. Glycerol was added as the major carbon source at a concentration of 100 or 150 mmol/l. During active cell growth, it was established that glycerol dissimilation by *Kl. planticola* DR3 led to the production of formate and ethanol at equimolar levels of 32 mmol/l and 30 mmol/l, respectively (Jarvis *et al.* 1997).

4.5 Genetic engineering *E. coli*

Although glycerol fermentation was described on the progress of microbial species belong to the genera *Klebsiella, Citrobacter, Enterobacter, Clostridium*, the potential for using these organisms at industrial level could be limited. Among the most relevant issues

are pathogenicity, requirement of strict anaerobic conditions, the need for supplementation with rich nutrients, and a lack of appropriate genetic tools and physiological knowledge necessary for their effective manipulation. A recent development in the microbial fermentation of glycerol is discovery of *E. coli* which known as an organism considered the workhorse of modern biotechnology, can anaerobically ferment glycerol. The use of *E. coli* as biocatalyst could help overcome the problems associated with the use of microorganisms that are not amenable to industrial applications, as discussed above. In addition, the feasibility of engineering *E. coli* for the production of chemicals and fuels from sugars has been extensively documented (Wendisch *et al*. 2006).

Therefore, metabolic engineering strategies can be used to develop *E. coli*-based platforms for the anaerobic production of reduced chemicals from glycerol at yields higher than those obtained from common sugars, such as glucose or xylose. The maximum production of the end products can be given by glycerol than sugars. Through strain- and process-based strategies, several folds improvements (yield and/or productivity) in the production of ethanol, formate, hydrogen, succinic acid, and the other products were achieved (Gonzalez *et al*. 2008).

5. Factors Affecting Ethanol Production from Glycerol

5.1 Microbial strain

As must fermentation is often conducted with select bacteria, it is important to determine the influence of the inoculated strain on the raw glycerol of biodiesel plant properties. Although many microorganisms are able to metabolize glycerol in the presence of external electron acceptors (respiratory metabolism), only few bacteria are able to convert glycerol to ethanol, some examples are given in Table 5.

From the literature, the present research relates to an ethanol producing microbial strain, such as *Aerobater aerogenes* (Magasanik *et al*. 1953), *Enterobacter aerogenes* HU-101 (Ito *et al*, 2005), and *Klebsiella Planticola* DR3 (Jarvis *et al*. 1997) which able to grow and produce ethanol as a main product on glycerol fermentation. Moreover, the other bacteria such as *Enterobacter agglomerans* (Barbirato *et al*. 1997), *Clostridium pasteurianum* (Biebl, 2001), *Clostridium acetobutylicum*, *Clostridium butylicum*, *Clostridium beijerinckii, Clostridium kainantoi*, *Clostridium butyricum* (Forsberg, 1987), *Klebsiella Pneumoniae* (Du *et al*. 2006) had known as bacteria which could produce ethanol although as by-product from glycerol fermentation. It is suggest that the different

bacterial strains gave the different ethanol productions on glycerol fermentation, depend on the metabolic pathway that used by bacteria. Furthermore, the different culture conditions gave different amount of ethanol (Table 1).

Table 1. Examples of bacterial and condition for glycerol fermentation

Bacterial Strain	Condition	YE	EC (g/l)	Ref
Aerobacter aerogenes 1033	Initial pH 6.5, temperature 35^{0}C, 250 ml shake flask containing 50 ml basal medium in batch culture containing 10% glycerol for 18 h	0.86 mol/mol glycerol	0.54 g/l	Magasanik (1953)
Enterobacter aerogenes HU101	Initial pH 6.8, temperature 37^{0}C, complex medium with 25 g/l purified glycerol and 160 mM phosphate buffer using batch culture in shake flask 250 ml for 12 h. Initial pH 6.8, temperature 37^{0}C, complex medium and 10 g/l waste glycerol using continuous culture with packed bed reactor and porous ceramics as a support material for 35 h.	0.8 mol ethanol/mol purified glycerol 0.85 mol ethanol/mol waste glycerol	0.51 g/l 0.54 g/l	Ito *et al* (2005)
Enterobacter agglomerans CNCM 1210	Control pH 7.0, temperature 30^{0}C, complex medium with 20 g/l glycerol using chemostat cultures for 24 h (D = 0.05 h^{-1})	0.23 mol/0.05 mol glycerol	2.91 g/l	Barbirato *et al* (1997)
Klebsiella planticola DR3	Initial pH 7.2-7.4, temperature 37^{0}C using liquid medium without yeast extract and acetate added with 10 g/l glycerol for 48 h	30 $mmol^{-l}$	2.76 g/l	Jarvis *et al* (1996)
Clostridium butylicum B593	Initial pH 6.5, temperature 35^{0}C in chemostat culture with 1% glycerol (w/v) for 96 h (D = 0.05 h^{-1})	0.54 mM	0.002 g/l	Forsberg (1986)
Klebsiella pneumoniae M5a1	Control pH 6.8, temperature 37^{0}C, agitation speed 150 rpm, sparging speed 0.25 vvm of N_2, using complex medium with 20 g/l in 5 l stirrer reactor for 8 h	34.0 $\pm$ 0.4 mmol/l	1.57 g/l	Lin *et al* (2005)
Clostridium pasteurianum DSM 525	Initial pH pH 7.5, temperature 30^{0}C using complex medium with 50 g/l glycerol in shake flask 500 ml for 22 h	26.5 mol / 100 mol glycerol	0.17 g/l	Biebl (2001)
Citrobacter freundii DSM 30040	Control pH pH 7.2, temperature 37^{0}C, using complex medium with 27.63 g/l glycerol for 30 min (D = 0.36 h^{-1})	14.8 mM/171mM glycerol	0.06 g/l	Boenigk *et al* (1993)
Klebsiella oxytoca NRCC 3006	Initial pH 7.0, temperature 30^{0}C using complex medium with 20 g/l glycerol in 1 l fermentor for 12 h	100 mM l^{-1}/543 mM l^{-1} glycerol	4.61 g/l	Homann *et al* (1990)

Remark: YE: Yield of ethanol EC: Ethanol concentration D=Dilution rate

5.2 Glycerol concentration

Ethanol production of *Enterobacter aerogenes* HU-101 was carried out with biodiesel waste glycerol. Ethanol yield of 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 of initial glycerol concentration, respectively. Therefore, the yields of ethanol decreased with the increased glycerol concentration (Ito *et al*, 2005). This is also the case for *Enterobacter agglomerans* that 0.15 and 0.02 mol ethanol / mol glycerol were obtained from the batch culture with the initial glycerol concentration of 20 and 70 g/l respectively (Barbito *et al*. 1997).

5.3 Impurities in waste glycerol

Effect of sodium chloride on ethanol production using *Enterobacter aerogenes* HU-101 on pure glycerol was studied. Ethanol concentration (80 mM ethanol) at 1% of sodium chloride was higher than that at without sodium chloride (70 mM ethanol). When the effect of sodium chloride using glycerol from biodiesel waste was studied, the maximum ethanol (55 mM) was obtained from glycerol without sodium chloride added, and 40 mM ethanol was produced in the presence of 1% sodium chloride (Ito *et al*. 2005). Effect of waste glycerol from biodiesel derived from alkali (KOH) and lipase catalyzed were studied using *Klebsiella pneumoniae* (Mu *et al*. 2006). The final ethanol concentration (11.9 g/l) on crude glycerol derived from the alkali-catalyzed reaction was higher 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).

5.4 Effect of nitrogen source

Nitrogen is an essential nutrient for all life forms. Nitrogen can be inorganic such as ammonium salts, or organic such as amino acids, proteins or urea. Different types and concentrations of nitrogen source can be added to the medium to obtain the optimum productivity (Stanbury *et al*. 1995). The culture fermentation using nitrogen as sparging gas in order to check its influence on the fermentation balance of *C. pasteurianum* on glycerol in medium containing only 0.1 g yeast extract/l, was known that the fermentation time was longer than an equally inoculated ammonium-grown culture. Ethanol as a by product was yielded 2.9 mol/100 mol glycerol during 37 hour cultivation which was known as better than using nitrogen gas that only yielded 0.8 mol/100 mol glycerol during

49 hour cultivation. It was suggested that the additional of NH_4 (ammonium) as nitrogen source could increase the ethanol production (Biebl, 2001).

5.5 Effect of vitamins and minerals

Nutritional strategy to produce a higher ethanol was achieved by implementing exponential feeding of vitamins throughout the fermentation process. An addition of vitamins can increase the amount of ethanol. This study was known when *C. pasteurianum* DSM 525 was growth in a chemically defined medium in batch culture. Glycerol was fermented by addition a mineral medium supplemented of biotin and yeast extract. The ethanol production was almost the same amount. But, the additional of yeast extract could reduce cultivation times three times as short as equally inoculated culture with biotin. In this case suggest that *C. pasteurianum* DSM 525 still needed some vitamins and minerals which contain on yeast extract which had effect to reduce cultivation time (Biebl, 2001).

Tryptone is an enzymatic digest of casein used as a nitrogen source in culture media. Casein is the main protein of milk, and a rich source of amino-acid nitrogen. Tryptone is rich in tryptophan, making it valuable for use in detecting indole production. Peptone S or papaic digest of soybean meal is a nitrogen source and contains the naturally occurring high concentrations of vitamins and carbohydrates of soybean. Malt extract is obtained from barley and have a high concentration of carbohydrates, particularly maltose. This product is generally employed in concentrations of 1–10%, and provides carbon, protein, and other nutrients. Yeast extract is the water soluble portion of autolyzed yeast containing vitamin B complex. It is an excellent stimulator of bacterial growth and used in culture media. It is also provides vitamins, nitrogen, amino acids, and carbon in microbiological culture media (http://www.neogen.com).

5.6 Effect of initial pH

The role of pH was investigated in batch cultures of *Clostridium pasteurianum* DSM 525 controlled at values between 4.5 and 7.5 using glycerol at concentration of 50 g/l. At pH 4.5, a greater part of glycerol was converted to butanol as a main product (38.8 mol/100 mol glycerol) and produce ethanol (2.4 mol/100 mol glycerol) as a by-product. The maximum of ethanol yield was obtained from the cultivation at pH 7.5 with 26.5 mol ethanol/100 mol glycerol as a main product and 22.2 mol butanol/100 mol glycerol was produced in the same condition (Biebl, 2001). The suitable pH for ethanol production from glycerol was reported to be in the neutral pH range (6.5-8.0); pH 6.8 for *Enterobacter aerogenes* HU-101 (Ito *et al*. 2005), pH 7 for *Klebsiella pneumoniae* (Mu *et al*. 2006), and

Klebsiella oxycota (Homann *et al*. 1990), and pH 8.0 for mixed culture from distillery wastewater treatment plant (Temudo *et al*. 2007).

5.7 Effect of aeration rate

Klebsiella pneumoniae was studied in batch culture under N_2 flow and four levels of air flow rates (0.1, 0.2, 0.4, and 0.6 vvm). They suggest that more high air flow rate could decrease ethanol production, because that the highest concentration of ethanol (about 5 g/l) was obtained from aerobic batch fermentation. Increase of air flow rate resulted in the decrease of ethanol formation to be lower than 5 g/l (Cheng *et al*. 2004).

6. Comparison on batch and semi continuous fermentation

Ethanol can be produced by four types of industrial operations such as, batch, continuous, fed-batch, and semi-continuous (Keim, 1983). 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. 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 product contains ethanol, cells, and residual sugar (Maiorella *et al.* 1981). 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. In semi-continuous processes, a portion of the culture is withdrawn at intervals and fresh medium is added to the system, so there is volume variation. There is no need to separate inoculums vessel, except at the initial startup. Time is also not wasted in non-productive idle time for cleaning and resterilization. Another advantage is not much control is required.

7. Response surface methodology (RSM)

Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors, and searching optimum conditions of factors for desirable responses. It includes experimental design and regression analysis which better for multifactor experiments and effective to find the optimum point of the factors and assessing the effects of the factors (Montgomery. 1991; Myers. 1976). The method was introduced by Box and Wilson (1951). The main idea of RSM is to use a set of designed experiments to obtain an optimal response. It used a first-degree polynomial model to do the optimization. An easy way to estimate a first-degree polynomial model is to use a factorial experiment or a fractional factorial designs. This is sufficient to determine which explanatory variables have an impact on the response variables of interest.

Some extensions of response surface methodology deal with the multiple response problem. Multiple response variables create difficulty because what is optimal for one response may not be very optimal for other responses. Other extensions are used to reduce variability in a single response while targeting a specific value, or attaining a near maximum or minimum while preventing variability in that response from getting too large. Significant criticisms of RSM include the fact that the optimization is almost always done with a model for which the coefficients are estimated, not known. An optimum value may only look optimal, but be far from the truth because of variability in the coefficients. A contour plot is frequently used to find the responses of two variables to find these coefficients by including a large number of trials in each and combinations of them, and using some sort of interpolation to find potentially better intermediate values between them (Box and Wilson, 1951).

RSM was known as a powerful and efficient mathematical approach widely applied in the optimization of fermentation process. It can give information about the interaction between variables, provide information necessary for design and process optimization, and give multiple responses at the same time. Box-Behnken designs are experimental designs for response surface methodology to achieve the following goals (Box and Behnken, 1960):

- Each factor, or independent variable, is placed at one of three equally spaced values. (At least three levels are needed for the following goal.)

- The design should be sufficient to fit a quadratic model, that is, one containing squared terms and products of two factors.
- The ratio of the number of experimental points to the number of coefficients in the quadratic model should be reasonable (in fact, their designs kept it in the range of 1.5 to 2.6).
- The estimation variance should more or less depend only on the distance from the centre (this is achieved exactly for the designs with 4 and 7 factors), and should not vary too much inside the smallest (hyper) cube containing the experimental points.

CHAPTER 2
MATERIALS AND METHODS

Materials

1. Microorganism

Enterobacter aerogenes TISTR 1468 was obtained from Environmental Biotechnology Laboratory, Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Thailand. The bacterium was maintained at tryptone soy agar (TSA) medium and kept in refrigerator.

2. Glycerol

Purified glycerol was kindly provided by a biodiesel production plant at Krabi Province. This glycerol was analyzed and found to contain about 99% glycerol (unpublished data).

3. Medium

Cultivation medium (per liter) contained 20 g 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 $MgSO_4 \cdot 7H_2O$, 0.021 g $CaCl_2 \cdot 2H_2O$, 0.12 g $Na_2MoO_4 \cdot 2H_2O$, 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 $MnCl_2 \cdot 4H_2O$, 0.1 g H_3BO_4, 0.01 g $AlK(SO_4)_2 \cdot H_2O$, 0.001 g $CuCl_2 \cdot 2H_2O$ and 0.5 g Na_2EDTA (per litre)), pH 6.8 (Ito *et al*, 2005).

Tryptone Soy Agar (TSA) (per liter) contained 15 g casein, 5 g soy peptone, 5 g NaCl, and 15 g agar (Iversen, 2004).

Analytical Methods

1. Determination of cell concentration

Cell concentration was determined by optical density with spectrophotometer at wavelength of 660 nm and dry cell weight. The optical density of cells suspension was directly related to dry cell weight. In this case, original medium was used as blank. Dry cell weight was obtained from standard curve (modified from Ito *et al*, 2004).

2. Determination of ethanol concentration, yield and productivity

Ethanol concentration of glycerol fermentation was determined by gas chromatography (GC) using a capillary column (model number J&W 123-3232 DB-FFAP) with flame ionization detector (Hewlette Packard 6890) to separate and determine the

amount of volatile components of a very small sample (http://pages.pomona.edu). The column was operated at a temperature of 250^0C, 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 yield (Yp/s) was calculated from product formation divided substrate and productivity (P_{max}/t) was found by divided the maximum product formation by time (Pirt, 1975).

3. Determination of glycerol concentration

Glycerol was spectrophotometrically determined by chromotropic acid method (modified from Handel, 1961). 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, after that 0.1 ml of 5% sodium metabisulfite was added and mixed and standing for 10 min. Chromotropic acid solution (3.0 ml) was added, shaked, and putted in boiling water bath (100°C, 30 min), allowed to cool to room temperature, then 0.3 ml of thiourea was added. After that, samples will be determined for optical density of 570 nm within 2 h using spectrophotometer. In case of blank reagent, distilled water was used instead the samples.

4. Determination of other end-products

The other end products such as acetate, butanol and butyric were determined by GC using a capillary column (model number J&W 123-3232 DB-FFAP) with flame ionization detector (Hewlette Packard 6890) to separate and determine the amount of volatile components of a very small sample (http://pages.pomona.edu). The column was operated at a temperature of 250^0C, with He (helium) as carrier gas. Each 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).

For detection of 1,3-propanediol as the end product was determined by HPLC (Agilent 1100). The apparatus included a quaternary pump, a manual injector, a compartment, an Aminex HPX-87H ion exclusion coloumn (300mm x 7.8mm) (Bio-rad Laboratories, Hercules, CA, USA) and Chemstation Software. Operating conditions were: sample volume 20 µl; mobile phase a 65:35 mixture of filtrated deionized water and

HPLC-grade acetonitrile (Tedia-USA) containing 0.00005 M H_2SO_4, flow rate of 0.5 ml/min, column temperature at 25^0C (Hongwen *et al*, 2005).

Experimental Methods

1. Factors affecting ethanol production from *Enterobacter aerogenes* TISTR 1468

In order to make starter, *Enterobacter aerogenes* TISTR 1468 was cultivated in 250 ml Erlenmeyer flasks containing 100 ml of cultivation medium for 12 hour at 37^0C under microaerobic condition (120 rpm). After finished, the culture was adjusted with fresh medium to obtain OD_{600} of 0.5 before using as a starter (ref).

In order to analyze all of the parameter in conventional method, cultivation was done at 37^0C under microaerobic condition in 250 ml of Erlenmeyer flasks containing 100 ml of cultivation medium which it contained each parameter under studied. Then, 10% of the starter culture was inoculated into the sample and cultivated at the same condition for 48 hour. Samples were taken every 4 hour to determine for cell growth using 2 ml of sample, pH using 3 ml of sample, glycerol and ethanol concentration. Then, calculated for glycerol consumption, ethanol yield (Yp/s) and productivity (P_{max}/t) (Pirt, 1975).

The influence of the following nutrients was investigated whereas the original or previous medium was used as a control. The experiments were conducted as described above and samples were taken every 4 hour till the time that reaches the highest ethanol production. The suitable source was selected for the subsequent studies.

1.1 Effect of cultivation condition

Effect of cultivation condition was determined under static condition and shaking condition at 120 rpm and 150 rpm.

1.2 Effect of glycerol concentration

In order to find the effect of glycerol concentration, the purified glycerol concentrations were varied at 0, 2, 5, 10, 20, and 40 g/l.

1.3 Effect of organic nitrogen source and concentration

The medium composition from results of section 1.2 was used to determine the effect of organic nitrogen sources with 5 g/l each of yeast extract and tryptone as a control and compared with using only yeast extract (without tryptone in the medium) at the concentrations of 0, 0.1, 0.5, 1, 5 and 10 g/l. Commercial yeast extract and tryptone from Difco were used in this research. Yeast extract was choosing in this research because it knew to stimulate microbial growth. One of the benefits of yeast extract should probably be related in some

way to the presence of compounds that have the ability to complex iron and thus make it more readily available to microorganisms (King *et al*, 1954). Sommer *et al* (1996) explained that the properties of yeast extract were 73 - 75 % protein contents, sodium and polysaccharide contents do not exceed 0.5 % or 5 % respectively. Oligosaccharide contents are less than 1 %. Fat contents less than 0.5 % do not have significant effects on the fermentation of microorganisms. Yeast autolysates serve as essential nutrients in fermentations. However, details of the exact mechanisms have still to be identified.

1.4 Effect of inorganic nitrogen sources

The medium composition from results of section 1.3 was used for study the effect of inorganic nitrogen sources; $(NH_4)_2HPO_4$, $(NH_4)_2SO_4$, and $NH_4C_2H_3O_2$ at 0.8 mmol each.

1.5 Effect of inorganic nitrogen concentration

The medium composition from results of section 1.4 was used for study the effect of selected inorganic nitrogen concentration at 0.4, 0.8 and 1.2 mmol of nitrogen.

1.6 Effect of mineral sources

The medium composition from results of section 1.5 was used for study the effect of mineral salts ($Na_2MoO_4 \cdot 2H_2O$, Na_2SeO_3, $NiCl_2$) and trace element solution by comparison the cases of with and without these sources.

1.7 Effect of initial pH

The medium composition from results of section 1.6 was used for study the effect of initial pH at pH 5.5, 6.0, 6.5, 7.0, and 7.5.

2. Optimization for ethanol production from *Enterobacter aerogenes* TISTR 1468 by Response Surface Methodology (RSM)

The optimization was conducted using statistical analysis to determine the optimum concentrations of the selected nutrients such as glycerol concentration, organic nitrogen concentration and inorganic nitrogen concentration. Box-Behnken Design-Expert Software (Stat-ease, USA) was used to determine the optimum concentration whereas each sample was varied in three levels (-1, 0, +1). Finally, response surface analysis was used to know the optimum condition for ethanol production.

3. Ethanol production using batch and semi-continuous fermentation

3.1 Batch fermentation

The medium obtained from the response surface methodology (RSM) analysis with the optimum concentration of glycerol concentration, organic nitrogen source and inorganic nitrogen source. A 10% of starter was added into a 3 L fermentor containing 2 L medium and cultivation was carried out at 37^0C for 8 hour (Fig. 5). Concentration of ethanol, glycerol and the other end products such as acetate, butanol, butyric and 1,3-propanediol were determined by GC and HPLC.

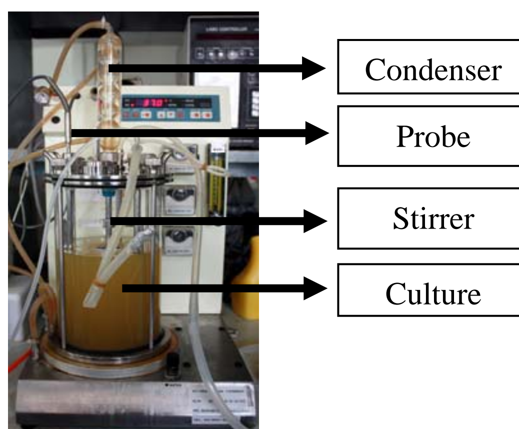


Figure 5. Set-up of batch fermentation

3.2 Semi-continuous fermentation

Cultivation is carried out in batch culture for the period that reaches the highest ethanol production during 8 hour. Then, 1/3 of the culture broth was removed and the same quantity of fresh growth medium was added by peristaltic pump under aseptic condition. This process was conducted 3 times during cultivation (Fig. 6). Samples were taken every 8 hour for determination of ethanol, glycerol, acetate, butanol, butyric and 1,3-propanediol concentrations. The results were compared with those of batch culture.

Figure 6. Set-up of semi-continuous fermentation

Statistical Analysis

Optimization on ethanol production by one factor at the time, each treatment was conducted in triplicate and statistical analysis of the data was determined using SPSS (Ver. 16.0) software. Optimization on ethanol production by response surface methodology (RSM) was conducted by Box-Behnken Design-Expert Software (Stat-ease, USA).

CHAPTER 3
RESULTS AND DISCUSSION

1. Factor affecting ethanol production by *Enterobacter aerogenes* TISTR 1468

The microbial conversion of glycerol can be naturally produced under anaerobic or microaerobic conditions by bacteria belonging to the genera *Klebsiella, Clostridia, Citrobacter* and *Enterobacter* (Chen *et al*, 2003). Especially for *E. aerogenes*, glycerol can be used as carbon source and yield ethanol as main product (Ito *et al*, 2005; Jarvis *et al*, 1997). During fermentation, glycerol was oxidized to glyceraldehyde-3-phosphate via dihydroxyacetone and via pyruvate converting to ethanol as well as the other products such as acetate, formate, CO_2, butanol, etc (Barbirato *et al*, 1997).

1.1 Effect of glycerol concentration and shaking speed

In order to know the effect of cultivation time for ethanol production by *E. aerogenes* TISTR 1468 at different glycerol concentrations (0-40 g/l) and shaking speeds (120 and 150 rpm), several batch fermentations were conducted using 250 ml shake flasks containing 100 ml of the cultivation medium for 48 hours. After starting fermentation, at lower initial glycerol concentrations of 0, 2, and 5 g/l (Fig. 7), the pH decreased from 6.8 to 6.63 within the first 4 hour cultivation, after that it increased steadily until the pH reached 7-7.3. The glycerol as carbon source was consumed completely during 4 hour cultivation (Fig. 8A). During fermentation, it could produce acid products such as succinate, lactate, acetate, and butyric acid at the same time (Barbirato *et al*. 1995). The result was the same with using shaking speed of 120 rpm and 150 rpm whereas the pH decreased rapidly after 4 hour cultivation, to pH 6.18 (Fig. 7B) and 5.95 (Fig. 7C).

Barbirato *et al* (1996) explained that the amount of glycerol consumed was dependent on the culture pH. Under batch fermentations by *Enterobacter agglomerans* at 720 mM glycerol, the pH values ranged from 6 to 8, only 10% of the initial glycerol content was metabolized at pH 6. Glycerol consumption increased with the increased pH and was complete at pH 8. The result was similar to this experiment, where the cultivation of *E. aerogenes* TISTR 1468 in batch culture using initial pH 6.8 could completely consumed glycerol at lower concentrations (0-5 g/l) during 4 hour cultivation, but in the highest concentration, it needed more time to consume completely.

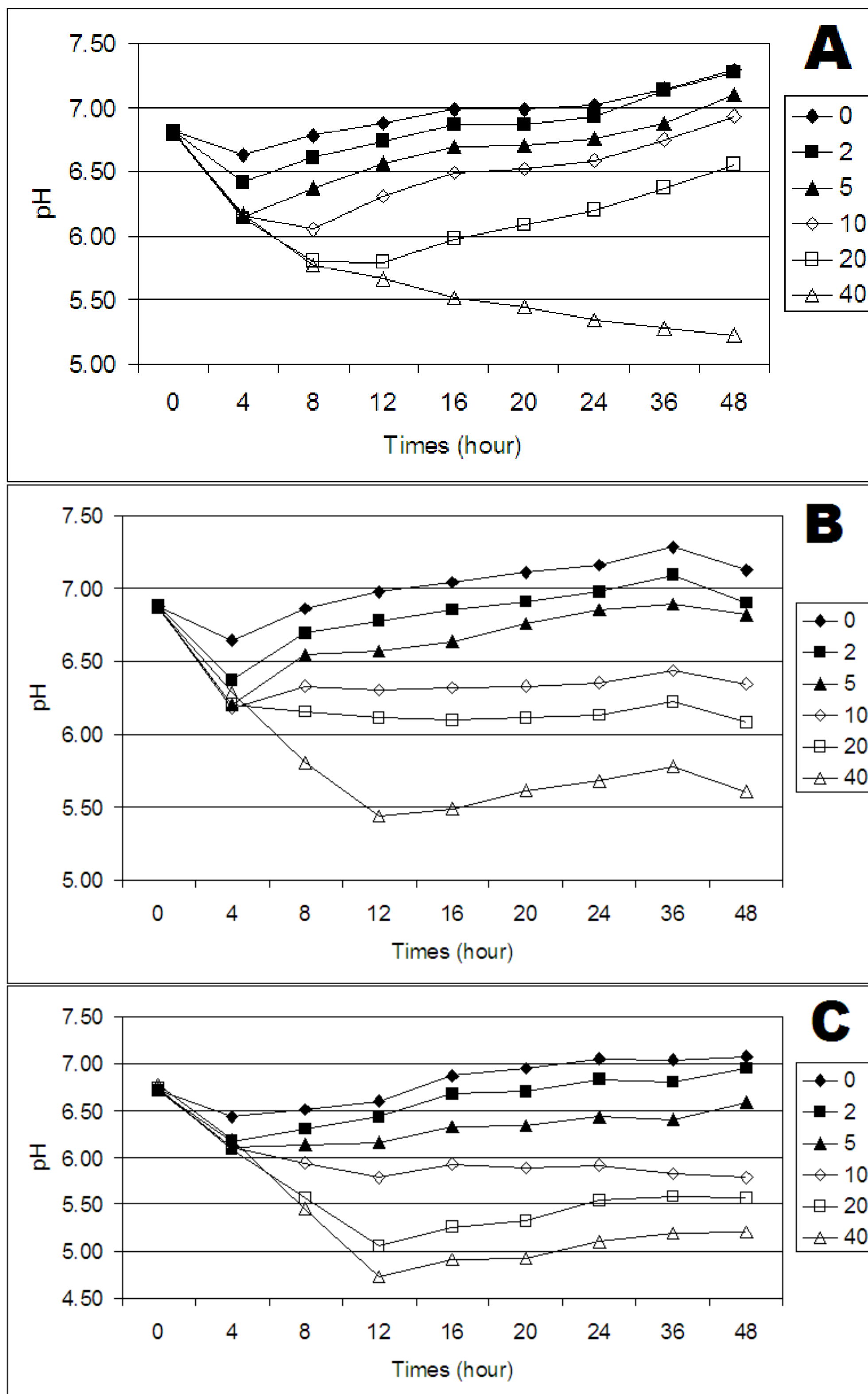


Figure 7. Changes of pH on cultivation of *Enterobacter aerogenes* TISTR 1468 in the medium (initial pH 6.8) with different initial glycerol concentrations (0-40 g/l) and shaking speds at (A) 0 rpm (static condition), (B) 120 rpm and (C) 150 rpm at 37^0C

Cultivation in the medium with 10 g/l glycerol without shaking, the pH decreased from 6.8 to 6.05 after 8 hour cultivation and increased again to 6.93 at 48 hour cultivation. It was found that, the initial glycerol was consumed completely during 8 hour cultivation (Fig. 8A). Cultivation of *E. aerogenes* TISTR 1468 in the medium with an glycerol concentration of 20 g/l under static condition (0 rpm) resulted in the decrease of pH from 6.80 to 5.80 at 12 hour cultivation, after that the pH increased again to 6.55 at the final pH after 48 hour cultivation (Fig. 7A). At 120 rpm of shaking speed, the pH of the medium decreased rapidly from pH 6.8 to 6.21 after 4 hour cultivation and changed slowly to 6.11 and it was stable until 48 hour cultivation at the pH reached 6.08-6.11 (Fig. 7B). The results were different from using 150 rpm shaking speed where the pH decreased rapidly to 5.05 within 12 hour of cultivation, after that it increased again to obtain the final pH at 5.57 after 48 hour cultivation (Fig. 7C).

When *E. aerogenes* TISTR 1468 was cultivated in the medium with an initial glycerol concentration of 40 g/l under static condition, the pH decreased from 6.8 to 5.23 (Fig. 7A). At 120 rpm shaking speed, it decreased rapidly within 12 hour cultivation to 5.44 and increased again until the end of cultivation time to 5.78 (Fig. 7B). At 150 rpm of shaking speed, it decreased rapidly within 12 hour cultivation to 4.73 and increased to 5.21 after 48 hour cultivation (Fig. 7C).

In the lower glycerol concentrations up to 5 g/l of initial glycerol concentrations tested, the glycerol almost completely consumed within 4 hour cultivation. At higher initial glycerol concentrations (10-40 g/l), at 40 g/l of glycerol could completely consumed except at 40 g/l under static condition (Fig.8).

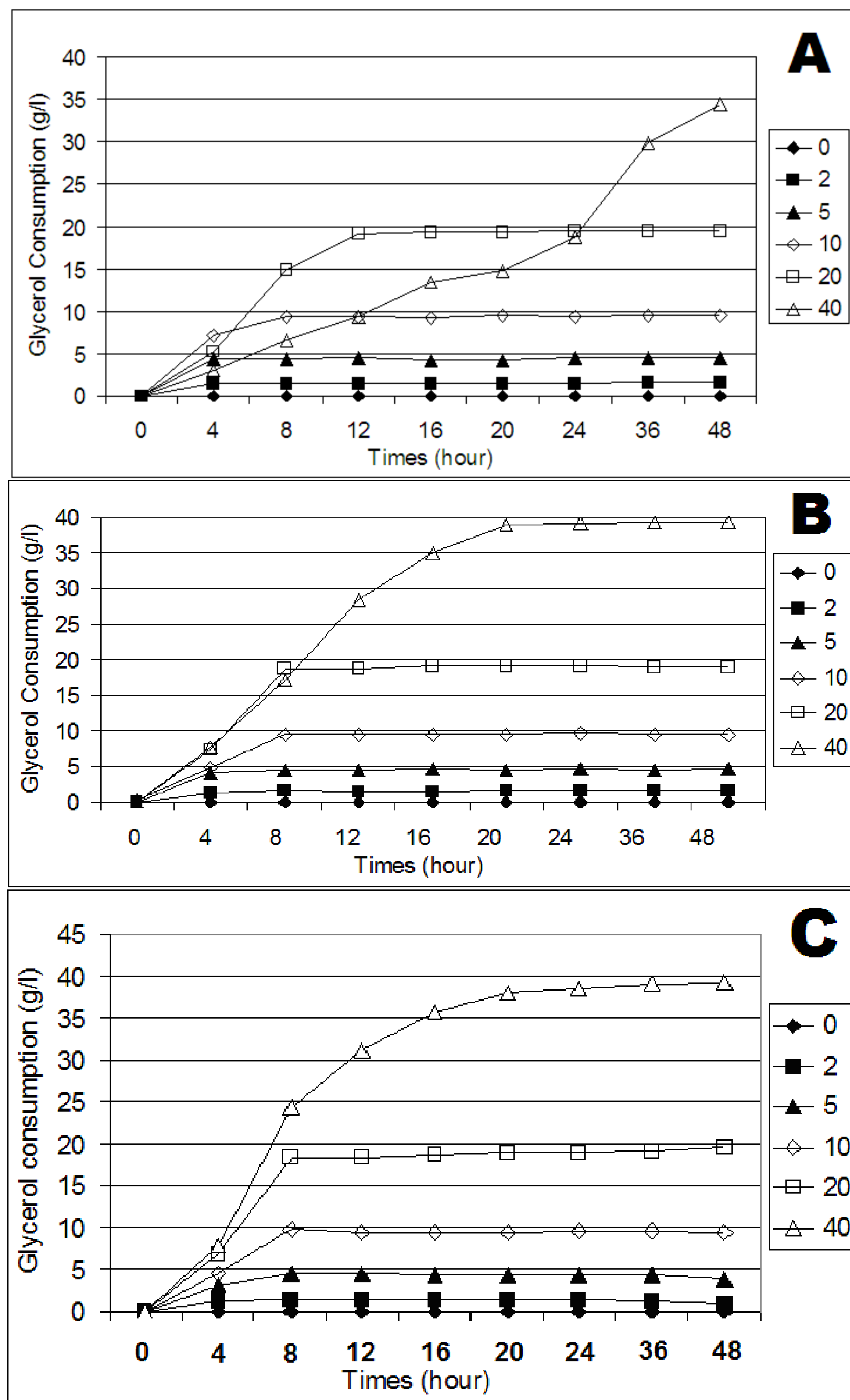


Figure 8. Glycerol consumptions on cultivation of *Enterobacter aerogenes* TISTR 1468 in the medium (initial pH 6.8) with different initial glycerol concentrations and agitation at (A) 0 rpm (static condition), (B) 120 rpm and (C) 150 rpm at 37^{0}C

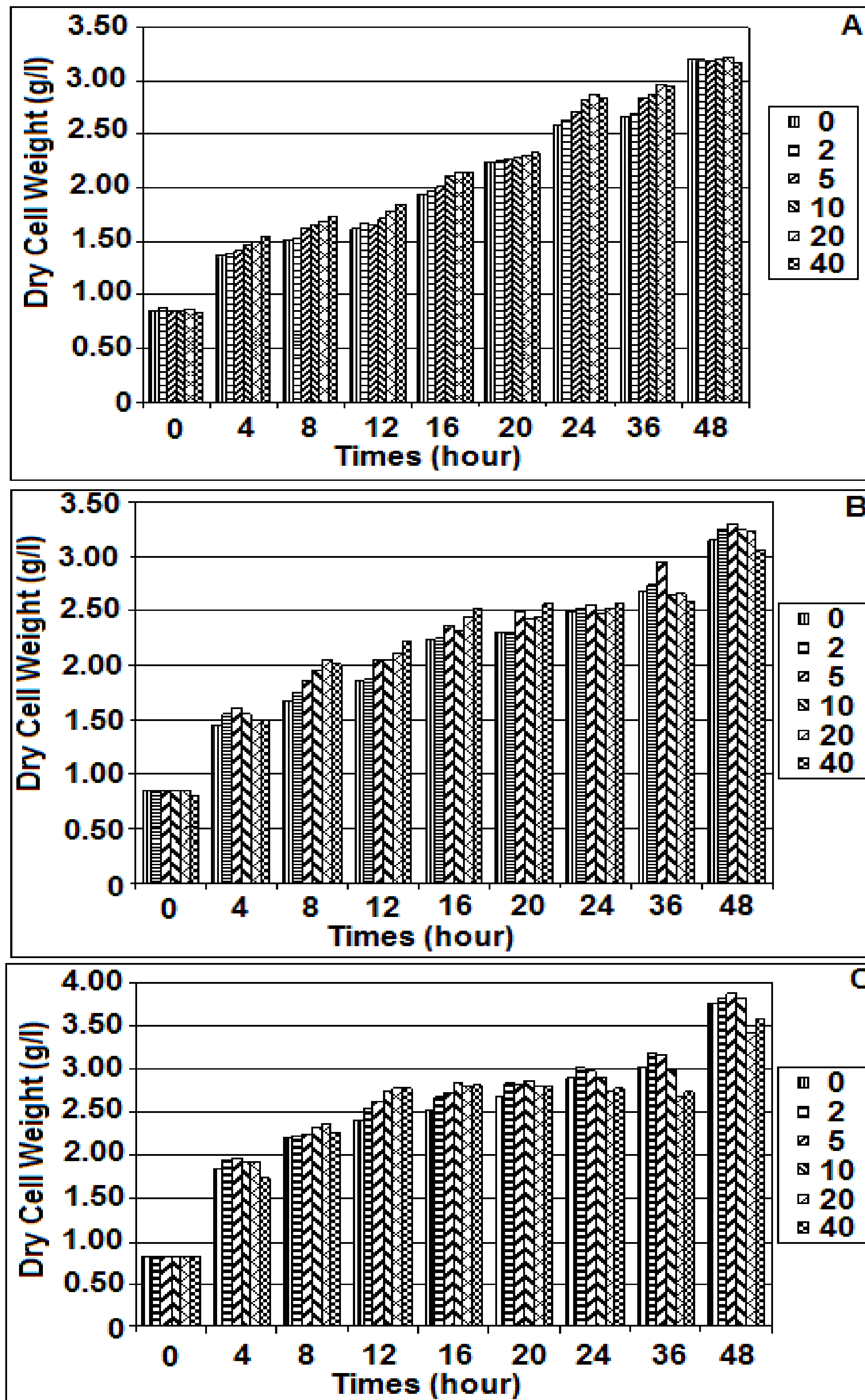


Figure 9. Microbial growth on cultivation of *Enterobacter aerogenes* TISTR 1468 in the medium (initial pH 6.8) with different initial glycerol concentrations and agitation at (A) 0 rpm (static condition), (B) 120 rpm and (C) 150 rpm at 37^0C

Effect of the initial glycerol concentrations and shaking speeds on growth is given in Fig. 9. In general, the growth of *E. aerogenes* TISTR 1468 increased with the increase of all initial glycerol concentrations tested (up to 40 g/l). The dry cell weight (DCW) was increased from 0.8 g/l to 3.0-3.8 g/l after 48 hour cultivation. The highest microbial biomass of 3.8 g/l was obtained at 150 rpm, 3.0 and 3.2 g/l at 120 rpm and 0 rpm (static condition), respectively. Glycerol was used as a substrate for bacteria belongs to the genera *Klebsiella, Clostridia, Citrobacter* and *Enterobacter* under anaerobic or microaerobic conditions (Chen *et al,* 2003). Nevertheless, the results from Fig. 9 showed that at 0 g/l of initial glycerol, *E. aerogenes* TISTR 1468 still could grow. It was due to the presence of yeast extract in the cultivation medium. Yeast extract has the essential components such as vitamins, nitrogen, amino acids and carbon source which important in microbiological and cell culture media (Sommer *et al,* 1996) important for *E. aerogenes* TISTR 1468 growth.

Cheng *et al* (2005) observed that by products of fermentation had an affect on microbial growth. Acetate as a by product of glycerol fermentation by *K. pneumoniae* M5al had been demonstrated to inhibit the growth of the bacteria. *K. oxytoca* (Fond *et al*, 1985), *C. butyricum* and *K. pneumoniae* (Zeng *et al*, 1994) were reported to be affected by an inhibitory effect of acetate in its undissociated form. However, it should be pointed out that acetate might not the only factor causing cell growth inhibition because the glycerol broth contained not only acetate but also some inorganic salts (such as KCL, NaCl, etc) from glycerol or glucose fermentation medium (Neves *et al*, 1997). The similar results had been reported by other researchers under anaerobic fermentation of glycerol.

The ethanol concentration and ethanol yield during cultivation of *E. aerogenes* TISTR 1468 at different initial glycerol concentrations and shaking speeds is shown in Table 2 and Table 3. The highest ethanol production was 12.30 g/l after 24 hour cultivation at 40 g/l of initial glycerol and 150 rpm of shaking speed. The highest ethanol yield of 0.98 g ethanol/g glycerol was achieved at 4 hour cultivation at 20 g/l of initial glycerol concentration and 150 rpm of shaking speed. The highest productivity of 1.36 g/l/h was obtained after 8 hour cultivation at 20 g/l of initial glycerol concentration and 150 rpm shaking speed (Table 4).

To know the best time for next experiment, there is a need to select the specific variable using statistical analysis by SPSS program. Overall, data of ethanol concentrations (Table 2), ethanol yields (Table 3) and productivity (Table 4) were used in this program. The results concluded that the best cultivation time was 8 hour as the highest productivity

and its ethanol concentration and ethanol yield were not significantly difference from their highest values.

Table 2. Ethanol concentration during cultivation of *Enterobacter aerogenes* TISTR 1468 for 48 h at 37 ^{0}C at different initial glycerol concentrations and shaking speeds

Initial glycerol concentration (g/l)	Time (hour)								
	0	4	8	12	16	20	24	36	48
A. Ethanol concentration (g/l) using static condition									
2	0	0	0.34	0.37	0.42	0.40	0.64	0.49	0.35
5	0	1.01	0.46	0.47	0.48	0.29	1.08	0.74	0.73
10	0	0.30	0.51	0.50	0.52	0.55	1.27	0.93	0.94
20	0	0.36	0.50	0.69	0.69	0.69	1.95	1.69	1.65
40	0	0.35	0.43	0.52	0.61	0.64	2.36	2.32	2.24
B. Ethanol concentration (g/l) using 120 rpm of shaking speed									
2	0	1.20	1.10	0.95	0.79	0.60	0.41	0	0
5	0	1.48	1.49	1.39	1.08	0.85	0.56	0.90	0
10	0	1.53	2.08	1.96	1.81	1.68	1.20	0.68	0.30
20	0	1.32	3.00	2.72	2.50	2.27	1.84	1.31	1.00
40	0	1.09	3.05	6.32	5.59	5.99	4.89	4.50	3.52
C. Ethanol concentration (g/l) using 150 rpm of shaking speed									
2	0	1.22	1.28	1.85	0.88	1.46	0.83	0.17	0.15
5	0	1.74	3.08	3.12	3.06	3.44	3.57	1.02	0.81
10	0	0.69	2.96	5.46	5.15	5.04	4.37	3.87	2.76
20	0	1.90	10.84	11.17	10.69	10.36	10.26	10.56	9.96
40	0	1.79	10.66	10.98	11.60	12.07	12.30	12.37	12.14

Table 3. Ethanol yield during cultivation of *Enterobacter aerogenes* TISTR 1468 for 48 h at 37 ^{0}C at different initial glycerol concentrations and shaking speeds

Initial glycerol concentration (g/l)	Time (hour)								
	0	4	8	12	16	20	24	36	48
A. Ethanol yield (g ethanol / g gycerol) using static condition									
2	0	0	0.22	0.25	0.29	0.27	0.43	0.31	0.23
5	0	0.23	0.10	0.10	0.11	0.07	0.24	0.16	0.16
10	0	0.04	0.06	0.05	0.06	0.06	0.13	0.10	0.10
20	0	0.07	0.03	0.04	0.04	0.04	0.10	0.09	0.08
40	0	0.11	0.07	0.06	0.05	0.04	0.13	0.08	0.07
B. Ethanol yield (g ethanol / g gycerol) using 120 rpm of shaking speed									
2	0	0.92	0.67	0.65	0.55	0.39	0.25	0	0
5	0	0.36	0.33	0.31	0.24	0.19	0.12	0.20	0
10	0	0.32	0.22	0.21	0.19	0.18	0.12	0.07	0.03
20	0	0.40	0.16	0.15	0.13	0.12	0.10	0.07	0.05
40	0	0.29	0.18	0.17	0.14	0.15	0.13	0.12	0.09
C. Ethanol yield (g ethanol / g gycerol) using 150 rpm of shaking speed									
2	0	0.96	0.88	1.29	0.67	1.06	0.62	0.14	0.18
5	0	0.56	0.67	0.70	0.68	0.79	0.81	0.23	0.21
10	0	0.17	0.30	0.57	0.54	0.53	0.46	0.40	0.29
20	0	0.38	0.59	0.61	0.57	0.55	0.54	0.55	0.51
40	0	0.33	0.44	0.35	0.33	0.32	0.32	0.32	0.31

Table 4. Productivity during cultivation of *Enterobacter aerogenes* TISTR 1468 for 48 h at 37 ^{0}C at different initial glycerol concentrations and shaking speeds

A. Productivity (g/l/h) using static condition									
Initial glycerol concentration (g/l)	Time (hour)								
	0	4	8	12	16	20	24	36	48
2	0	0	0.04	0.03	0.03	0.02	0.03	0.01	0.01
5	0	0.25	0.06	0.04	0.03	0.01	0.05	0.02	0.02
10	0	0.08	0.06	0.04	0.03	0.03	0.05	0.03	0.02
20	0	0.09	0.06	0.06	0.04	0.03	0.08	0.05	0.03
40	0	0.09	0.05	0.04	0.04	0.03	0.10	0.06	0.05
B. Productivity (g/l/h) using 120 rpm of shaking speed									
2	0	0.30	0.14	0.08	0.05	0.03	0.02	0	0
5	0	0.28	0.19	0.12	0.07	0.04	0.02	0.03	0
10	0	0.38	0.26	0.16	0.11	0.08	0.05	0.02	0.01
20	0	0.33	0.38	0.23	0.16	0.11	0.08	0.04	0.02
40	0	0.27	0.38	0.53	0.35	0.30	0.20	0.13	0.07
C. Productivity (g/l/h) using 150 rpm of shaking speed									
2	0	0.31	0.16	0.15	0.05	0.07	0.03	0.00	0.00
5	0	0.43	0.38	0.26	0.19	0.17	0.15	0.03	0.02
10	0	0.17	0.37	0.45	0.32	0.25	0.18	0.11	0.06
20	0	0.47	1.36	0.93	0.67	0.52	0.43	0.29	0.21
40	0	0.45	1.33	0.92	0.73	0.60	0.51	0.34	0.25

Results on the effect of shaking speed on ethanol production from *E. aerogenes* TISTR 1468 after 8 hour cultivation at 37^{0}C is shown at Fig. 10. Zheng *et al* (2008) confirmed that the rate of stirring on glycerol fermentation using *Klebsiella pneuminiae* IC 15 showed a significant effect on fermentation process. They suggested the rate of stirring conferred the more influence to dissolved oxygen. Kirkpatrick *et al* (2001) concluded that the availability of oxygen and the nature and quantity of the carbon source dictate the status of the TCA cycle. If the oxygen utilization is sufficient, the reduced cofactors generated by glycerol consumption would be reoxidized in the electron transport chain and the redox balance could be maintained. The high stirring rate enhances oxygen transfer rate to a culture and facilitates diffusion. The enterobacteria could adapt their metabolism to oxygen availability via a group of global repression regulators, which include the one-component Fnr protein, and the two-component Arc system. The main targets for repression are genes related to the electron transfer chain and the TCA cycle (Gunsalus, 1992). Moreover, it was found that the most significant role of ArcA functions under

microaerobic conditions, while that of FNR functions under more strictly anaerobic conditions. Oxygen could relieve cell of ArcA inhibitory influence (Shalel-Levanon *et al*, 2005).

Using SPSS software, it was concluded that the cultivation condition using 150 rpm shaking speed gave the highest values of ethanol concentration, ethanol yield and productivity. Therefore, the shaking speed of 150 rpm was selected for next experiment.

The effect of initial glycerol concentrations on ethanol production from *E. aerogenes* TISTR 1468 after 8 hour cultivation at 37^0C is shown in Fig. 11. The highest ethanol concentration and ethanol yield were achieved at 40 g/l, but the highest productivity was at 20 g/l initial glycerol concentration.

SPSS program showed that the best initial glycerol concentration for *E. aerogenes* TISTR 1468 was at 40 g/l because it had the highest ethanol concentration although it was not significantly difference from that at 20 g/l glycerol. However, its ethanol yield was better than that of 20 g/l glycerol, and the value was not significantly difference with that of 20 g/l of glycerol. Although initial glycerol of 20 g/l gave the highest productivity, the value was not significantly difference with that of 40 g/l of glycerol. In order to find the selected parameter for the subsequent experiment, the result from SPSS program was compared with the results in Table 2, 3 and 4. Although glycerol concentration was almost completely consumed at 20 g/l initial glycerol concentration, the best parameter from analytical statistic using SPSS program was 40 g/l glycerol which could produce the highest ethanol concentration, ethanol yield and not significant different in productivity at 10.66 g/l, 0.44 g ethanol/g glycerol and 1.33 g/l/h, respectively. It was chosen for the further study although glycerol concentration still remained at 15.65 g/l.

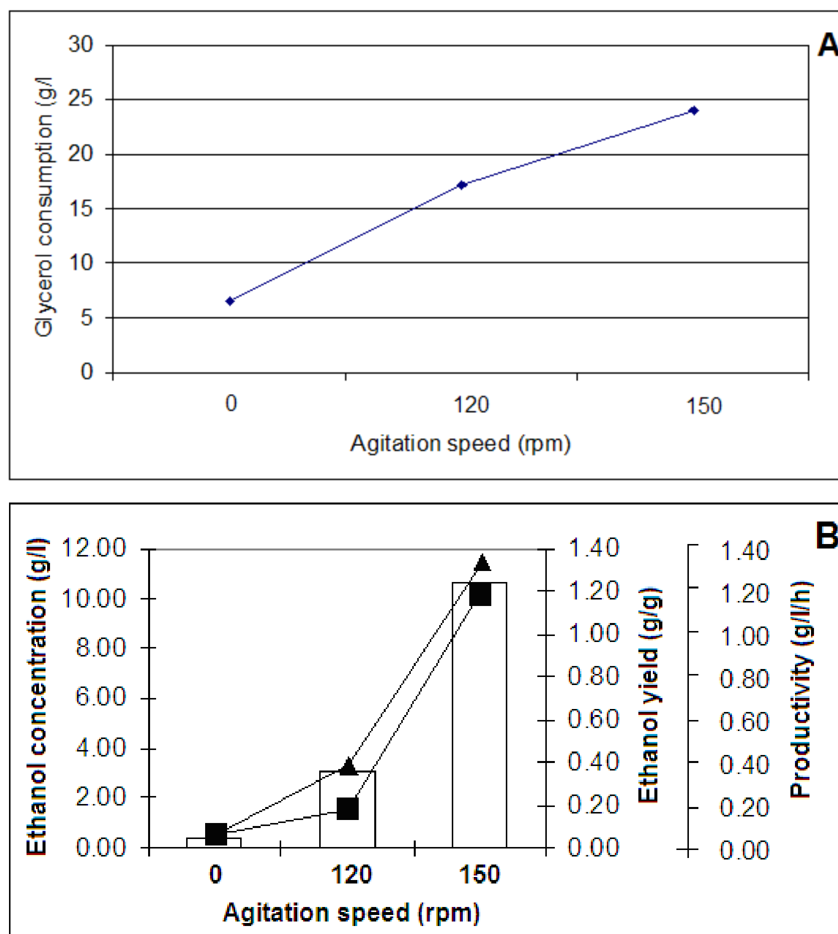


Figure 10. (A) Glycerol consumption and (B) Ethanol concentration (column), ethanol yield (■) and productivity (▲) from cultivation of *Enterobacter aerogenes* TISTR 1468 at 37 ^{0}C for 8 h at different shaking speed

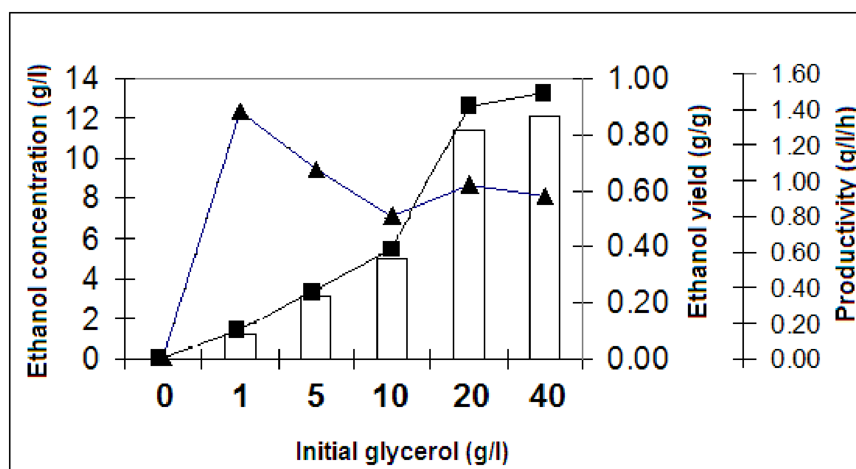


Figure 11. Effect of initial glycerol concentration on ethanol concentration (column), ethanol yield (■) and productivity (▲) from cultivation of *Enterobacter aerogenes* TISTR 1468 at 37 ^{0}C for 8 h

The experiment using initial glycerol higher than 40 g/l (60, 80 and 100 g/l) had been carried out in the preliminary studies. *E. aerogenes* TISTR 1468 could not grow better than the lower initial glycerol concentration. This result agreed with that of Ciptanto *et al*. (2008) where ethanol concentration increased with the increasing of initial glycerol concentration up to 40 g/l. At higher glycerol concentrations 60, 80 and 100 g/l, the ethanol concentrations were 8.04, 7.31 and 7.03 g/l, respectively. Cultivation of *Clostridium pasteurianum* under fed-batch fermentation at different initial glycerol concentrations with controlled pH at 6.0, the substrate was used only up to 60 g/l initial glycerol concentration. At higher substrate concentrations, conversion was slower, and some glycerol remained in the culture broth. In addition a certain amount of glycerol always has to be reduced to product formation like 1,3-propanediol, butanol and ethanol because glycerol is more reduced than the cell mass formed along with the fermentation products, additional reducing equivalents are released and need an acceptor (Biebl, 2001).

The *E. aerogenes* HU-101 completely consumed 5 g/l or 10 g/l pure glycerol within 6 h and 25 g/l after 12 h. Although the yields of ethanol and hydrogen were 1 mol/mol glycerol using 5 g/l glycerol, they decreased with the increase in glycerol concentration (as also observed in biodiesel wastes) (Ito *et al*, 2005). 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). At 100 g/l of commercial glycerol, growth of *C. butyricum* DSM 5431 was inhibited by 59% (Petitdemange *et al,* 1995).

1.2 Effect of organic nitrogen source and concentration

Nitrogen is an essential component of protein and other constituents of cellular protoplasm, part of nucleotide bases. Nitrogen is an essential nutrient for all life forms. Evolutionary selective pressure has thus likely favored the early emergence of cells able to transport and catabolize a wide variety of nitrogenous compounds as well as to synthesize endogenously all essential nitrogen-containing molecules (Magasanik *et al*, 1987). If it is not present, it can be a limiting factor for that bacterium's viability. Nitrogen can be in the organic form such as amino acids, proteins, urea, or in the inorganic form such as NH_4 and NO_3 salt. Different types and concentrations of nitrogen source can be added to the medium to obtain the optimum productivity (Stanbury *et al*, 1995).

Effect of organic nitrogen source and concentrations in the medium tested using yeast extract at 0, 0.1, 0.5, 1, 5 and 10 g/l was studied and compared to the control using 5 g/l

yeast extract and 5 g/l tryptone (Fig. 12). The best conditions obtained from the above experiments were used (40 g/l glycerol and cultivation using 150 rpm shaking speed for 8 hour). *E. aerogenes* TISTR 1468 in the medium with purified glycerol at 37^0C grew very fast in the first 8 hour (log phase) and slowly at 12 hour (stationary phase) (Fig 12.A). During cultivation, the initial pH of 6.8 decreased to 4.9 - 5.9 after 8 hour cultivation and to more acidic pH of 4.9 - 5.9 after 12 hour cultivation, where cell growth reached stationary phase (Fig. 12B).

It means that in the fermentation process, the bacteria produced some acids which affected on pH of the medium. This case was similar *on E. aerogenes* HU-101 that convert glycerol to ethanol as a main product and also produced by-products such as hydrogen, formate, lactate, acetate and 1,3-propanediol (Ito *et al*, 2005), where the products could affect the pH in the medium.

E. aerogenes TISTR 1468 could not consume 40 g/l glycerol completely (Fig. 12C). Control (5 g/l of yeast extract and 5 g/l of tryptone) and 10 g/l of yeast extract exhibited the highest glycerol consumption of 28.48 g/l and 27.99 g/l, respectively. At 5 g/l yeast extract, glycerol of 23.77 g/l was consumed and for the other initial organic nitrogen sources only 15 - 17 g/l glycerol was consumed. This indicated that *E. aerogenes* still need more nitrogen for growth.

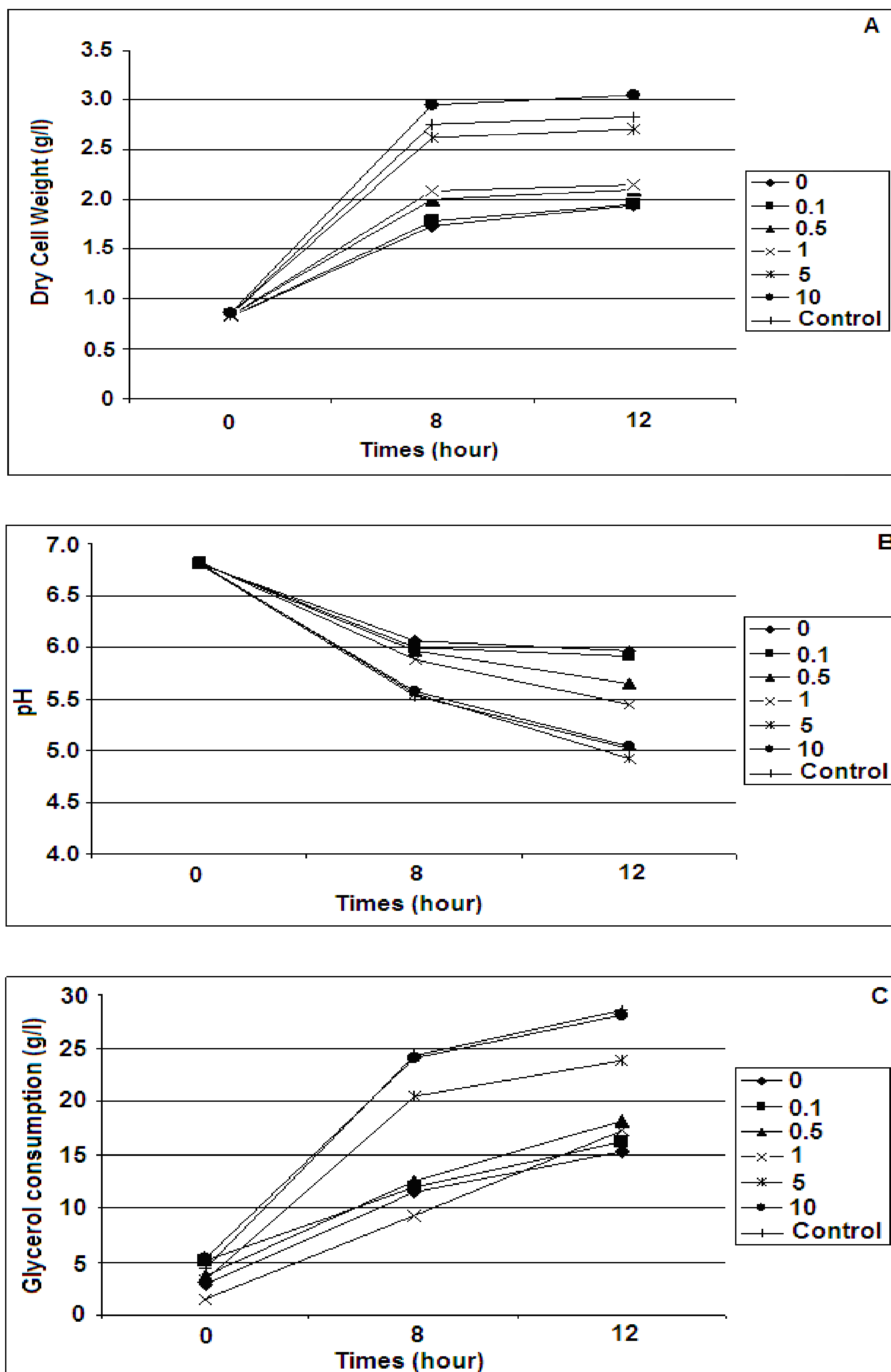


Figure 12. Effect of initial organic nitrogen source (yeast extract) concentration on microbial growth (A), pH values (B) and glycerol consumption (C) during cultivation of *Enterobacter aerogenes* TISTR 1468 and compared to the control (yeast extract and tryptone)

The highest ethanol concentration was obtained at 12 h cultivation using 10 g/l yeast extract as an organic nitrogen source (Table 5). The highest ethanol yield was at 1 g/l of yeast extract after 8 hour cultivation while the highest productivity was obtained at 8 hour cultivation at 10 g/l yeast extract. Yeast extract is a good substrate for many microorganisms (Jackson *et al*, 1998) because it contains amino acids and peptides, water soluble vitamins and carbohydrates (Peppler, 1982).

Table 5. Effect of organic nitrogen source on ethanol concentration, ethanol yield and productivity during cultivation of *Enterobacter aerogenes* TISTR 1468 at 37^0C for 8 and 12 h

Yeast extract (g/l)	Ethanol concentration (g/l)		Ethanol yield (g ethanol/g glycerol)		Productivity (g/l/h)	
	8	12	8	12	8	12
0	2.25^{d}	3.36^{d}	0.20^{bc}	0.22^{c}	0.28^{d}	0.28^{d}
0.1	2.82^{d}	3.78^{d}	0.24^{b}	0.23^{c}	0.35^{c}	0.31^{d}
0.5	3.90^{c}	4.96^{c}	0.31^{b}	0.27^{b}	0.49^{bc}	0.41^{c}
1	4.52^{c}	5.42^{c}	0.49^{a}	0.31^{ab}	0.57^{b}	0.45^{c}
5	8.73^{b}	8.86^{b}	0.43^{ab}	0.37^{ab}	1.09^{b}	0.74^{b}
10	11.16^{a}	12.49^{a}	0.46^{a}	0.45^{a}	1.39^{a}	1.04^{a}
Control*	10.82^{a}	11.70^{a}	0.45^{a}	0.41^{a}	1.35^{a}	0.97^{a}

Where: * = 5 g/l yeast extract and 5 g/l tryptone

SPSS program of analytical statistics was used to find the best concentration of organic nitrogen source for the next experiment. The highest ethanol concentration was obtained from using either the control (yeast extract and tryptone) or 10 g/l yeast extract, as their values were not significantly difference. Yeast extract has a buffering capacity and this might contribute to the high productivity using the media containing yeast extract (Gaudreau *et al* (1997). Furthermore, the initial yeast extract at 1 g/l, 5 g/l, 10 g/l, and the control gave the highest ethanol yield, because their values were not significantly difference. The highest productivity was obtained at initial nitrogen source of 10 g/l at 8 hours. Moreover, to choose the best cultivation time between 8 and 12 hours, analytical SPSS was used. It was concluded that ethanol concentration, yield and productivity were not significantly difference. For further study, 8 hour was chosen.

Analytical statistic by SPSS program indicated that 10 g/l yeast extracts and the control (5 g/l of yeast extract and 5 g/l of tryptone) exhibited the highest ethanol concentration, ethanol yield, and productivity. The results were the same as that reported

by Ito *et al* (2005) where addition of both yeast extract and tryptone to the synthetic medium was effective in increasing the rates of H_2 and ethanol production. It suggested that some nutrients such as specific amino acids and vitamins that are still unknown were needed for the better growth of *E. aerogenes* HU-101. For the next experiment, this initial organic nitrogen source would be used, because it gave the highest ethanol concentration, ethanol yield and productivity.

It was also reported that the fermentation time of *Clostridium pasteurianum* DSM 525 was prolonged when it used 25 µg/l biotin compared to the medium containing 0.1 g/l yeast extract. In batch, the addition of 25 µg/l biotin needed 59 hour to complete consumption of glycerol compare with 1 g/l of yeast extract which only need 19 hour (Biebl, 2001).

1.3 Effect of inorganic nitrogen sources

Nitrogen can be found in inorganic source such as ammonium salts and can be used for biocontrol agents that able to assimilate ammonium and to reduce nitrate (Stanbury *et al*, 1995). These inorganic nitrogen sources probably contain only the nutrients that satisfy no more than the minimal requirement for growth (Gibbins, 1978).

E. aerogenes TISTR 1468 was grown in the cultivation medium (pH 6.8) at 37 ^{0}C with 40 g/l glycerol and cultivated on a shaker (150 rpm) for 8 hour. In order to know the effect of different inorganic nitrogen sources, the medium was made without the addition of any organic nitrogen source, either 10 g/l yeast extract or 5 g/l of a yeast extract and tryptone (the control). Three types of inorganic nitrogen source, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ and $NH_2C_2H_3O_2$, were tested at the same concentration of 0.8 mmol nitrogen.

The results indicated that the addition of organic nitrogen source besides inorganic source had significant effect on microbial growth (Table 6). In the presence of inorganic nitrogen source supplemented with yeast extract or the control (5 g/l of yeast extract and 5 g/l of tryptone) could increase the microbial growth with the highest dry cell weight of 2.8 g/l and 2.3 g/l compared to 1.7 g/l without adding any supplement of organic nitrogen sources, respectively. During the cultivation, the pH decreased from 6.8 to 5.2 when it used only inorganic nitrogen sources. The pH was changed to 5.6 and 5.8 when the medium was added with yeast extract and the control (yeast extract and tryptone), respectively.

Table 6. Effect of inorganic nitrogen sources on microbial growth, pH and glycerol consumption during cultivation of *Enterobacter aerogenes* TISTR 1468 at 37 ^{0}C for 8 h using initial pH 6.8

Nitrogen source (mmol)	DCW (g/l)	pH	GC (g/l)	EC (g/l)	EY (g/g)	P (g/l/h)
$(NH_4)_2SO_4$	1.653	5.25	17.30	2.43^c	0.15^e	0.30^c
$(NH_4)_2HPO_4$	1.707	5.37	11.62	2.44^c	0.20^d	0.30^c
$NH_2C_2H_3O_2$	1.683	5.45	10.93	2.70^c	0.23^d	0.34^c
$(NH_4)_2SO_4$ +YE	2.823	5.64	25.83	11.13^a	0.43^b	1.39^c
$(NH_4)_2HPO_4$ + YE	2.831	5.73	23.97	11.34^a	0.48^a	1.42^c
$NH_2C_2H_3O_2$ + YE	2.795	5.83	25.15	10.76^{ab}	0.43^b	1.35^{bc}
$(NH_4)_2SO_4$ + YE&T	2.319	5.81	25.18	10.51^{ab}	0.42^{bc}	1.31^{bc}
$(NH_4)_2HPO_4$ + YE&T	2.269	5.87	26.42	10.62^{ab}	0.40^{bc}	1.33^{bc}
$NH_2C_2H_3O_2$ + YE&T	2.245	5.98	26.03	10.15^b	0.39^c	1.27^b

Where: DCW : Dry cell weight GC: Glycerol consumption EC: Ethanol concentration
EY : Ethanol yield P : Productivity YE: Yeast extract
T : Tryptone

Moreover, the glycerol consumption at the same time was not completely utilized within 8 hour. The highest glycerol consumption was found when the medium was added the control (5 g/l of yeast extract and 5 g/l of tryptone) at 26.42 g/l, 26.03 g/l and 25.18 g/l of $(NH_4)_2HPO_4$, $NH_2C_2H_3O_2$ and $(NH_4)_2SO_4$, respectively. This values were not significantly different when 10 g/l yeast extract was added in the medium, whereas the glycerol consumption was obtained at 25.83 g/l, 25.15 g/l and 23.97 g/l of $(NH_4)_2SO_4$, $NH_2C_2H_3O_2$ and $(NH_4)_2HPO_4$, respectively. Furthermore, without the addition of organic nitrogen sources, the glycerol consumption was lower than 20 g/l, whereas the addition of $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ and $NH_2C_2H_3O_2$ was achieved at 17.30 g/l, 11.62 g/l and 10.93 g/l, respectively. It also concluded the highest ethanol concentration, ethanol yield, and ethanol productivity which given using $(NH_4)_2HPO_4$ with 10 g/l of yeast extract as organic nitrogen source (Table 6).

Analytical statistic using SPSS program showed that the lowest ethanol concentration, ethanol yield and productivity were obtained from using only inorganic nitrogen sources without the addition of yeast extract and control (yeast extract and tryptone). The highest ethanol concentration, ethanol yield and ethanol productivity were achieved at all of inorganic nitrogen sources with the addition of yeast extract. It indicated that *E. aerogenes* TISTR 1468 still need much of nitrogen source. On the other hand, organic nitrogen source have many components which are needed for microbial growth. Combination of $(NH_4)_2HPO_4$ with 10 g/l of yeast extract was chosen for the next

experiment because it produced the highest ethanol concentration, ethanol yield and ethanol productivity.

1.4 Effect of inorganic nitrogen concentration

E. aerogenes TISTR 1468 was cultivated under the optimum condition; 40 g/l glycerol concentration, 10 g/l yeast extract, 150 rpm shaking speed, at 37 ^{0}C for 8 hour. Effect of $(NH_4)_2HPO_4$ concentrations studied at 0.5 g/l, 1 g/l and 1.5 g/l which corresponded to 0.4, 0.8 and 1.2 mmol of nitrogen in $(NH_4)_2HPO_4$, respectively.

After 8 hour cultivation, initial growth of *E. aerogenes* TISTR 1468 increased from 0.8 g/l to 2.86 - 2.93 g/l dry cell weight. The initial pH of 6.8 decreased to 5.67, 5.68 and 5.72 at 0.4 mmol, 0.8 mmol and 1.2 mmol nitrogen of $(NH_4)_2HPO_4$, respectively. At the same condition, the initial glycerol concentration of 40 g/l was reduced to 25.87 g/l, 24.13 g/l and 25.15 g/l, respectively. This indicated that the bacteria could utilize glycerol only 35.3%, 39.6% and 37.1%, respectively (Table 7). This was due to the fact that the cultivation time was 8 hour. The highest ethanol concentration, ethanol yield and ethanol productivity were 11.25 g/l, 0.48 g ethanol/g glycerol and 1.41 g/l/h at 0.8 mmol nitrogen concentration, respectively. Cultivation of *Pantoea agglomerans* CPA-2 in the medium added with inorganic nitrogen sources such as ammonium chloride, ammonium sulphate and ammonium nitrate revealed that the microbial growth had the same concentration of 10^6 cfu/ml (Costa *et al*, 2002). Similarly with this experiment, whereas the adding of different concentration of $(NH_4)_2HPO_4$, the microbial growth and product formation were achieved at the same range.

Table 7. Effect of $(NH_4)_2HPO_4$ conentration on microbial growth, pH, glycerol consumption, ethanol concentration, ethanol yield and productivity during cultivation of *Enterobacter aerogenes* TISTR 1468 at 37^{0}C for 8 h using initial pH 6.8

Nitrogen content as $(NH_4)_2HPO_4$ (mmol)	MG (g/l)	pH	GC (g/l)	EC (g/l)	EY (g/g)	P (g/l/h)
0.4	2.86	5.67	25.87	10.39^a	0.40^a	1.30^a
0.8	2.93	5.68	24.13	11.34^a	0.47^a	1.42^a
1.2	2.91	5.72	25.15	9.49^a	0.38^a	1.19^a

Where: MG : Microbial growth GC : Glycerol consumption
EC : Ethanol concentration EY : Ethanol yield P : Productivity

Analytical statistics using SPSS program showed that there was not significantly difference between the yield of 0.8 mmol with the other nitrogen concentrations (0.4 and 1.2 mmol), whereas ethanol concentrations were 11.25 g/l, 11.14 g/l and 10.93 g/l at 0.8,

0.4 and 1.2 mmol nitrogen concentrations, respectively. Similar composition of inorganic nitrogen concentration was obtained for ethanol yield and productivity, whereas ethanol yield were 0.48, 0.43 and 0.43 g ethanol/g glycerol and productivity were 1.41, 1.39 and 1.37 g/l/h, respectively. For the next experiment, 0.4 mmol nitrogen in $(NH_4)_2HPO_4$ was chosen.

1.5 Effect of mineral salts

Various mineral salts; $Na_2MoO_4.2H_2O$, Na_2SeO_3, $NiCl_2$, and trace elements (0.5 g $MnCl_2 \cdot 4H_2O$, 0.1 g H_3BO_4, 0.01 g $AlK(SO_4)_2 \cdot H_2O$, 0.001 g $CuCl_2 \cdot 2H_2O$ and 0.5 g Na_2EDTA (per liter) were tested for their importance on ethanol production from *E. aerogenes* TISTR 1468 (Table 8). The cultivation was conducted under the optimum condition, obtained from previous experiment such as 40 g/l glycerol concentration, 10 g/l yeast extract, 0.4 mmol nitrogen in $(NH_4)_2HPO_4$, 150 rpm shaking speed, at 37 ^{0}C for 8 hour.

Table 8. Effect of mineral salts on microbial growth, pH, glycerol consumption, ethanol yield and productivity during cultivation of *Enterobacter aerogenes* TISTR 1468 at 37 ^{0}C for 8 h using initial pH 6.8

Mineral Sources	MG (g/l)	pH	GC (g/l)	EC (g/l)	EY (g/g)	P (g/l/h)
Control with mineral salts	2.85	5.65	27.57	5.94[e]	0.22[e]	0.74[e]
Control without mineral salts	2.64	5.81	19.92	3.44[d]	0.17[d]	0.43[d]
Without $Na_2MoO_4.2H_2O$	3.09	5.44	34.43	11.84[b]	0.34[b]	1.48[b]
Without Na_2SeO_3	2.55	5.53	29.49	8.55[c]	0.29[c]	1.07[c]
Without $NiCl_2$	2.98	5.41	33.09	13.53[a]	0.41[a]	1.69[a]
Without trace element	3.09	5.46	31.65	13.33[a]	0.42[a]	1.67[a]

Where: MG : Microbial growth GC : Glycerol consumption
EC : Ethanol concentration EY : Ethanol yield P : Productivity

The highest microbial growth 3.09 g/l dry cell weight was obtained without trace element solution and without $Na_2MoO_4.2H_2O$. The lowest of cell growth 2.55 g/l was obtained from using the medium without Na_2SeO_3. The values 2.64 g/l, 2.85 g/l and 2.98 g/l were obtained in the medium without mineral sources, with all mineral sources and without $NiCl_2$, respectively (Table 8). Therefore, *E. aerogenes* TISTR 1468 only need some mineral salts for the growth. During 8 hour cultivation, the pH changed from 6.8 to pH range 5.41 - 5.81, results from the acidic product during fermentation. It was suggested by Cheng *et al* (2007) that fermentation products such as lactic acid, acetic acid, 1,3-

propanediol, 2,3-butanediol and ethanol using *Klebsiella pneumonia*e at pilot scale experiment decreased the pH in the medium.

The highest glycerol consumption of 34.43 g/l was found without the addition of $Na_2MoO_4.2H_2O$ from 40 g/l glycerol concentration. The lowest glycerol consumption of 19.92 g/l was found without the addition of mineral salts. For the other parameters such as without $NiCl_2$, without trace element, without Na_2SeO_3 and with all mineral salts, the highest glycerol consumptions were 33.09 g/l, 31.65 g/l, 29.49 g/l and 27.57 g/l, respectively. The highest ethanol concentration (13.533 g/l) and productivity (1.69 g/l/h) were achieved without the addition of $NiCl_2$. The highest ethanol yield (0.42 g ethanol/g glycerol) was obtained without the addition of trace element solution (Table 8).

SPSS analysis indicated that both parameters; without $NiCl_2$ and without trace element, were not significantly different. They gave higher ethanol concentration, ethanol yield, and productivity. Therefore, without trace element solution was chosen for the next experiment.

1.6 Effect of initial pH

The initial pH is highly significant in fermentation in this experiment. Batch cultures at values between 5.5 and 7.5 using the best parameters from the previous results were conducted. The highest microbial growth was found at the initial pH 7.5 followed by initial pH 6.8, 7.0, 5.5, 6.5 and 6.0 giving the dry cell weight of 3.25 g/l, 3.09 g/l, 3.02 g/l, 3.00 g/l, 2.84 g/l and 2.71 g/l, respectively (Table 9). At the same condition and cultivation time, although they had different initial pH, the pH value decreased at the same pH range of 5.15 to 5.68 after 8 hour cultivation. The highest glycerol consumption of 33.84 g/l was found at the initial pH 6.8. The lowest glycerol consumption of 21.81 g/l was found at the initial pH 5.5. From the range of glycerol consumption, it indicated that the higher initial pH resulted in the increase of glycerol consumption and microbial growth. Therefore, the higher initial pH (7.5) was the appropriate condition for microbial growth and it affected on glycerol consumption.

Table 9. Effect on initial pH on microbial growth, pH, glycerol consumption, ethanol yield and productivity during cultivation of *Enterobacter aerogenes* TISTR 1468 at 37 ^{0}C for 8 h

Initial pH	MG (g/l)	pH	GC (g/l)	EC (g/l)	EY (g/g)	P (g/l/h)
5.5	3.00	5.15	21.81	7.31^{e}	0.34^{bc}	0.91^{e}
6.0	2.71	5.20	26.58	7.84^{de}	0.30^{d}	0.98^{de}
6.5	2.84	5.28	28.71	8.30^{d}	0.29^{d}	1.03^{d}
6.8	3.09	5.44	33.84	12.55^{bc}	0.37^{cd}	1.66^{bc}
7.0	3.02	5.43	29.43	13.26^{b}	0.45^{b}	2.04^{a}
7.5	3.25	5.68	31.65	16.31^{a}	0.52^{a}	1.57^{c}

Where: MG : Microbial growth GC : Glycerol consumption
EC : Ethanol concentration EY : Ethanol yield P : Productivity

The highest ethanol concentration (16.31 g/l), ethanol yield (0.52 g ethanol/ g glycerol) and productivity (2.04 g/l/h) from *E. aerogenes* TISTR 1468 were achieved in the medium using initial pH 7.5 (Table 9). It had relationship between pH and ethanol production, whereas ethanol concentration, ethanol yield, and productivity increased with the increase of pH. At initial pH 5.5, the bacteria could produce 6.31 g/l ethanol, when the pH increased to 6.0, 6.5, 6.8, 7.0 and 7.5, the ethanol concentration increased to 6.84, 7.30, 8.88, 9.26 and 16.31 g/l, respectively. The pH range from 5.5 to 6.8 gave no significant effect where the ethanol yields were the range of 0.25 to 0.29 g ethanol / g glycerol. For productivity, pH had an effect where it increased at the same time when the initial pH values increased. Initial pH at 5.5, the productivity was 0.79 g/l/h, when it increased to 6.0, 6.5, 6.8, 7.0 and 7.5, the productivity increased to 0.86, 0.91, 1.1, 1.16 and 2.04 g/l/h, respectively.

This experiment was similarly with Biebl (2001). Using *Clostridium pasteurianum* in batch cultures controlled pH 4.5 and 7.5 using 50 g/l glycerol concentration, the cultures grew more or less equally well over the whole pH range with a slight preference for the weakly acidic range. It increased from pH 6 to 7.5 and required up to 25% of the glycerol. On the other hand, a high variability in product formation was observed when the influence of pH between 5 and 7.5 was examined. Small variations appeared in the culture conditions or slight differences in the pre-cultures were able to change the product selectivity considerably. Such a weak regulation between the fermentation pathways seems to be a general feature of this fermentation. Biebl *et al* (1998) demonstrated anaerobic fermentation in comparative batch fermentation without pH control start at 6.2 using *Klebsiela pneumoniae* GT1, the results indicated that low pH was coupled to incomplete

glycerol utilization in the experiment described; one might ask whether the observed changes in the product spectrum are really an effect of acid accumulation or whether they have to be taken as a more general growth inhibition consequence. Other stress conditions that lead to incomplete utilization of glycerol in the culture contribute to it as well.

SPSS analysis indicated that initial pH gave the different significant value. The highest ethanol concentration and ethanol yield was achieved at initial pH 7.5 and the highest productivity was obtained at initial pH 7.0. Therefore, initial pH 7.5 was chosen as the best initial pH and used in further experiment.

1.7 Confirmation experiment

Under the optimum condition from 'one factor at the time', confirmation experiment conducted in order to know the difference between before and after optimization. The cultivation was obtained 40 g/l glycerol concentration, 10 g/l yeast extract, 0.4 mmol nitrogen in $(NH_4)_2HPO_4$, 150 rpm shaking speed, without trace element solution, 7.5 as initial pH at 37 ^{0}C for 8 hour.

By comparing the original cultivation medium with the optimum condition, the microbial growth, glycerol consumption, ethanol concentration, ethanol yield and productivity (Table 10) increased 1.59, 1.69, 5.44, 3.25 and 4.13 folds, respectively.

The highest ethanol production was concluded at 17.07 g/l after 16 hour in the optimum condition. At the same time, glycerol concentration almost completely consumed with the highest microbial growth at 3.58 g/l of dry cell weight after 24 hour (Fig. 13B). On the other hand, in original medium, the highest ethanol concentration was 3.34 g/l after 24 hour, although glycerol consumption almost completely consumed with the microbial growth at 2.56 g/l (Fig. 13A).

This meant that a substantial optimization by 'one factor at the time' in the batch fermentation process was realized. It increased the ethanol production from original medium to highest production in the last results.

Table 10. The yield of ethanol production before and after optimization

Product yield	Before optimization	After optimization
Microbial growth (g/l)	2.05	3.25
Glycerol consumption (g/l)	18.69	31.65
Ethanol concentration (g/l)	3.00	16.30
Ethanol yield (g ethanol/g glycerol	0.16	0.52
Productivity (g/l/h)	0.38	1.57

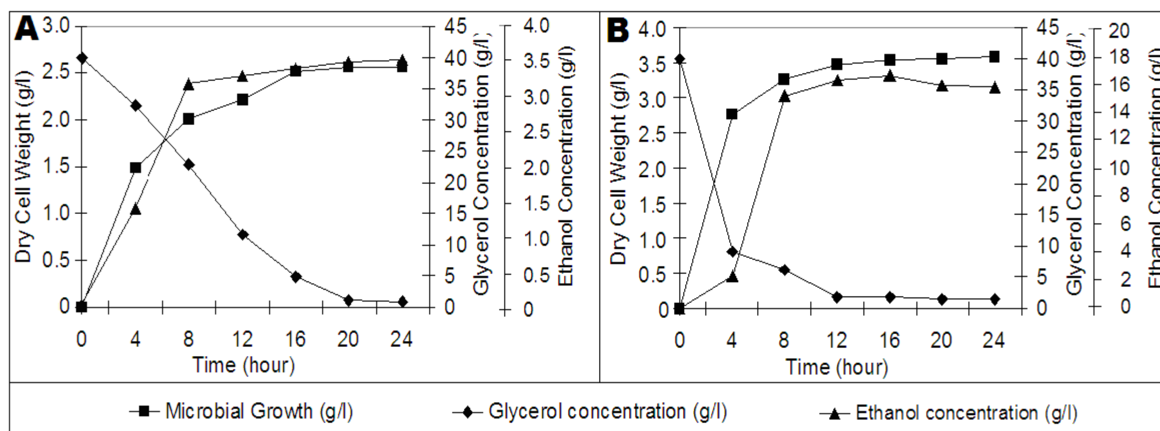


Figure 13. Comparison of time course of ethanol production during cultivation of *Enterobacter aerogenes* TISTR 1468 in the original cultivation medium (A) and in the optimum condition after optimization using 'one factor at the time' (B)

2. Optimization for ethanol production from *Enterobacter aerogenes* TISTR 1468 by Response Surface Methodology (RSM)

RSM mainly consisted of the central composite design (CCD), the Box-Behnken design (BBD), the one factor design, the D-optimal design, the user-defined design, and the historical data design. CCD and BBD were the most used response surface design methods, which had 5 levels and 3 levels, respectively for one numeric factor. BBD (Box and Wilson, 1951) was used to optimize the levels of glycerol concentration, organic nitrogen concentration and inorganic nitrogen concentration in this study.

The experiment was performed in 250 ml shake flask containing 100 ml cultivation medium with 10% inoculum. The concentration of glycerol ranged from 20 to 60 g/l, organic nitrogen concentration and inorganic nitrogen concentration ratios ranged from 5 to 15 g yeast extract and 0.2 to 0.6 mmol $(NH_4)_2HPO_4$. The experiment was conducted using triplicate shake flasks where they were incubated at 37°C with an initial pH of 7.5 adjusted with 5M NaOH or 5M HCl. The sample was collected after 8 hour cultivation and measured for ethanol. A confirmation experiment was carried out in 250 ml shake flask containing 100 ml cultivation medium with 10% inoculum and the optimal concentrations of glycerol, organic nitrogen and inorganic nitrogen were added.

Response surface methodology combined with BBD was used to describe the relationship between the independent variables and the responses as well as to determine the optimum condition of nutrient factors. A three-factor, three-level BBD with three

replicates at the center point was selected to build response surface models. Three factors are used in this research from conventional method, glycerol concentration (X_1), organic nitrogen sources (X_2) and inorganic nitrogen sources (X_3). Experiments were run according to design in a single block with 3 central points. The 3 central points represent the same value of the parameter. Ethanol concentration (Y_1), ethanol yield (Y_2) and ethanol productivity (Y_3) was used as the response to analyze the design.

The optimum response was predicted using the above mentioned polynomial model equation. The mathematical relationship of the independent variables and the response can be calculated by the quadratic polynomial equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_1^2 + \beta_5 X_2^2 + \beta_6 X_3^2 + \beta_7 X_1 X_2 + \beta_8 X_1 X_3 + \beta_9 X_2 X_3$$

Where β_0 is an intercept and β_1- β_9 are the coefficients of respective factors and their interaction terms. The response surfaces were constructed using the software package Design Expert software (version 5.0, Stat-Ease Inc., Minneapolis, U.S.A.). A suitable polynomial model was selected based on the estimation of several statistical parameters such as the multiple correlation coefficient (R_2), adjusted multiple correlation coefficient (adjusted R_2).

Table 11. The high and low values of the variables for response surface methodology

Factor	Name	Units	Low Actual	Middle Actual	High Actual
X_1	Glycerol	g/l	20	40	60
X_2	Yeast extract	g/l	5	10	15
X_3	$(NH_4)_2HPO_4$	mmol	0.2	0.4	0.6

Using Design Expert software, three factors (Table 11) were calculated to get the experiment design and response of statistical analysis to know the effect of each factor for ethanol production. 17 samples was concluded to used for glycerol fermentation based on different glycerol concentration, yeast extract concentration and $(NH_4)_2HPO_4$ concentration. The results of ethanol concentration, ethanol yield and productivity were explained in Table 12. The significant relation effect of each factor for ethanol production were explained in model coefficient estimated by multiple linear regression whereas it were get from the software.

Table 12. Experiment design and response of Box Behnken Design for ethanol concentration, ethanol yield, and productivity

Std	Glycerol (g/l)	Yeast extract (g/l)	$(NH_4)_2HPO_4$ (mmol)	EtOH conc (g/l)	EtOH yield (g/g)	Productivity (g/l/h)
1	20	5	0.4	10.26	0.57	1.28
2	60	5	0.4	7.73	0.20	0.97
3	20	15	0.4	11.17	0.63	1.40
4	60	15	0.4	8.27	0.21	1.03
5	20	10	0.2	11.26	0.65	1.41
6	60	10	0.2	8.38	0.23	1.05
7	20	10	0.6	10.90	0.62	1.36
8	60	10	0.6	7.37	0.21	0.92
9	40	5	0.2	8.14	0.53	1.02
10	40	15	0.2	10.37	0.58	1.30
11	40	5	0.6	10.54	0.54	1.32
12	40	15	0.6	11.36	0.52	1.42
13	40	10	0.4	15.47	0.59	1.93
14	40	10	0.4	15.48	0.56	1.93
15	40	10	0.4	16.14	0.58	2.02
16	40	10	0.4	15.47	0.55	1.93
17	40	10	0.4	16.38	0.63	2.05

Remark: EtOH: Ethanol

2.1 Effect of glycerol, organic nitrogen and inorganic nitrogen concentration on ethanol production

Effects of nutrients factors for fermentative ethanol production from purified glycerol based on previous knowledge obtained by Ito *et al* (2005), Barbirato *et al* (1997), and Jarvis *et al* (1997) were investigated. The ethanol production ranged from 7.37-16.38 g/l (Table 12). Low ethanol production at 7.37 g/l was obtained using the highest glycerol concentration (Table 12, trial 8). A maximal ethanol production was obtained at trial number 17 (16.38 g/l) using the middle glycerol concentration, yeast extract and $(NH_4)_2HPO_4$.

To evaluate the results, data in Table 12 were subjected to regression analysis, using the following quadratic equation:

$$Y_1 = 15.7858 - 1.4814 \text{ x } X_1 + 0.5621 \text{ x } X_2 + 0.2525 \text{ x } X_3 - 3.5282 \text{ x } X_1^2 - 2.9047 \text{ x } X_2^2 - 2.7819 \text{ x } X_3^2 - 0.0930 \text{ x } X_1X_2 - 0.1608 \text{ x } X_1X_3 - 0.3538 \text{ x } X_2X_3$$

Where Y_1 is the ethanol concentration response and X_1, X_2 and X_3 are the coded values of glycerol concentration, organic nitrogen concentration and inorganic nitrogen concentration, respectively.

The model presented a high determination coefficient ($R^2 = 0.9746$) explaining 97% of variability in the response. The value of the adjusted determination coefficient (adjusted $R^2 = 0.9420$) was also high, indicating a high significance of the model (Dabrock *et al*, 1992). The ANOVA quadratic regression model demonstrated that the model was highly significant whereas the low probability ($P<0.0001$) and lack of fit model were not significant ($P=0.0629$). At the same time, a lower variation coefficient value (CV = 6.6486%) indicated a high precison and reliability of the experiments.

As shown in Fig. 13, the response surface of ethanol production indicated that glycerol concentration had significant interactive influence and individual influence on ethanol production. The significant of each coefficient was determined by probability values. It was obvious that all variables had significant effect on ethanol production ($P<0.05$). It also showed that linear (X_1^2, X_2^2, X_3^2) are significant ($P<0.05$), demonstrating that the microorganisms responsible for ethanol production required both a supplementary yeast extract and $(NH_4)_2HPO_4$.

Similarly with the experiment of Ito *et al* (2005), they were demonstrated the sole effect of glycerol concentration on substrate utilization and product formation. The complex medium was supplemented with 5 g/l, 10 g/l and 25 g/l purified glycerol concentrations in batch culture. The results confirmed that *E. aerogenes* HU-101 could consumed completely 5 g/l or 10 g/l purified glycerol concentrations within 6 h and for 25 g/l after 12 h. For ethanol production, it decreased with the increase in glycerol concentration at 1.00 g/l, 0.86 g/l and 0.80 g/l from 5 g/l, 10 g/l and 25 g/l, respectively. It indicated that the higher glycerol concentration could inhibit ethanol production. In this experiement, the highest ethanol concentration was obtained from 30 g/l glycerol (15.204 g/l) and the increase of glycerol up to 50 g/l caused the decrease of ethanol production to 2.945 g/l.

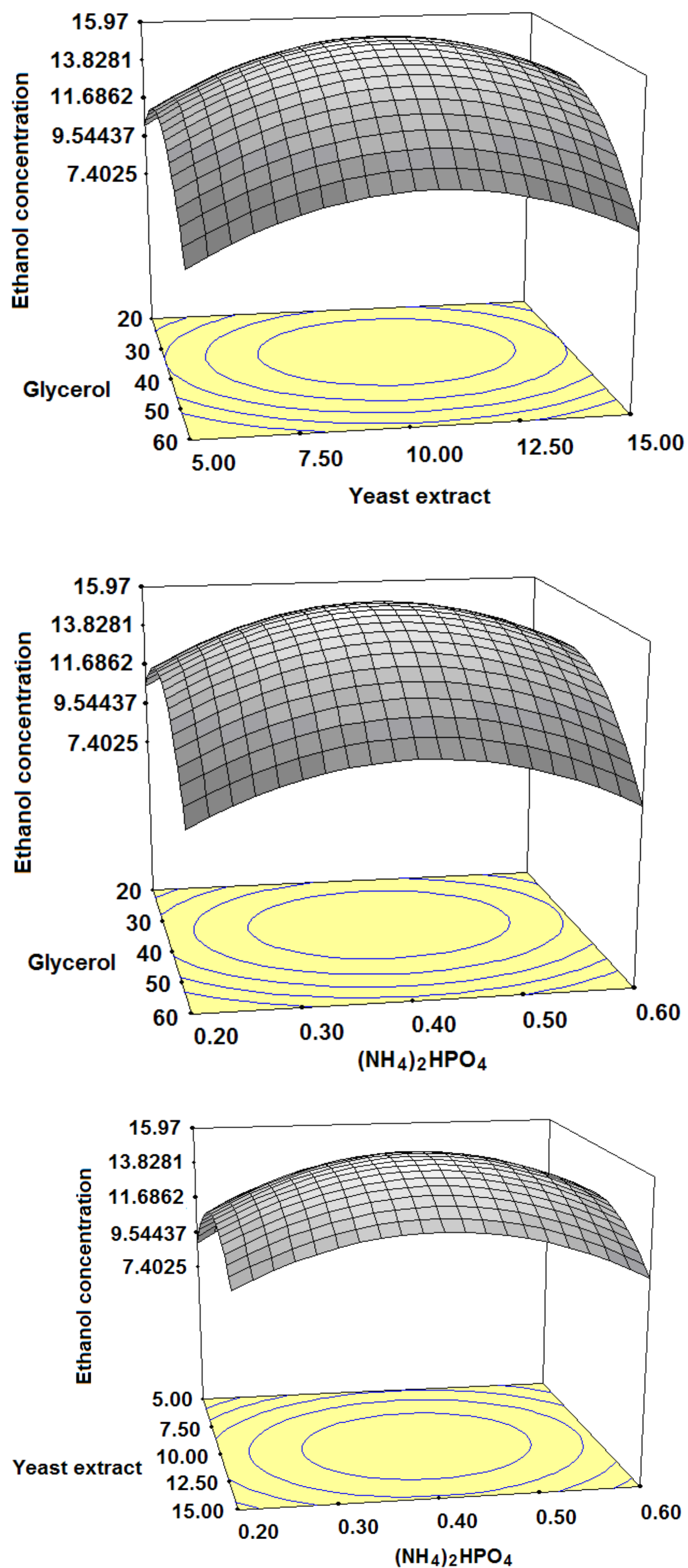


Figure 14. Effect of glycerol, yeast extract and $(NH_4)_2HPO_4$ in response surface plot of ethanol concentration during cultivation of *Enterobacter aerogenes* TISTR 1468

2.2 Effect of glycerol, organic nitrogen and inorganic nitrogen concentration on ethanol yield

Ethanol yields ranged from 0.21 - 0.65 g ethanol/g glycerol were obtanied (Table 12). Low ethanol yield at 0.21 g ethanol/g glycerol was obtained from using the highest glycerol concentration (60 g/l), the lowest concentration of yeast extract (5 g/l) and the middle concentration of $(NH_4)_2HPO_4$ (0.4 mmol) (Table 12, trial 2). A maximum ethanol production (0.65 g ethanol/g glycerol) was obtained at trial number 5 using the lowest concentration of glycerol (20 g/l), the middle concentration of yeast extract (10 g/l) and the lowest concentration of $(NH_4)_2HPO_4$ (0.2 mmol). Results in Table 12 were evaluated by regression analysis, using the following quadratic equation:

$$Y_2 = 0.5806 - 0.2037 \text{ x } X_1 + 0.0107 \text{ x } X_2 - 0.0123 \text{ x } X_3 - 0.1462 \text{ x } X_1^2 - 0.0324 \text{ x } X_2^2 - 0.0072 \text{ x } X_3^2 - 0.0124 \text{ x } X_1X_2 + 0.0034 \text{ x } X_1X_3 - 0.0175 \text{ x } X_2X_3$$

Where Y_2 is the ethanol yield response and X_1, X_2 and X_3 are the coded values of glycerol concentration, organic nitrogen concentration and inorganic nitrogen concentration, respectively.

The model presented a high determination coefficient ($R^2 = 0.9907$) explaining 99% of variability in the response. The value of the adjusted determination coefficient (adjusted $R^2 = 0.9787$) is high. The ANOVA quadratic regression model demonstrated that the model was highly significant whereas the probability was $P<0.0001$ and lack of fit model was not significant ($P=0.9380$). At the same time, a lower variation coefficient value (CV = 4.8937%) indicated a high precison and reliability of the experiments.

The response surface of ethanol yield in Fig. 14 indicated that glycerol concentration had significant interactive influence on ethanol yield. It was concluded from the model coefficient estimates by multiple linier regressions. At 95% significant level, only glycerol concentration had individual significant effect on ethanol yield at <0.0001. It was obvious that the variables with a significant effect on ethanol yield was the term of glycerol concentration (X_1) where $P<0.05$. It also showed that the interaction (X_1^2 and X_2^2) were significant ($P<0.05$).

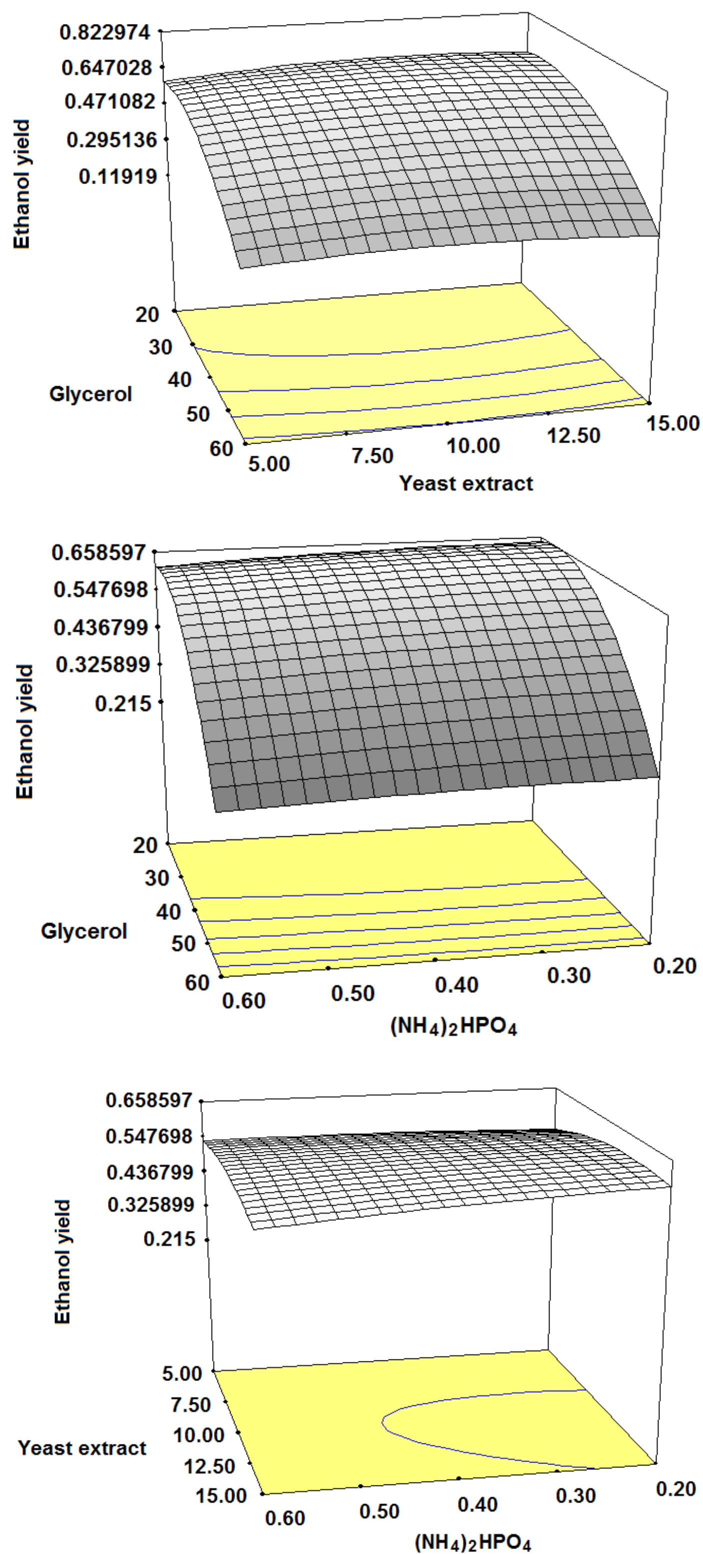


Figure 15. Effect of glycerol, yeast extract and $(NH_4)_2HPO_4$ concentration on response surface plot of ethanol yield during cultivation of *Enterobacter aerogenes* TISTR 1468

2.3 Effect of glycerol, organic nitrogen and inorganic nitrogen concentration on productivity

The experiment showed that the ethanol productivity from *E. aerogenes* TISTR 1468 was in the range of 0.92 – 2.05 g/l/h (Table 12). Low productivity was obtained using the highest glycerol concentration (60 g/l) and $(NH_4)_2HPO_4$ concentration (0.6 mmol) but using middle value of yeast extract concentration (10 g/l) (Table 12, trial 8). Maximum productivity was obtained at trial number 17 using the middle glycerol concentration (40 g/l), yeast extract concentration (10 g/l) and $(NH_4)_2HPO_4$ concentration (0.4 mmol). To evaluate the results in Table 12, the data were subjected to regression analysis using the following quadratic equation:

$$Y_3 = 1.9732 - 0.1852 \times X_1 + 0.0703 \times X_2 + 0.0316 \times X_3 - 0.4410 \times X_1^2 - 0.3631 \times X_2^2 - 0.3477 \times X_3^2 - 0.0116 \times X_1X_2 - 0.0201 \times X_1X_3 - 0.0442 \times X_2X_3$$

Where Y_3 is the productivity response and X_1, X_2 and X_3 are the coded values of glycerol concentration, organic nitrogen concentration and inorganic nitrogen concentration, respectively.

The model presented a high determination coefficient ($R^2 = 0.9746$) explaining 97% of variability in the response. The value of the adjusted determination coefficient (adjusted $R^2 = 0.942$) was accepted as significant of the model. The ANOVA quadratic regression model demonstrated that the model was highly significant whereas had low probability ($P<0.0001$) and lack of fit model was not significant ($P=0.0629$). At the same time, a lower variation coefficient value (CV = 6.6486%) indicated a high precison and reliability of the experiments.

The response surface of productivity indicated that glycerol concentration had significant interactive influence on productivity (Fig. 15). However, glycerol had an individual significant influence on productivity, whereas from model coefficient estimates by multiple linier regressions at 95% significant level, it had the values at 0.0009. It was shown that the variables with a significant effect on productivity was the term of glycerol concentration (X_1) ($P<0.05$). The interaction (X_1^2, X_2^2, X_3^2) had significant affect ($P<0.05$).

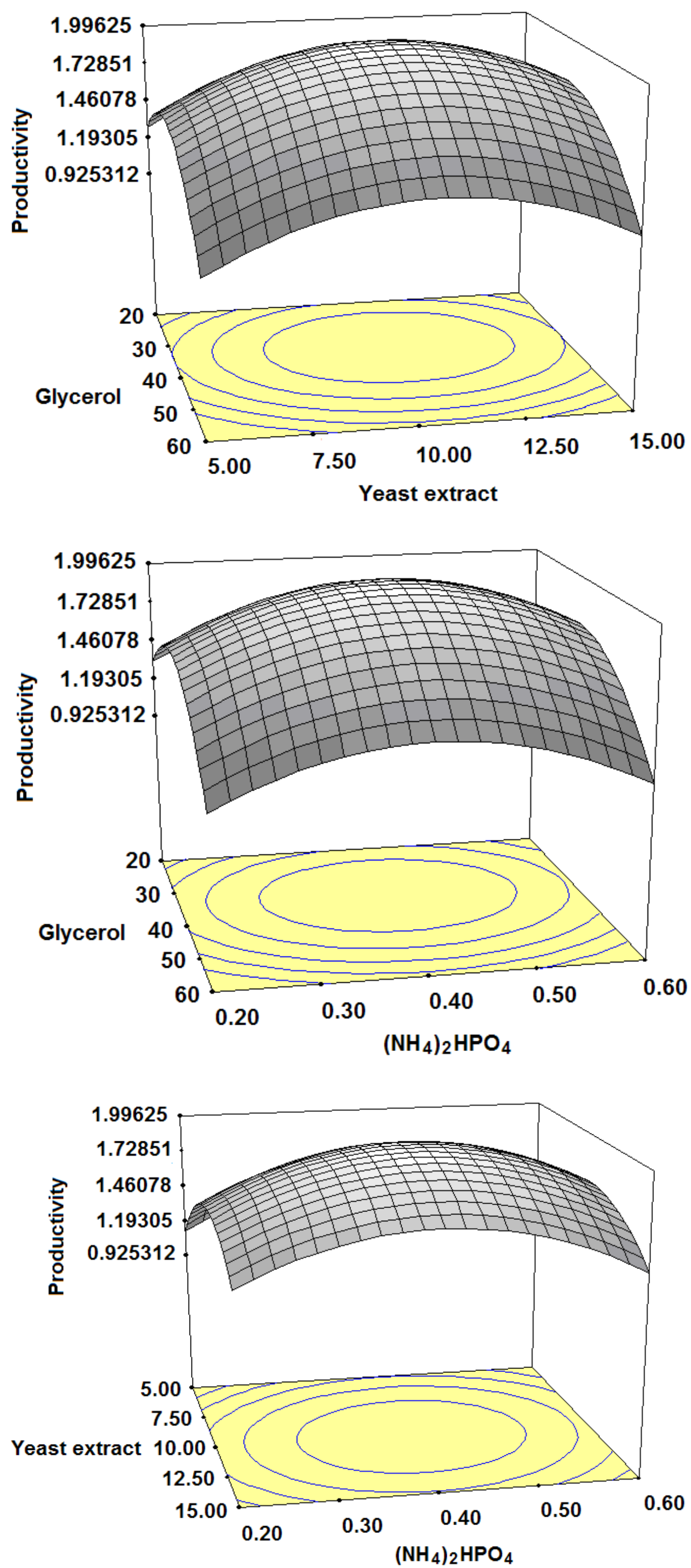


Figure 16. Effect of glycerol, yeast extract and $(NH_4)_2HPO_4$ on response surface plot of productivity during cultivation of *Enterobacter aerogenes* TISTR 1468

2.4. Confirmation experiment

After optimization process using response surface methodology by BBD, the maximum ethanol production, ethanol yield and productivity were illustrated at a glycerol concentration of 30 g/l, yeast extract concentration of 8.73 g/l and $(NH_4)_2HPO_4$ concentration of 0.30 mmol. Under this condition, the model predicted ethanol production, ethanol yield and productivity of 14.32 g/l, 0.64g ethanol/g glycerol, and 1.79 g/l/h, respectively (Table 13).

Table 13. Comparison of predict values and actual values of ethanol production, ethanol yield and productivity after optimization process

Standard order	Predicted values	Actual Values
Ethanol production (g/l)	14.32	16.38
Ethanol yield (g ethanol / g glycerol)	0.64	0.63
Productivity (g/l/h)	1.79	2.05

To confirm the validation of the statistical experiments and to improve the understanding of ethanol production, ethanol yield and productivity, three replicates of batch experiments were performed under the optimal conditions. The results are summarized in Table 14, with ethanol production, ethanol yield and productivity of 16.473 g/l, 0.791 g ethanol/g glycerol and 2.059 g/l/h, respectively. It showed that the results were not significantly difference with the predicted values.

Table 14. Confirmation experimental design and results obtained at 48 h (data are given as mean; n=3)

Trial	Condition	Glycerol concentration (g)	Organic nitrogen (g)	Inorganic nitrogen (mmol)	Ethanol Concentration (g/l)	Ethanol yield (g/g)	Productivity (g/l/h)
-	Optimum[a]	30	8.73	0.30	16.473	0.791	2.059
1	Maximum	40	10	0.4	15.146	0.566	1.893
7	Middle	40	15	0.6	11.875	0.429	1.484
6	Minimum	60	10	0.6	9.876	0.172	1.235
-	Control	30	0	0	0.035	0.102	0.004

[a]Based on the highest ethanol production

RSM have been shown to optimize the process earlier by many workers (Gu et al., 2005). Under experiment using Response Surface Methodology, Zheng *et al* (2008) was confirmed that RSM is qualified to optimize culture conditions on glycerol fermentation using *Klebsiella pneumoniae* IC 15 at batch culture, whereas it could increase the product

formation until 19.78% of 1,3-propanediol and deceased cultivation time from 48 h to 30 h. This result was similar to this research, whereas after optimization process under RSM using BBD, the initial glycerol could decrease from 60 g/l to 29 g/l. The yeast extract concentration and $(NH_4)_2HPO_4$ concentration could be decreased from 15 to 10.25 g/l and 0.6 to 0.29 mmol, respectively.

Ethanol production from biodiesel waste glycerol using *E. aerogenes* HU-101 gave the ethanol yields of 0.96, 0.83, 0.67, and 0.56 mol ethanol / mol glycerol from the initial glycerol concentration of 1.7, 3.3, 10, and 25 g/l, respectively. Therefore, the yields of ethanol decreased with the increase of glycerol concentration (Ito *et al*, 2005). This is also the case for *E. agglomerans* that 0.15 and 0.02 mol ethanol/mol glycerol were obtained from the batch culture with the initial glycerol concentration of 20 and 70 g/l respectively (Barbito *et al*, 1997). Comparing with the result of this research, the highest glycerol concentration (50 g/l) in the medium only could convert to ethanol in the range of 2.945-5.899 g/l and from the confirmation experiment, the ethanol production was 16.9 g/l at 30.13 g/l initial glycerol concentration.

In the case of nitrogen source and concentration, Biebl (2001) suggested that in batch fermentation using *C. pasteurianum* with the addition of 1 g/l yeast extract and nitrogen sparging gas, the ethanol as a by product was higher (0.029 mol/mol glycerol) with the adding of 0.1 g yeast extract/l during 37 hour cultivation than using nitrogen gas (0.008 mol/mol glycerol) during 49 hour cultivation. It was suggested that the additional of NH_4 (ammonium) as nitrogen source could increase the ethanol production (Biebl, 2001). Similarity with this experiment, the highest ethanol concentration (15.204 g/l) was produced with the combination of 5 g/l yeast extract and 0.4 mmol $(NH_4)_2HPO_4$. Furthermore, the highest concentration of yeast extract (15 g/l) and $(NH_4)_2HPO_4$ (0.6 mmol) could not increase the ethanol production (5.640 g/l). It suggested that nitrogen sources and concentration had the effect on *E. aerogenes* TISTR 1468 growth. Yeast extract as organic nitrogen source not only gave nitrogen source but also gave the other compound including carbohydrate, nitrogen, amino acids, vitamins, inorganic salts, etc, which important for microbial growth. On other hand, $(NH_4)_2HPO_4$ as inorganic nitrogen source was used as nitrogen source too. The combination of both nitrogen sources was necessary in order to know the maximum nitrogen of each source. From the confirmation experiment after optimization process, 5.06 g/l yeast extract and 0.58 mmol nitrogen as $(NH_4)_2HPO_4$ were found to be the maximum combination in order to produce the highest

ethanol production (16.9 g/l), ethanol yield (0.606 g ethanol/g glycerol) and productivity (2.112 g/l/h).

2.5 Time course after optimization

From the results of conventional techniques such as the 'one factor at a time' method and response surface methodology (RSM) for ethanol production, it was achieved the strongly influence by many factors, such as carbon source, organic and inorganic nitrogen source, mineral salts, pH and cultivation conditions, it is crucial to search for the time course using optimized formulation. The present study explored the time course of *Enterobacter aerogenes* TISTR 1468 in batch culture using shake flasks (250 ml) containing 10% of starter in 100 ml of culture broth. The medium composition were 30 g/l purified glycerol, 8.73 g/l yeast extract, 0.30 mmol nitrogen content as $(NH_4)_2HPO_4$, 150 rpm of shaking speed, initial pH at 7.5, without trace element solution in the culture broth and cultivation time for 24 hour. Samples were taken every 4 hour during 24 hour.

Time course study showed that glycerol dissimilation by *E. aerogenes* TISTR 1468 led to the production of ethanol at 15.640 g/l after 24 h cultivation. Moreover, microbial growth of 3.595 g/l dry cell weight levels and decreasing glycerol concentration to 29.765 g/l (Fig. 16) indicated that glycerol fermentation to ethanol supported growth of the culture. The rapid increasing of microbial growth was obtained after 8 hour cultivation, the same time for ethanol remained constant that indicating the rest cell conditions. Glycerol was completely consumed after 16 hour cultivation, with ethanol as the major product detected.

Similarly with Ito *et al* (2005) that explained the substrate utilization and product formation in the complex medium supplemented with commercially available glycerol (pure glycerol) using batch cultures. *E. aerogenes* HU-101 was completely consumed 5 g/l or 10 g/l pure glycerol within 6 h and 25 g/l after 12 h with the ethanol and hydrogen as main products. The other researchers found that the medium contained high levels of nitrogen and mineral salts like potassium and sodium phosphates, presumably used to maintain neutral to slightly alkaline conditions (pH in the 7–7.5 range) (Bouvet *et al*, 1995; Sprenger et al, 1989; Tang *et al*, 1982a, b). Moreover, the optimum medium from 'one-factor-at-a-time method' and response surface methodology included the high amount of nitrogen and mineral salts. It suggested that this medium supported for microbial growth and glycerol fermentation.

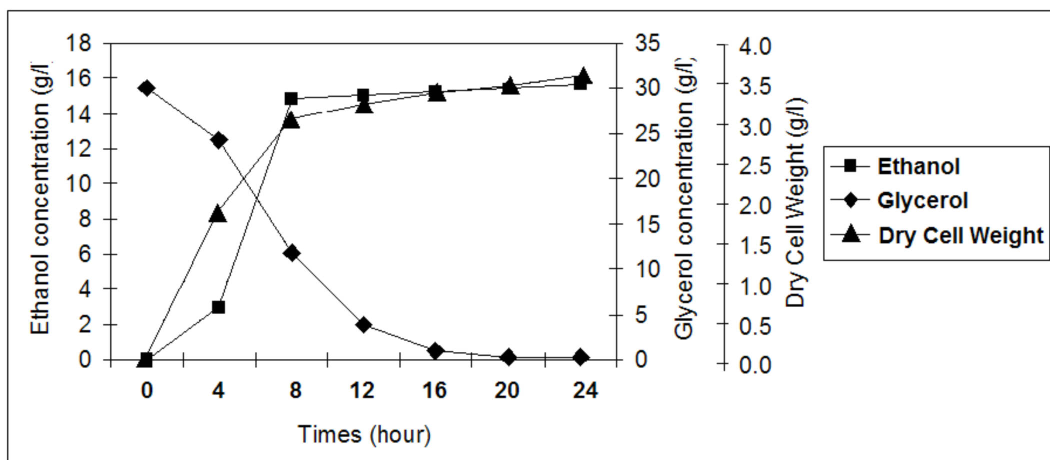


Figure 17. Time course of ethanol production during cultivation of *Enterobacter aerogenes* TISTR 1468 in optimum condition after optimization using one factor at the time and response surface methodology

3. Ethanol production using batch and semi continuous fermentation

3.1 Batch fermentation

Batch culture was conducted in a 3 L fermentor containing 2 L of the optimum medium containing 30 g/l glycerol, 8.73 g/l yeast extract and 0.30 mmol nitrogen as $(NH_4)_2HPO_4$ with 150 rpm stirring speed, the initial pH 7.5 and cultivated at 37^0C, for 8 hour. The inoculum was 10%, at the end of fermentation, all products such as ethanol, 1,3-propanediol, acetic acid, butyric acid and butanol were determined.

E. aerogenes TISTR 1468 grew rapidly to 2.17 g/l after 8 hour cultivation (Table 15). Similarly, previous results found that at this time, *E. aerogenes* TISTR 1468 was in the log phase and reached stationary phase after 12 hour cultivation. After 8 hour the pH decreased from 7.50 to 6.51 and about half glycerol (15.99 g/l) was consumed (the initial value was 30 g/l glycerol) by *E. aerogenes* TISTR 1468. This result was similar to that obtained from this research experiment using 250 ml shake flasks and the results reported by Ciptanto *et al* (2008) that the 40 g/l initial glycerol was consumed in the range of 19-30 g/l. After cultivation, ethanol production, ethanol yield and productivity were achieved at 15.51 g/l, 0.97 g ethanol/g glycerol and 1.94 g/l/h, respectively (Table 15). The by-products such as butanol, acetic acid, butyric acid were achieved in the lower concentration at 0.35 g/l, 0.02 g/l and 0.02 g/l, respectively, and 1,3-propanediol was not detect.

Table 15. Batch fermentation of *Enterobacter aerogenes* TISTR 1468 in the cultivation medium with glycerol as a carbon source cultivated at 37 ^{0}C for 8 h

Batch fermentation	Value
Microbial growth (g/l)	2.17
pH	6.51
Glycerol consumption (g/l)	16.00
Ethanol concentration (g/l)	15.51
Ethanol yield (g ethanol / g glycerol)	0.97
Productivity (g/l/h)	1.94

3.2 Semi continuous fermentation

Under the optimum condition based on response surface methodology, semi continuous fermentation was conducted using the 3 L fermentor, with the 150 rpm stirring speed and initial pH 7.5 at 37^{0}C for 24 h cultivation. Every 8 hour cultivation, 1/3 of the culture broth was replaced by fresh medium two times. Products formation was determined at the end of fermentation such as ethanol, acetate, butyric, butanol and 1,3-propanediol.

The results showed that *E. aerogenes* TISTR 1468 grew rapidly after inoculation. The value of dry cell weight of *E. aerogenes* TISTR 1468 increased from 0.81 g/l at the beginning to 2.19 g/l after 8 hour cultivation. After changing of 1/3 of fresh medium for the first time, the microbial growth reduced to 1.96 g/l and to 2.36 g/l after 16 hour cultivation. The last changing of 1/3 of the medium, the growth was 1.95 g/l and reached until 2.42 g/l after 24 hour cultivation. The results showed that, at 3 L fermentor containing 2 L optimum medium, the maximum microbial growth after 8 hour cultivation and changed for two times were found to be very similar (Fig. 17A).

From Fig. 17B, the initial pH decreased from 7.5 to pH of 6.52 after 8 hour cultivation. When part of the medium was replaced by the fresh medium and the pH increased slightly to pH 7.03 and 7.13 for the first and the second replacement, respectively. After twice changes, the final pH values were almost the same (6.56 and 6.57, respectively).

For glycerol consumption (Fig. 17C), the initial glycerol of 30.13 g/l was consumed 54.30% (16.36 g/l). After replacing with fresh medium, the glycerol consumption was 16.54 g/l and 16.57 g/l from the initial glycerol of 25.80 g/l and 24.85 g/l of the first and the second replacement, respectively. The initial microbial growth of 0.81 g/l was increased to 2.19 g/l after 8 hour cultivation and consumed 16.36 g/l of glycerol. This value was not significant different from those obtained from the first and the second

medium replacement (1.96 g/l and 1.95 g/l dry cell weight, respectively). The glycerol consumptions were 16.54 g/l and 16.57 g/l, respectively.

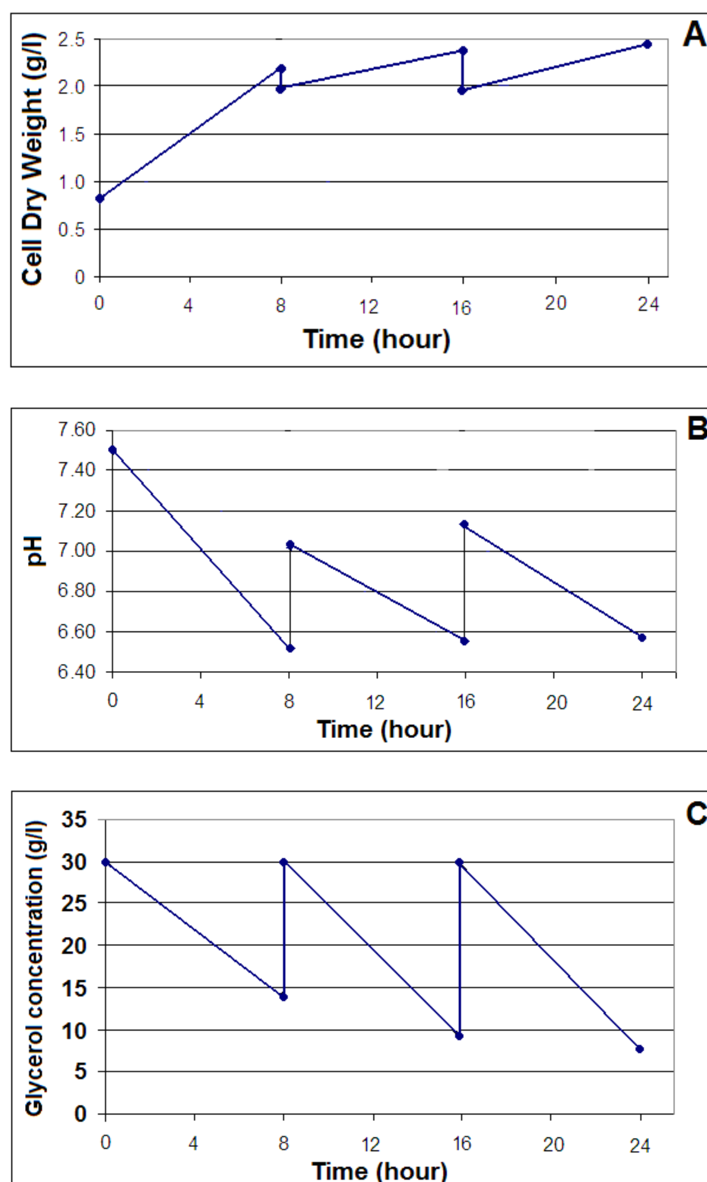


Figure 18. Microbial growth (A), pH (B) and glycerol consumption (C) during cultivation of *Enterobacter aerogenes* TISTR 1468 at 37 ^{0}C for 24 h using semi-continuous fermentation

3.3 Comparison of batch and semi-continuous fermentation

Ethanol production, ethanol yield, and productivity from the batch and the semi-continuous fermentations are given in Table 16. After 8 hour cultivation, the values of each parameter were not significant different. Ethanol production in the batch fermentation was 15.51 g/l whereas in the semi-continuous fermentation, they were 15.04, 15.31 and 15.69

g/l. This meant that *E. aerogenes* TISTR 1468 could consume glycerol and produce similar ethanol concentration using either batch or semi-continuous fermentation. On the other hand, it suggested that the main product was ethanol and the by-products were butanol, butyric acid, acetic acid, and 1,3-propanediol. The concentrations of each product had same amount in both of fermentation process, only 1,3-propanediol was not detected after the end of cultivation.

Table 16. Comparison of product formation in batch and semi-continuous fermentation process

Parameters	Batch	Semi-continuous		
		First	Second	Third
Ethanol concentration (g/l)	15.51	15.04	15.31	15.69
Ethanol yield (g ethanol/g glycerol)	0.97	0.92	0.93	0.95
Productivity (g/l/h)	1.94	1.88	1.91	1.96
Butanol (g/l)	0.35	0.37	0.40	0.45
Acetic acid (g/l)	0.02	0.02	0.03	0.03
Butyric acid (g/l)	0.02	0.02	0.03	0.03
1,3-Propanediol (g/l)	nd	nd	nd	nd

Where: nd = not detected

Comparison on the end products of glycerol fermentation from Enterobacteriaceae, *E. agglomerans* forms a relatively large amount of ethanol. As previously mentioned, this metabolic pathway is one means of maintaining a correct oxido-reduction balance during growth (Barbirato *et al*, 1995). Moreover, other research concluded that glycerol fermentation could produce the end product such as formate in large amount, whereas it was generated together with ethanol and acetate production via acetyl-CoA; it can be cleaved into CO_2 and H_2 by formate hydrogen-lyase (Gottschalk, 1987). Furthermore, the glycerol fermentation using *Klebsiella planticola* DR3 was reported to produce formate and ethanol as main product at equimolar levels nearly at 50 mmol/l and 40 mmol/l, respectively (Jarvis *et al*, 1997). The Batch and Semi-continuous fermentation in the further research was similar with *E. aerogenes* DSM 30053*, K. terriyena* DSM 2687, *K. trevisani* DSM 2688 and *K. planticola* DSM 3060 which formed limited amounts of ethanol as a main product at 36, 51, 66 and 72 mmol/l, but no propanediol. Three genera *Klebsiella* (*K. planticola* IAM 1133, *K. oxytoca* NRCC 3006 and *K. pneumoniae* DSM 202) formed 1,3-propanediol as a main product, ethanol and acetate were found as a by products (Homman *et al*, 1990).

CHAPTER IV
CONCLUSIONS

1. By 'one-factor-at-a-time method', the conditions that gave the maximum ethanol production by *Enterobacter aerogenes* TISTR 1468 were 20 g/l glycerol, 10 g/l yeast extract, 0.4 mmol nitrogen as $(NH_4)_2HPO_4$, with 150 rpm shaking speed and initial pH at 7.5. Trace element solution had no effect on ethanol production. Under the optimum condition, the highest ethanol concentration of 16.31 g/l, ethanol yield of 0.52 g ethanol/g glycerol, and productivity of 1.57 g/l/h were achieved. The results were significantly higher than those of using the original medium which yields 3.00 g ethanol/l, 0.16 g ethanol/g glycerol and 0.38 g/l/h, respectively. These resulted in ethanol concentration, ethanol yield, and productivity was 5.44, 3.25 and 4.13 folds increase, respectively.
2. Glycerol concentration had significant interactive effect on ethanol production from *Enterobacter aerogenes* TISTR 1468. Yeast extract and $(NH_4)_2HPO_4$ had no significant interactive effect. The RSM results indicated the optimum condition as following 30 g/l glycerol, 8.73 g/l yeast extract and 0.30 mmol $(NH_4)_2HPO_4$. The highest ethanol production, ethanol yield and productivity were 16.38 g/l, 0.63 g ethanol/g glycerol and 2.05 g/l/h, respectively.
3. Under the optimum condition from 'one-factor-at-a-time method' and RSM method, batch fermentation and semi-continuous fermentation gave no significant difference in ethanol yield. Ethanol of 15 g/l was the main product and the other by products were butanol, acetic acid and butyric acid that gave the lower concentrations of 0.3-0.4 g/l, 0.02-0.03 g/l and 0.02-0.03 g/l, respectively.

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Appendix

Figure 1-A. Standard curve of cell dry weight

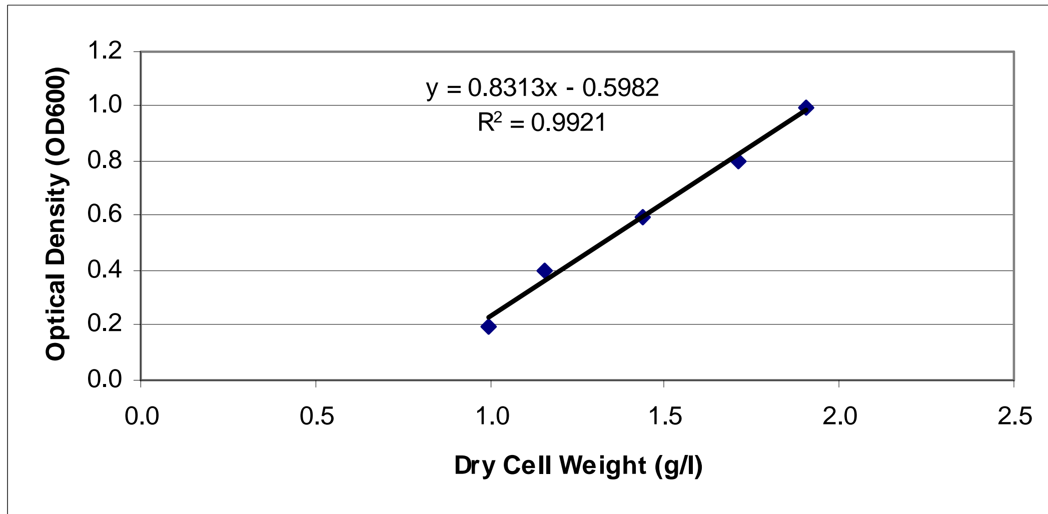


Figure 2-A. Standard curve of glycerol concentration

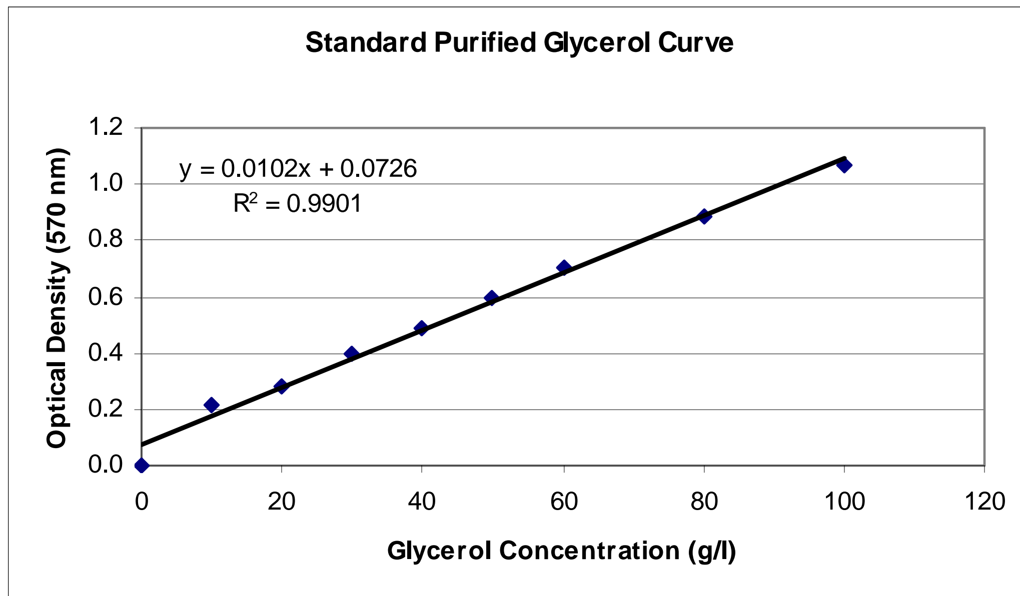
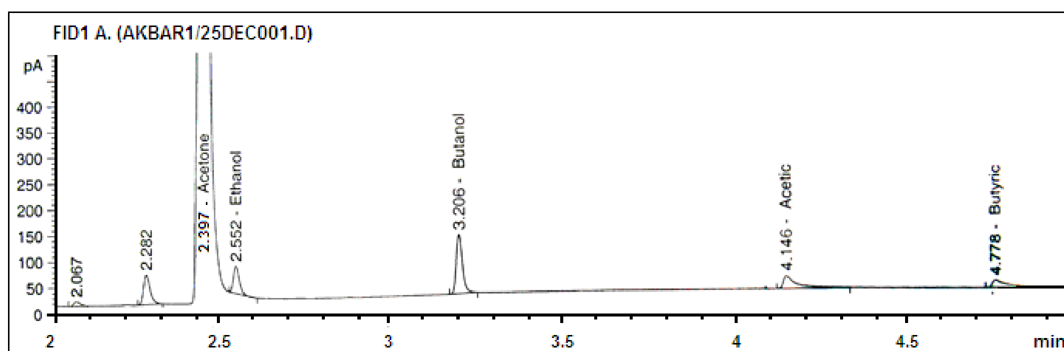


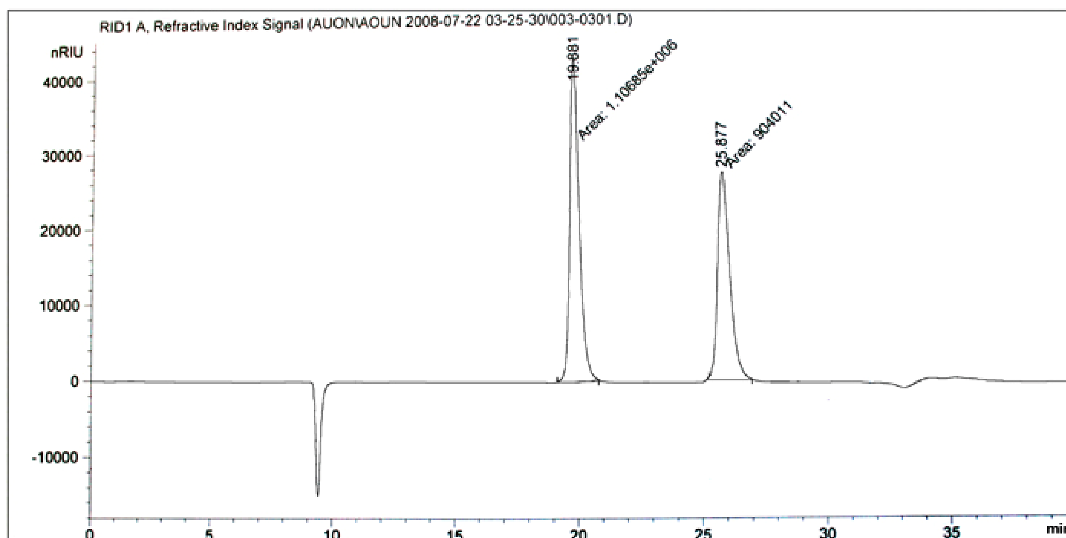
Figure 3-A. Peak of product formation by GC-FID



Signal 1: FID1 A,

Peak #	RetTime [min]	Type		Width [min]	Area [pA*s]	Area %	Name
1	2.067	VV		0.0175	12.02561	2.94795	?
2	2.282	VV		0.0196	78.21111	19.17265	?
3	2.397	BB	+	0.0190	2633.83961	99.52818	Acetone
4	2.552	BP	+	0.0186	62.09254	15.22135	Ethanol
5	3.206	BB	+	0.0180	131.56148	32.25095	Butanol
6	4.146	BB	+	0.0376	67.27885	16.49272	Acetic
7	4.778	BB	+	0.0499	56.76100	13.91438	Butyric
Totals :					3041.47020		

Figure 4-A. Peak of 1,3-propanediol by HPLC



Signal 1: RID1 A, Refractive Index Signal

Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Area %	Name
1	19.881	MM	0.4325	1.10685e6	55.0436	glycerol
2	25.877	MM	0.5550	9.04011e5	44.9564	1,3 propanediol

Totals : 2.01086e6

Figure 5-A. Standard curve of ethanol by GC-FID

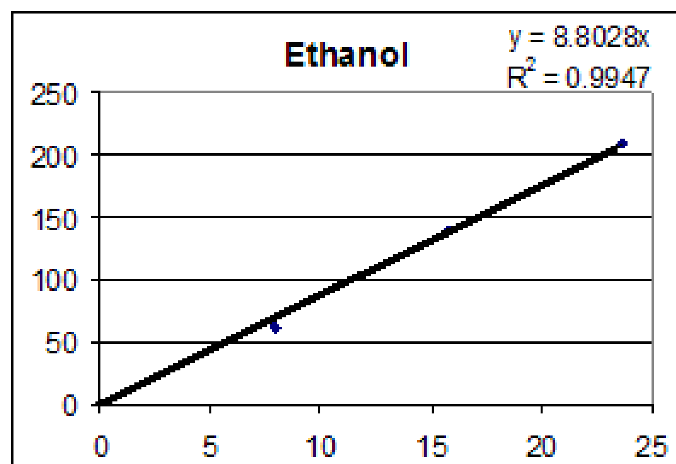


Figure 6-A. Standard curve of butanol by GC-FID

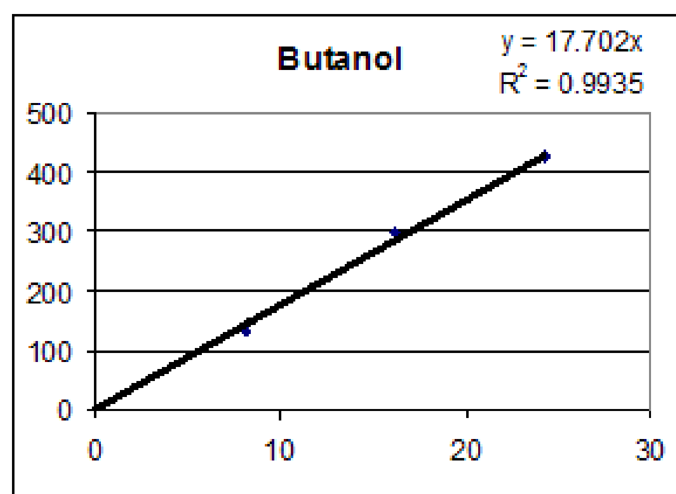


Figure 7-A. Standard curve of butyric acid by GC-FID

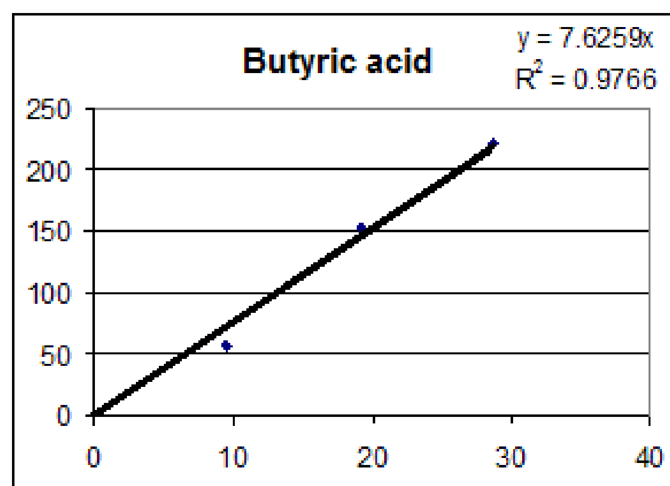


Figure 8-A. Standard curve of acetic acid by GC-FID

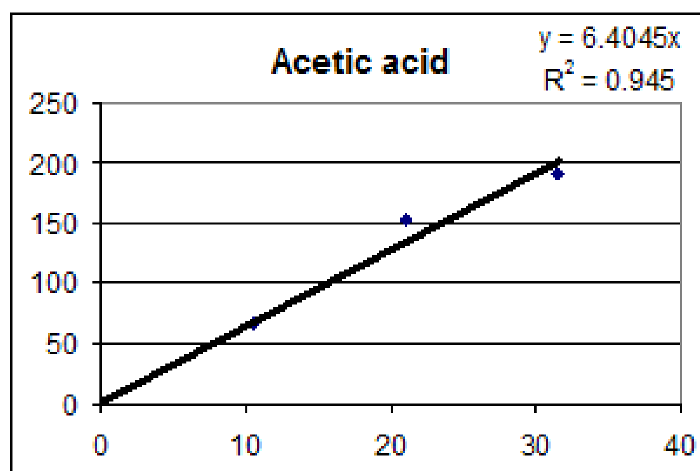


Table 1-B. Univariate analysis of variance by SPSS software for the effect of cultivation time for ethanol production

Ethanol Concentration			Ethanol Yield			Productivity				
Time	Subset		Time	Subset		Time	Subset			
	1	2		1	2		1	2	3	4
0	0.000		0	0.000		0	0.000			
4	0.888	0.888	48	0.138	0.138	48	0.042	0.042		
48	2.027	2.027	36	0.170	0.170	36	0.062	0.062		
20		2.201	24		0.238	24	0.098	0.098	0.098	
36		2.246	20		0.256	20	0.110	0.110	0.110	
8		2.344	16		0.295	16		0.159	0.159	0.159
24		2.352	4		0.319	4			0.217	0.217
16		2.548	8		0.331	12			0.224	0.224
12		2.692	12		0.347	8				0.293

Table 2-B. Univariate analysis of variance by SPSS software for the effect of shaking speed for ethanol production

Ethanol Concentration			Ethanol Yield			Productivity		
SS	Subset		SS	Subset		SS	Subset	
	1	2		1	2		1	2
0 rpm	0.583		0 rpm	0.090		0 rpm	0.035	
120 rpm	1.388		120 rpm	0.166		120 rpm	0.111	
150 rpm		3.795	150 rpm		0.442	150 rpm		0.256

Remark: SS: Shaking speed

Table 3-B. Univariate analysis of variance by SPSS software for the effect of initial glycerol concentration in the medium for ethanol production

Ethanol Concentration				Ethanol Yield				Productivity			
IG	Subset			IG	Subset			IG	Subset		
	1	2	3		1	2	3		1	2	3
0	0.000			0	0.000			0	0.000		
2	0.600	0.600		10		0.193		2	0.060	0.060	
5	1.087	1.087		20		0.228		5	0.099	0.099	
10		1.742		5		0.252		10		0.124	
20			3.430	40		0.300	0.300	20			0.234
40			4.672	2			0.423	40			0.287

Remark: IG: Initial glycerol (g/l)

Table 4-B. Univariate analysis of variance by SPSS software for the effect of organic nitrogen source and concentration in the medium for ethanol production

Ethanol concentration			Ethanol yield			Productivity		
Times (hour)	Subset		Times (hour)	Subset		Times (hour)	Subset	
	1	2		1	2		1	2
8		6.3131	12		0.3242	12		0.6013
12		7.2237	8		0.3675	8		0.7888

Table 5-B. Univariate analysis of variance by SPSS software for the effect of inorganic nitrogen with the addition of organic nitrogen in the medium

Nitrogen Source	Ethanol concentration		
	1	2	3
$(NH_4)_2SO_4$	2.42		
$(NH_4)_2HPO_4$	2.44		
$NH_4C_2H_3O_2$	2.70		
$NH_4C_2H_3O_2$ + yeast extract and tryptone		10.15	
$(NH_4)_2SO_4$ + yeast extract and tryptone		10.51	10.51
$(NH_4)_2HPO_4$ + yeast extract and tryptone		10.62	10.62
$NH_4C_2H_3O_2$ + yeast extract		10.77	10.77
$(NH_4)_2SO_4$ + yeast extract			11.12
$(NH_4)_2HPO_4$ + yeast extract			11.34

Nitrogen Source	Ethanol yield				
	1	2	3	4	5
$(NH_4)_2SO_4$	0.15				
$(NH_4)_2HPO_4$		0.20			
$NH_4C_2H_3O_2$		0.23			
$NH_4C_2H_3O_2$+ yeast extract and tryptone			0.39		
$(NH_4)_2HPO_4$+ yeast extract and tryptone			0.40	0.40	
$(NH_4)_2SO_4$+ yeast extract and tryptone			0.42	0.42	
$NH_4C_2H_3O_2$ + yeast extract				0.43	
$(NH_4)_2SO_4$ + yeast extract				0.43	
$(NH_4)_2HPO_4$ + yeast extract					0.48

Nitrogen Source	Productivity		
	1	2	3
$(NH_4)_2SO_4$	0.30		
(NH4)2HPO4	0.31		
$NH_4C_2H_3O_2$	0.34		
$NH_4C_2H_3O_2$ + yeast extract and tryptone		1.27	
$(NH_4)_2SO_4$ + yeast extract and tryptone		1.31	1.31
$(NH_4)_2HPO_4$ + yeast extract and tryptone		1.33	1.33
$NH_4C_2H_3O_2$ + yeast extract		1.35	1.35
$(NH_4)_2SO_4$ + yeast extract			1.39
$(NH_4)_2HPO_4$ + yeast extract			1.42

Table 6-B. Univariate analysis of variance by SPSS software for the effect of sole inorganic nitrogen concentration with the adding of yeast extract in the medium for ethanol production

Ethanol concentration		Ethanol yield		Productivity	
Nitrogen content as $(NH_4)_2HPO_4$	Subset 1	Nitrogen content as $(NH_4)_2HPO_4$	Subset 1	Nitrogen content as $(NH_4)_2HPO_4$	Subset 1
1.2 mmol	10.9292	0.4 mmol	0.4306	1.2 mmol	1.3661
0.4 mmol	11.1373	1.2 mmol	0.4346	0.4 mmol	1.3922
0.8 mmol	11.2508	0.8 mmol	0.4772	0.8 mmol	1.4064

Table 7-B. Univariate analysis of variance by SPSS software for the effect of mineral salts in the medium for ethanol production

Mineral salts	Ethanol concentration				
	1	2	3	4	5
Control with mineral salts	3.4354				
Control without mineral salts		5.9407			
Without Na_2SeO_3			8.5502		
Without $Na_2MoO_4.2H_2O$				11.8344	
Without trace element					13.3258
Without $NiCl_2$					13.5333
Mineral salts	Ethanol yield				
	1	2	3	4	5
Control with mineral salts	0.1723				
Control without mineral salts		0.2143			
Without Na_2SeO_3			0.2913		
Without $Na_2MoO_4.2H_2O$				0.3441	
Without $NiCl_2$					0.4078
Without trace element					0.4222
Mineral salts	Productivity				
	1	2	3	4	5
Control with mineral salts	0.4296				
Control without mineral salts		0.7414			
Without Na_2SeO_3			1.0686		
Without $Na_2MoO_4.2H_2O$				1.4788	
Without trace element					1.6665
Without $NiCl_2$					1.6913

Table 8-B. Univariate analysis of variance by SPSS software for the effect of initial pH in the medium for ethanol production

Initial pH	Ethanol concentration				
	1	2	3	4	5
5.5	6.3110				
6.0	6.8410	6.8410			
6.5		7.3020			
6.8			8.8837		
7.0			9.2603	9.2603	
7.5					16.3050

Initial pH	Ethanol yield			
	1	2	3	4
6.5	0.2543			
6.0	0.2575			
6.8	0.2627	0.2627		
5.5		0.2892	0.2892	
7.0			0.3145	
7.5				0.5153

Initial pH	Productivity				
	1	2	3	4	5
5.5	0.7889				
6.0	0.8551	0.8551			
6.5		0.9128			
7.5			1.1105		
6.8			1.1575	1.1575	
7.0					2.0380

Table 9-B. Model coefficient estimated by multiples linear regression (significance of regression coefficients)

Factor	Ethanol concentration		Ethanol yield		Productivity	
	Coefficient estimate	Probability	Coefficient estimate	Probability	Coefficient estimate	Probability
Intercept	15.7858	-	0.5806	-	1.9732	-
X_1	-1.4814	0.0009*	-0.2037	< 0.0001*	-0.1852	0.0009*
X_2	0.5621	0.0751	0.0107	0.2502	0.0703	0.0751
X_3	0.2525	0.3794	-0.0123	0.1919	0.0316	0.3794
X_1^2	-3.5282	< 0.0001*	-0.1462	< 0.0001*	-0.4410	< 0.0001*
X_2^2	-2.9047	0.0001*	-0.0324	0.0283*	-0.3631	0.0001*
X_3^2	-2.7819	0.0001*	-0.0072	0.5618	-0.3477	0.0001*
X_1X_2	-0.0930	0.8140	-0.0124	0.3398	-0.0116	0.8140
X_1X_3	-0.1608	0.6854	0.0034	0.7890	-0.0201	0.6854
X_2X_3	-0.3538	0.3836	-0.0175	0.1896	-0.0442	0.3836

* Significant level at 95%

Table 10-B. ANOVA for response surface quadratic model on effect of glycerol, organic nitrogen and inorganic nitrogen concentration on ethanol production

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	155.7060	9	17.3007	29.8559	< 0.0001
Residual	4.0563	7	0.5795		
Lack of Fit	3.2879	3	1.0960	5.7047	0.0629
Pure Error	0.7685	4	0.1921		
Cor Total	159.7630	16			
Root MSE	0.7612		R-Squared	0.9746	
Dep Mean	11.4495		Adj R-Squared	0.9420	
C.V.	6.6486		Pred R-Squared	0.6632	
PRESS	53.8065		Adeq Precision	14.3589	Desire > 4

Table 11-B. ANOVA for response surface quadratic model on effect of glycerol, organic nitrogen and inorganic nitrogen concentration on ethanol yield

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	0.4341	9	0.0482	82.8101	< 0.0001
Residual	0.0041	7	0.0006		
Lack of Fit	0.0004	3	0.0001	0.1290	0.9380
Pure Error	0.0037	4	0.0009		
Cor Total	0.4381	16			
Root MSE	0.0241		R-Squared	0.9907	
Dep Mean	0.4931		Adj R-Squared	0.9787	
C.V.	4.8937		Pred R-Squared	0.9736	
PRESS	0.0116		Adeq Precision	24.3140	Desire > 4

Table 12-B. ANOVA for response surface quadratic model on effect of glycerol, organic nitrogen and inorganic nitrogen concentration on productivity

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Model	2.4329	9	0.2703	29.856	< 0.0001
Residual	0.0634	7	0.0091		
Lack of Fit	0.0514	3	0.0171	5.7047	0.0629
Pure Error	0.0120	4	0.0030		
Cor Total	2.4963	16			
Root MSE	0.0952		R-Squared	0.9746	
Dep Mean	1.4312		Adj R-Squared	0.942	
C.V.	6.6486		Pred R-Squared	0.6632	
PRESS	0.8407		Adeq Precision	14.359	Desire > 4

VITAE

Name Mr. Akbar Ciptanto

Student ID 5011020065

Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Forestry	Mulawarman University	2004

Scholarship Award during Enrolment

Scholarship under the Department of National Education, Republic of Indonesia.

List of Publication and Proceedings

Publication of International Journal

Ciptanto, A., Prasertsan, P. and Hanpongkittikun, A. Optimization for ethanol production from *Enterobacter aerogenes* TISTR 1468 by Response Surface Methodology. (Under correction)

Ciptanto, A., Prasertsan, P., Hanpongkittikun, A and O-Thong, S. Ethanol Production from Purified Glycerol of Biodiesel Plant using Batch and Semi-continuous Fermentation by *Enterobacter aerogenes* TISTR 1468. (Under correction)

Proceedings of International Conference

Ciptanto, A., Prasertsan, P. and Hanpongkittikun, A. Optimization on ethanol production from glycerol using *Enterobacter aerogenes* TISTR 1468**.** The 20th Annual Meeting and International Conference of the Thai Society for Biotechnology. Taksila Hotel, Maha Sarakham, Thailand. October 14-17th, 2008.