Ethanol Production from Biodiesel-Derived Crude Glycerol by *Enterobacter aerogenes* with Tuna Condensate as a Nitrogen Source using Fed-batch Fermentation Process

Juli Novianto Sunarno

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology (International Program)
Prince of Songkla University
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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ABSTRACT

The ethanol production from crude glycerol by *Enterobacter aeroegenes* TISTR 1468 was investigated through the application of simple medium and fed-batch cultivation. Waste-based raw materials, crude glycerol from biodiesel plant and tuna condensate from tuna canning factory, were used to substitute the expensive complex medium. The optimum crude glycerol concentration was 21.43 g/L. Using only crude glycerol and tuna condensate (GT medium) as carbon and nitrogen source for studying the effect of initial C/N ratio (115 - 365 g/g) revealed the insignificant impact on ethanol production in every C/N ratio tested. Meanwhile, GT medium complemented with inorganic salts resulted in 2-folds ethanol production at C/N ratio of 115 g/g. Phosphate buffer were the most influential factors in inorganic salts with 77.6% contribution. However, phosphate buffer could be replaced by keeping the pH constant at 7.0. Under optimum operation (20 g/L glycerol, initial C/N of 115 g/g and the pH of 7.0), the glycerol to ethanol yield was 24% higher than the theoretical conversion of glycerol to ethanol or 12.33 g/L of ethanol due to the presence of carbon source

in tuna condensate. The ethanol selectivity was also 3-fold higher than the complex medium. Hence, cultivation under microaerobic conditions was optimized where redox potential (ORP) sensor was employed as aeration controller to replace the conventional oxygen sensor. The performance using batch cultivation was evaluated on various aeration: continuous aeration at 0.5 vvm; controlled aeration at -350 mV and -400 mV; and no aeration. The 72-h batch fermentation showed that a more reductive environment (lower aeration) increased ethanol and yield, but decreased the productivity. No aeration system achieved the highest ethanol (18.78 g/L; 0.94 g-ethanol/g-glycerol). Meanwhile, a more oxidative environment (higher aeration) fastened the cell growth, but ethanol concentration and yield decreased. The highest specific ethanol production rate was achived at B_{400}. Fed-batch with two-stage aeration strategy (-350 mV and -400 mV) acquired higher ethanol (30.31 g/L; 0.86 g-ethanol/g-glycerol) than with single-stage aeration (25.95 g/L) and batch process (12.33 g/L). This study has revived the potential of crude glycerol biotransformation to ethanol and opened up opportunities for optimization of related microaerobic systems.

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Even a small bridge was built from one small stone arranged piece by piece with ardor and persistence, so is like the completion of this thesis. This thesis would not have been completed without a number of supportive people. Therefore, I want to acknowledge and verbalize my deepest appreciation to all those who had contributed. I would like to express my sincere gratitude to my major advisor, Prof. Dr. Poonsuk Prasertsan for her patience and guidance. Words cannot describe my thankfulness to her for taking such good care of me throughout this journey. Thank you very much to believe in me and to let me be your student. This journey would not be more inspirational and aspiring without your support and inputs. And it will continually motivate and inspire me in the future.

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Like a bridge crossing the wide ocean to unite place to place; removing the barrier, giving a glimmer of hope and new insights. I wish this thesis would also be that small bridge that unifies science to the real-world practice.

Juli Novianto Sunarno

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Paper I

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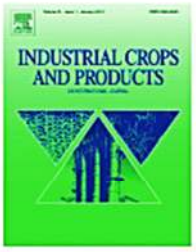

Title: Biodiesel derived crude glycerol and tuna condensate as an alternative low-cost fermentation medium for ethanol production by Enterobacter aerogenes

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1. INTRODUCTION

As the world's petroleum reserves depleting, the concerns in sustaining the supply of chemical commodities have been raising lately. Meanwhile, to continue relying on fossil fuels reserves to fulfill this demand is deemed undesirable along with the adverse of environmental impacts and human safety associated with the oil-drilling activity (Bakke *et al.*, 2013). Bio-refinery offers a more sustainable and ecologically beneficial solution in sustaining the supply of chemicals through the integration of biomass conversion via either thermochemical or biochemical process into fuels, power, heat, and value-added chemicals. In such a manner, to uphold the core value of sustainable bio-refinery, the practice of exploiting raw materials must be done sustainably. Low-value waste and byproducts of industries (e.g. food industry, pulp, paper industry, and 1st-generation biofuel production process) have emerged as the potential candidates. The integration of its kind into bio-refineries is possible technically and economically (Koutinas *et al.*, 2014) which has fastened the innovation of products derived from industrial waste and/or by-product at a significant pace.

1.1 The crude glycerol of the biodiesel industry

World biodiesel production has leap substantially in the last decade with concomitant byproduct generation such as crude glycerol. Approximately 4 million kL of crude glycerol surplus is being anticipated following the growth of biodiesel production to reach 40 million kl in 2026 (OECD-FAO, 2017). Glycerol is a popular chemical commodity in food and pharmaceutical.

However, both applications require glycerol to be free from impurities, which disqualifies crude glycerol derived from biodiesel industry. Meanwhile, the cost of purification is expensive and the quality of purified glycerol has been inconsistent lately due to variation in crude glycerol impurities. With the increase of biodiesel production, a glut of crude glycerol is flooding the market now and selling it has become popularly unprofitable, as the price has dropped significantly. Lack of demand from downstream industries may cause crude glycerol to lose its value and justify its presence as wastewater, forcing the biodiesel companies to spend an extra cost for the safe disposal of it. Hence, the development of crude glycerol bio-refinery in attempt to diversify glycerol into a more valuable product will be highly appreciated.

1.2 Ethanol production from crude glycerol

As the key solution for crude glycerol underutilization, focused has been made to find an effective conversion of crude glycerol into various products such as 1,3-propanediol (Yang *et al.*, 2018), 2,3-butanediol (Almuharef *et al.*, 2019), ethanol (Stepanov *et al.*, 2017), hydrogen (Chookaew *et al.*, 2014), succinic acid (Li *et al.*, 2018), and lactic acid (Murakami *et al.*, 2016). Ethanol is one of the most popular bioenergy commodities nowadays with a total production of 102 million kiloliters in 2017 (RFA, 2017). The growing popularity of ethanol as energy also foster interest in its use as feedstock for bio-based chemicals such as biodiesel (Martinez-Guerra *et al.*, 2014), bio-ethylene (Mohsenzadeh *et al.*, 2017) and ethyl tert-butyl ether (Umar *et al.*, 2013).

Consequently, the consumption of ethanol will be double in the upcoming year to meet the demand for energy and chemicals.

The ongoing bio-based ethanol production mostly relies on precious-yet-limited food crops feedstock such as corn and sugar cane. Recently, the exploration of the feedstock has been focused on the utilization of industrial waste or by-product. Crude glycerol from the biodiesel industry can be converted to ethanol (Stepanov *et al.*, 2017) via microbial fermentation. Ethanol production using crude glycerol resulted in a 40% lower price than the conventional corn-derived ethanol (Yazdani and Gonzalez, 2007). Glycerol as a carbon source for microbial fermentation is more advantageous than glucose or other sugars like sorbitol and xylose. Due to its higher reduction state, it can produce double amounts of reducing agents than xylose or glucose. The available electrons will then facilitate the production of reduced metabolites like ethanol. The application of crude glycerol as feedstock for ethanol is intriguing, considering that other non-food alternatives such as lignocellulose are still much expensive to be actualized. Importantly, the integration of the by-product stream of biodiesel industry with bio-refinery will also improve the sustainability of the biodiesel industry.

The previous study reported optimum ethanol production from crude glycerol (Saisa-ard, 2012). The study showed that *E. aerogenes* TISTR1468 could produce higher ethanol than other bacteria such as *Citrobacter freundii*, *Klebsiella oxytoca*, *Klebsiella terrigena*, and *Klebsiella pneumonia*. *E.*

aerogenes is a Gram-negative, oxidase negative, catalase positive, citrate positive, indole negative, rod-shaped bacterium. The highest ethanol concentration of *E. aerogenes* was reported at 7.24 g/L and further improved to 14.53 g/L after the application of the optimum medium (7.2 g/L yeast extract, 2.1 g/L di-ammonium phosphate and 38.28 g/L crude glycerol). The environment conditions were also optimized at the 3-L reactor. Aeration rate and initial pH arose as the influential parameters where 14 g/L of ethanol was achieved at an initial pH of 8.0 and aeration rate of 0.5 vvm. Three reactor configurations were studied, including two-stage (micro-aerobic and anaerobic) batch, fed-batch and continuous cultivation. The highest ethanol concentration, yield, and productivity were 24.5 g/L, 0.56 g-ethanol/g-glycerol and 1.17 g/L/h using two-stage batch process. The ethanol obtained from this study was still lower than 5% (± 50 g/L), the minimum concentration to achieve efficient downstream process (Vane, 2008). As for that concern, an attempt to increase the ethanol concentration must be conducted.

1.3 The challenge of utilizing crude glycerol for microbial fermentation

In the premises that glycerol acts as carbon and energy source, still, bacteria are found not to consume glycerol completely and were found to be inhibited at high glycerol concentration. Two inhibitive mechanisms had been reported during glycerol assimilation in bacteria including catabolic repression and glycerol kinase inhibition (Lin, 1976). Catabolic repression is the condition where the activity of certain operons is suppressed due to the presence of certain

carbon sources in the medium, most remarkably by glucose. The regulation of catabolic repression in bacteria is controlled by the phosphorylation of Enzyme IIA^{Glc}, which involves during phosphorylation transfer system (PTS) (Yao *et al.*, 2016). The unphosphorylated form of Enzyme IIA^{Glc} is thought to inhibit adenylate cyclase and to stimulate it when phosphorylated. Since adenylate cyclase controls the level of intracellular cAMP, catabolite activator protein or CAP (cAMP receptor protein) will also be influenced by PTS activity of the cells (Eppler *et al.*, 2002). The un-phosphorylation of Enzyme IIA^{Glc} occurs when glucose is present in the substrate. Accordingly, a cAMP/CAP that is associated with controlling the transcription process of individually inducible catabolite-sensitive operons such as glycerol operon will inhibit the expression of glycerol operon in the presence of glucose and then reduce the glycerol uptake rate (Yao *et al.*, 2016). The glucose-free medium will then desirable for bacterial fermentation of glycerol.

For the case of feedback inhibition (Figure 1), glycerol kinase controls the activity of almost glycerol operon by producing metabolite intermediate, which is glycerol-3-phosphate. The activity of glycerol kinase, however, is controlled by fructose-1,6-diphosphate and enzyme IIA^{Glc} that the binding of both into glycerol kinase reduces its affinity toward glycerol (Yu *et al.*, 2003). This phenomenon was observed to occur only during over-phosphorylation of glycerol (Lin, 1976). Therefore, the glycerol concentration

must be maintained in medium to maintain fructose-1,6-diphosphate concentration as low as possible to avoid this inhibition to occur.

Since glycerol could inhibit the growth of *E. aerogenes* (Saisa-ard *et al.*, 2012), the challenge to improve ethanol concentration lies on the threshold concentration of glycerol at which cells remain viable and to maintain this concentration throughout the cultivation. The study of effect initial crude glycerol concentration showed that the concentration ± 40 g/L demonstrated the highest cell growth rate (0.43 g/L/h) and glycerol uptake rate (2.22 g/L/h), whereas the highest ethanol production rate (1 g/L/h) achieved at 50 g/L and higher initial crude glycerol concentration further reduced the cell's performance (Saisa-ard, 2012). Due to the different nature of glycerol tolerance, each of *E. aerogenes* strain grew optimally at different glycerol concentration such as Chanthooma *et al.* (2016) and Ito *et al.* (2005) produced ± 9 g/l ethanol by using 25 g/l glycerol.

1.4 Fed-batch cultivation to counter glycerol inhibition

The earlier section has mentioned that glycerol is inhibitive toward cell performance. The reported optimum glycerol concentration for *E. aerogenes* TISTR1468 (± 40 g/L) could only produce 24.5 g/L ethanol. Improving ethanol production to at least 50 g/l, for instance, will require 100 g/l glycerol where this concentration has been above the tolerance threshold of *E. aerogenes* TISTR1468 toward glycerol. This limitation makes simple batch cultivation no longer relevant for further study. Hence, the fed-batch cultivation is developed.

Fed-batch cultivation is selected mostly to handle the process with substrate inhibition and high viscosity substrate (Henry C and Hwa Sung, 2013). The fed-batch operation is a semi-batch process which one or more fresh substrates are fed during the course of cultivation. By regulating the feed rates and concentration, it is possible to regulate glycerol concentration in the reactor to maximize the rate of ethanol production.

Saiasa-ard (2012) was successfully increased glycerol consumption up to 71.15 g/L throughout 48 h of cultivation, but the maximum ethanol concentration, yield, and productivity retarded at 19.21 g/L, 0.27 g-ethanol/g-glycerol, and 0.46 g/L/h, lower than the result of batch cultivation. The study indicated that the cell concentration dropped when the cultivation approaching the endpoint (72 h) followed by the decreased in glycerol utilization and ethanol production. Unfortunately, the study provided no information concerning the cause of this reduced performance.

1.5 Operational parameters affecting *E. aerogenes* performance

The earlier report studied that the substrate pH was a significant parameter on the production of ethanol from crude glycerol using *E. aerogenes* (Lee *et al.*, 2012; Saisa-ard, 2012). Both studies showed that very acidic (pH < 5) and very alkaline (pH < 8) environment had an adverse impact on ethanol production. *E. aerogenes* also produces organic acid as secondary metabolites such as acetic acid, succinic acid, malic acid, and citric acid, which could affect the pH of the medium during cultivation (Lee *et al.*, 2012).

The previous report also mentioned the importance of aeration strategy for ethanol production using *E. aerogenes* (Saisa-ard *et al.*, 2012; Lee *et al.*, 2012). The study revealed that micro-aerobic environment (0.5 vvm) gave the highest ethanol production (11.66 g/L), productivity (0.73 g/L/h) and yield (0.58 g/L). Meanwhile, during anaerobic fermentation, ethanol production, yield, and productivity reduced to 4.95 g/L, 0.41 g-ethanol/g-glycerol and 0.25 g/L/h. During the progression of fed-batch fermentation under micro-aerobic condition, the addition of substrate into the reactor increases the volume of the reactor and changes the hydrodynamics of the system (Zeng *et al.*, 1994). Controlling oxygen supply to maintain identical micro-aerobic condition at the initial stage of cultivation must be considered or else the oxygen will be depleted (fermentation approaching less efficient anaerobic cultivation). The indication such as lower ethanol production and substrate consumption rate at the endpoint of fed-batch cultivation may be associated with this anaerobic fermentation. Therefore, re-optimizing the micro-aerobic condition with respect to the dissolved oxygen concentration and maintain this concentration throughout the fermentation can be an important parameter on the production of ethanol.

1.6 Redox potential control for micro-aerobic fermentation

Redox potential or oxidation-reduction potential (ORP) reflects net electron transfer and redox balance involved in intracellular metabolism (Liu *et al.*, 2013). Oxidation-reduction reactions occur simultaneously within cells no matter cultures are growing in an aerobic, microaerobic or anaerobic

environment, making ORP detected in the extracellular environment an indicator of the net intracellular metabolism. Compared to pH value that provides information about protons, ORP represents activities of electrons. Therefore, ORP is more sensitive to delicate changes in intracellular metabolism (Liu *et al.*, 2013). When the oxidizing power is greater than the reducing counterpart, the redox potential rises and when reducing power is more dominant than oxidizing power, the redox potential declines. In this case, the dissolved oxygen introduced by sterile air is the major contributor to the oxidizing power; whereas, the reducing power is mainly attributed to glycolysis and cell propagation during ethanol fermentation. When the fermentation redox potential (due to active cell growth) is lower than the set point, external supplementation of oxidizing power is required; as such, the redox potential during fermentation can be controlled. When bacteria cannot generate enough reducing power to counteract the oxidizing power, the redox potential control is stopped. Hence, maintaining and prolonging the capability of the cell to generate reducing power is a prerequisite for operating a redox potential-controlled fermentation.

The range of optimum ORP for ethanol production was strain specific. Wong *et al.* (2014) demonstrated that controlling ORP of ethanol production by *E. coli* using fed-batch cultivation at -390 to -350 mV exhibited higher ethanol production compared with the cultivation without ORP. The ethanol production, yield, and productivity were 46.5 g/l, 0.44 g-ethanol/g-glycerol and 1.03 g/l/h. Liu *et al.* (2016) observed ORP level between -200 and

-50 mV as a microaerobic condition during very-high-gravity glucose fermentation to ethanol using *Saccharomyces cerevisiae*. Although ORP theory and measurement have been well established with relatively simple chemical reaction systems, investigation on ORP's impact on intracellular metabolism, and applications of ORP control in bioprocess engineering are still very limited.

1.7 Tuna condensate from seafood processing industry as a source of nutrients

Most of the studies on microbial fermentation of glycerol have used media containing complex growth supplements to achieve high product concentration, such as yeast extract, peptone, or tryptone (Adnan *et al.*, 2014; Choi *et al.*, 2011; Lee *et al.*, 2017; Saisaard *et al.*, 2011). The cell performance during glycerol fermentation to ethanol largely depends on media composition. Saisa-ard (2012) simplified the medium used by Ito *et al.* (2005) and achieved an ethanol production of 20.7 g/l. The medium composed only crude glycerol (38.28 g/l), yeast extract (7.2 g/l), and di-ammonium phosphate (2.1 g/l). However, Chanthooma *et al.* (2016) that used medium from Ito *et al.* (2005) without yeast extract and tryptone achieved lower ethanol concentration than Saisa-ard (2012). Ito *et al.* (2005) accentuated the importance of organic nitrogen such as yeast extract and tryptone including cell growth acceleration, hydrogen, and ethanol production enhancement. However, the cost of these complex mediums is impractical for industrial scale application.

The exploration for low-cost supplement sources has raised a wide range of waste-based raw materials with equivalent nutritional value. Tuna condensate, the discharged from steam cooker after tuna precooking, is a byproduct from the tuna processing factories with high nitrogen content (Prasertsan *et al.*, 1997). The waste is considered underutilized and the volume is quite significant considering that Thailand is the biggest canned tuna importer worldwide. The application of tuna condensate as a growth supplement during microbial fermentation has barely been popular though it is rich in nutrition and cheap. The application of tuna condensate currently ranges from food products and animal feeds (Gamarro, 2013). However, due to stricter environmental regulation, the tuna canning industries have to provide a new approach for handling their waste. Utilizing this waste for the production of a more valuable product could be one of the potential solutions. At present, some seafood factories use tuna condensate as feedstock for the production of fish extract as a flavoring agent but limited to the Japanese market only (Prasertsan, 2015). The application of tuna condensate is considered limited and its volume remains significant considering that Thailand is the world's largest canned tuna exporter.

Tuna condensate is associated with high COD content (Prasertsan *et al.*, 1997). Within the corridor to open various opportunities for waste management, tuna condensate utilization for microbial fermentation were investigated. Prasertsan *et al.* (1993) reported the highest *Rhodocyclus gelatinosus* growth when using tuna condensate as substrate and achieved 86%

COD removal accompanied by 5.6 g/l cells concentration (containing 50% protein). The increase of biomass concentration was reported four-folds by repeated addition of the fresh tuna condensate for every 40 h (Prasertsan *et al.*, 1997). Tuna condensate also found its application for the production of Gamma-Aminobutyric acid (GABA), an important dietary supplement, and the GABA productivity could reach 135.42 mg/l/h when using the enriched natural glutamic acid tuna condensate containing 1% glucose as a medium, including both encapsulated *Candida rogusa* 8YB and *Lactobacillus futsaii* CS3 as starter cultures (Sanchart *et al.*, 2018). The effectiveness of tuna condensate as culture medium lies within its composition that rich in organic and nitrogenous compound (Table 9). In addition, it contains various inorganic salts such as Cl-, Mg2+, Fe3+, and HPO-4 that plays an important role as a buffer, cofactor, or phosphorous sources. This potential has driven the motivation to further investigate the effect of tuna condensate supplementation on the production of ethanol from crude glycerol.

2. Objectives

The objectives of this study were:

1. To develop a minimal medium for ethanol production of *E. aerogenes* TISTR1468 using crude glycerol and tuna condensate.
    a. To optimize crude glycerol concentration.
    b. To optimize tuna condensate concentration by employing initial C/N ratio as a parameter.
    c. To screen important inorganic salts constituents.
2. To study the effect of various aeration control for batch ethanol production under microaerobic condition using ORP controller.
3. To develop an effective aeration strategy for fed-batch cultivation during ethanol production.

3. Results and Discussion

3.1 Biodiesel-derived crude glycerol and tuna condensate as an alternative low-cost fermentation medium for ethanol production by *Enterobacter aerogenes*

Crude glycerol and tuna condensate are wastes from different industries; non-food (biodiesel) and food (canned tuna) plants, respectively, hence varied in composition. Crude glycerol had alkaline pH (9.57) and density of 1055 g/L. It contained about 70 % of the total mass (259,460 mg/L glycerol) with the residual methanol at 28% (102,953 mg/L). The crude glycerol had very high COD (1,175,000 mg/L) but very low total nitrogen (TKN) (1120 mg/L) without ammonium-nitrogen (NH4-N) and giving the C/N ratio of 1049:1. Other chemicals were phosphorous (129 mg/L), iron (15.4 g/L), and magnesium (574 mg/L). The tuna condensate contained lower COD (79,000 mg/L) but higher total nitrogen (6527 mg/L) than those of the crude glycerol with 400 mg/L of ammonium-nitrogen. The C/N ratio was 12:1 which indicated the more suitable for being substrates for microbial growth. Unlike crude glycerol, it had an acidic pH (5.67) and contained higher phosphorous (665 mg/L) but lower magnesium (264 mg/L) and iron (2.4 mg/L).

This study aims to simplify the medium for ethanol production using crude glycerol and tuna condensate. Two sequential experiments were performed to optimize the composition of crude glycerol and tuna condensate (in

terms of initial C/N ratio). Then, a final experiment was carried out to re-evaluate the importance of inorganic salts composition on ethanol production based on the complex medium developed by Ito *et al.* (2005).

Optimization of crude glycerol concentration was evaluated in the range of 9.32 g/L to 59.77 g/L (about 10 - 60 g/L) using the complex medium developed by Ito *et al.* (2005). Three metabolites were produced with ethanol being the major compounds (2.04-6.72 g/L) followed by acetic acid (1.01-3.09 g/L) and 2,3-butanediol (0.63-0.74 g/L). The optimum concentration of the crude glycerol was 21.43 g/L, giving the highest ethanol concentration of 6.72 ± 0.59 g/L. The ethanol yield (0.51 g-ethanol/g-glycerol) and selectivity (1.42 g-ethanol/g-byproducts) observed at this concentration were also the highest amongst the other counterparts. This optimum concentration of crude glycerol was similar to those previously reported (20 g/L glycerol and ~6 g/L ethanol, respectively) (Chantoom *et al.*, 2014; Lee *et al.*, 2012).

Increasing initial crude glycerol concentration caused an inhibition in cell growth as the final cell concentration gradually decreased. Glycerol consumption and ethanol production were also adversely affected. A complete crude glycerol consumption and fast glycerol uptake rate were occurred at 10 g/L and decreased thereafter. The ethanol yield dropped below the theoretical value after 31.44 g/L glycerol. Reduced performance at increasing glycerol concentration has been associated with feedback inhibition of glycerol kinase which in charges in glycerol dissimilation (Zwaig and Lin, 1966). The inhibition

of glycerol kinase is characterized by slow glycerol uptake rate and a lower tendency toward cell production as shown in the result of this study. Fed-batch fermentation used to reduce the impact of substrate inhibition could be an alternative for the conventional batch fermentation (Oh *et al.*, 2011).

Furthermore, crude glycerol impurities such as inorganic salts (NaCl and/or KCl) (37,250 mg/L chloride) and methanol (102,953 mg/L) might also act as an inhibitor for the cell performance (Venkataramanan *et al.*, 2012). The inorganic salts were reported to inhibit *E. aerogenes* growth at the concentration higher than 1% wt. (±10 g/L) (Lee *et al.*, 2012). However, after the dilution to the expected concentration, the initial salts concentration dropped in the range of 1.34 – 8.58 g/L, which was below the threshold concentration reported. So hypothetically, inorganic salts inhibition might not be significant during the present study.

Methanol is one of the inhibitors in the crude glycerol. Our preliminary studies indicated that methanol concentration from 1% to 3% (w/v) decreased the ethanol production as much as 7% to 20%, respectively (Sunarno and Prasertsan, 2018). Methanol higher than 2.3 g/L was inhibitive for *E. aerogenes* by reducing substrate utilization (Sarma *et al.*, 2012). The dilution of crude glycerol during medium preparation was expected to overcome this problem. However, even the CG medium at 21.43 g/L glycerol (optimum concentration) still contained methanol higher than the reported threshold (3.46 g/L). Consequently, the total glycerol consumption decreased at increasing

glycerol concentration following the increase of methanol residue. Plausibly, methanol became inhibitive when glycerol concentration increased. Though longer chain alcohol (such as ethanol, which is the product in this study) could vent a more dangerous impact on the cell, the fact that methanol encounters the cell earlier at the beginning of the fermentation allowing it to control the progression of the fermentation (Patra *et al.*, 2006). Sarma *et al.* (2013) stated that if the effect of methanol was unavoidable by dissolving it, then purification of glycerol by heating at a temperature of 65 ºC was permissible. The removal of methanol may improve the performance of fermentation. However, in certain cases, the cell performance in pure glycerol and crude glycerol medium did not show a significant difference, it could even produce higher ethanol (Chantoom *et al.*, 2014). This happens if the inhibition due to glycerol is more dominant than the inhibitor. So that even though the impurities have been eliminated, no improvement will be visible. Thus, the purification of crude glycerol must be decided by knowing information about the critical concentration of each inhibitor. However, since crude glycerol purification is cost intensive, the purification of crude glycerol will not be performed in this study to keep the production cost as minimum as possible. Alternatively, the potential of using crude glycerol without pretreatment was further investigated by optimizing the nutrient composition only.

Tuna condensate replaced the rest of complex medium composition as an alternative nutrient leaving only crude glycerol and tuna

condensate. Due to heterogeneous nature of tuna condensate composition, the experiment was focused solely on its total nitrogen (TKN) and total organic carbon (TOC) (calculated from COD). The initial carbon to nitrogen (C/N) ratio was chosen as a control variable. The initial crude glycerol concentration was maintained at its optimum level ~20 g/L (the actual concentration was 23.33 ± 0.48 g/L) where the tuna condensate was varied to comply the initial C/N ratio of 115, 200, 257 and 365 (g/g).

During the fermentation with GT medium, ethanol was the major product alongside with two others byproduct and the highest ethanol concentration was observed at the initial C/N ratio of 257 g/g. The overall difference between the control and GT medium was not significant ($p>0.05$) as the control experiment was statistically classified into the same group by Tuckey's pairwise test. This finding suggested that the complex composition (organic nitrogen, inorganic salts, and trace elements) of the complex medium could be replaced by GT medium without causing a significant impact on the ethanol production.

Furthermore, cell growth was also affected by the initial C/N ratio value. The highest cell concentration of 1.65 ± 0.02 g/L was obtained at the C/N ratio of 115 g/g and decreased as the ratio increase. Nutrient sufficiency is vital for cell growth. As the cell grew prosperously with the decrease of C/N ratio (increasing tuna condensate concentration), evidentially, tuna condensate might be rich in growth promoting substances. This finding is also observed by

Prasertsan *et al.* (1997) on the growth of *Rhodocyclus gelatinosus* where repeated addition of fresh tuna condensate increased the cell concentration up to four-folds. Consequently, when the C/N ratio increased (decreasing tuna condensate concentration), the cell concentration dropped and the assimilated carbon was moved toward the formation of the metabolite such as 2,3-butanediol (2,3-BDO; $C_4H_{10}O_2$). The advent of 2,3-BDO was eminent at the C/N ratio of 200– 365 where up to 5.71 ± 0.15 g/L of 2,3-BDO was acquired along with the decrease in cell concentration.

Since ethanol is a growth-associated product, enhancing cell growth is important. Our result showed that the complex medium (control) accumulated 18% higher cell concentration than GT medium (C/N ratio of 115 g/g) suggesting that the former was still a better medium for growth. Possibly, some components in the complex medium such as inorganic salts and trace elements are complementary, not substitutive to GT medium. Hence, to elaborate the function of inorganic salts and trace elements further, the fermentation on the GT medium supplemented with both components was performed.

The addition of inorganic salts and trace elements could increase the production of ethanol (1.2-2.1 folds) and acetic acid (1.0-1.5 folds) but reduced the 2,3-BDO (to only about 1/3). Therefore, the ethanol production increased by about 2-fold as compared with the control and GT medium only. The rise of ethanol production was possible following the reduction of 2,3-BDO production. Considering it is a 4-carbon compound, the advent of 2,3-BDO was

actually a loss of carbon flux toward ethanol. Reducing 2,3-BDO synthesis is, therefore, important as for each molecule of 2,3-BDO not produced, 2 ethanol molecules will be produced. Besides, during the biosynthesis of 2,3-BDO, the cell used up 1 out of 2 NADH molecules obtained during the catabolism of glycerol while at the same time, ethanol synthesis required 2 NADH molecules (Um *et al.*, 2017). By the addition of inorganic salts and trace elements into the GT medium, the carbon predisposition toward ethanol pathway (the main product) increased and resulted in lower byproduct formation than using sole GT medium (1.5 to 3 times higher ethanol selectivity).

The effect of the initial C/N ratio on cell growth with the addition of inorganic salts exhibited the same predisposition. The lower the C/N ratio was, the higher the cell concentration. The highest cell concentration of 2.03 ± 0.02 g/L was obtained at the C/N ratio of 115 g/g and this result was comparable to the control (complex medium). It proved that one or more components of inorganic salts and trace elements were growth promoting substances. Furthermore, glycerol consumption improved from 51-78% (control) to 79% - 100%, suggesting that the combination of GT medium and inorganic salts-trace elements was improving the cell performance. From this result, it is justifiable to conclude that the correlation of inorganic salts and tuna condensate was more toward complementarity than replacing each other as what might initially be perceived.

The addition of inorganic salts and trace elements to the GT medium had improved the performance of *E. aerogenes* TISTR1468. In this way, it is important to select only the components with the most contribution instead of using altogether since the purpose of the study was to simplify the medium. Plackett-Burman experimental design accompanied by an analysis of variance (ANOVA) was performed to screen the significant factor upon ethanol production (Ps).

K_2HPO_4 was the most significant constituents with 56.9% contribution for ethanol production while KH_2PO_4 came second with 20.6% contribution. Both combinations made up the phosphate buffer with a total contribution of 77.5%. The confirmation experiment was performed using GT medium at the C/N ratio 115 g/g with the addition of phosphate buffer. Using only phosphate buffer, the difference in ethanol production was insignificant compared with complete inorganic salts-trace element addition and both were classified into one group (A) by Tuckey's test. Complete substrate consumption was also achieved. In fermentation, phosphate buffer is used to maintain the medium pH from fluctuation or substantial drop due to acid generation from microbial activity. Being the most significant factor has emphasized the fact that *E. aerogenes* is a pH-sensitive strain and ethanol production may occur in merely narrow pH range. $(NH_4)_2SO_4$ and $CaCl_2$ were also significant variables with 9.843% and 7.961% contribution, respectively. Meanwhile, Na_2MoO_4 and nicotinic acid were insignificant with positive effects. However, to maintain the

simplicity of the medium, these four later constituents were ignored because their contribution was far below the phosphate buffer.

As phosphate buffer is barely cost-effective for the industry, the GT medium without phosphate buffer fermented under pH controlling scheme was performed. The pH of the medium was controlled at ~ 7 by the addition of 3 N NaOH for 72 h of fermentation (Lee *et al.*, 2012). The performance of the cell was maintained after the pH-controlling scheme. The difference in ethanol production was insignificant as it also belongs to group A according to the result of Tuckey's pairwise. This result confirmed that the phosphate buffer could be eliminated and alternatively, the pH would be controlled by the addition of sodium hydroxide only. At particular pH of 7.0, the cell was able to convert most of the carbon available into ethanol either glycerol or another carbon. This resulted in higher ethanol yield (0.62 g-ethanol/g-glycerol) than other treatment done before (0.37 -0.55 g-ethanol/g-glycerol), and even 24% higher than the theoretical yield of glycerol to ethanol. Indicated by high COD content, tuna condensate and crude glycerol may contain another carbon besides glycerol. This extra carbon could be assimilated by *E. aerogenes* to produce ethanol or to maintain cell viability resulting in a yield higher than the theoretical yield (Nwachukwu *et al.*, 2012). The increase in yield during pH control indicated that pH control was very effective to increase glycerol consumption and ethanol production. Furthermore, the cell concentration was increased by 20%. Cell at controlled pH can effectively use their energy for cell growth. A wide pH

gradient between inside and outside the cell requires more ATP for the extrusion or intrusion of a proton to maintain pH homeostasis (Zeng *et al.*, 1990). This causes the depletion on the amount of ATP for cell growth. Controlling the pH near neutral zone hereby helps the cell to conserve more energy so it can be allocated for growth as well as ethanol since it is the growth-associated product (Zeng *et al.*, 1990). This could explain why the growth was better under pH controlling scheme. Furthermore, it was also able to decrease the formation of acetic acid and 2,3-BDO as much as 88.9% and 100%, respectively. It might be because the two byproducts were not produced predominantly at pH 7. For example, the optimal production of 2,3-BDO was reported in the pH range of 5.5 – 6.5, above which lower production rate was observed (Um *et al.*, 2017; Zeng *et al.*, 1990). This led to an increase in ethanol selectivity up to 2-folds higher than using phosphate buffer (at the initial C/N ratio of 115 g/g).

The sensitivity analysis showed that crude glycerol had the most significant impact on the ethanol production in compare to the effect of C/N ratio itself. The effect of C/N ratio became significant after the addition of inorganic salts constituent, which lead to the discovery that buffer phosphate was the main contributor. In fact, the effect of C/N ratio at controlled pH showed that it was better than the result of phosphate buffer supplementation. The performance of cell at constant pH showed how important pH is to the metabolism pathway of *E. aerogenes*, signifying the importance of pH control scheme. Initially, phosphate buffer had failed to maintain the pH in all previous experiments that

could be seen from the final pH of fermentation which all were below 7.0. This infers that buffer must be supplemented excessively into the medium, which could result in a more expensive production cost (Ito *et al.*, 2005). The pH-controlling scheme is alternatively more cost-friendly than phosphate buffer for industrial application. The sensitivity analysis showed that the pH controlling was more effective than just adding phosphate buffer and thereby, the fermentation medium can be further simplified consisting only crude glycerol and tuna condensate. The simple medium in the previous study produced ethanol as much as 4.96 g/L with a yield of 0.415 g ethanol/g-glycerol (Lee *et al.*, 2017). Nevertheless, this medium still used peptone and citrate buffer. The results of this study produced 2.5 times more ethanol (12.33 g/L) with higher yields (0.62 g-ethanol/g-glycerol). The yield achieved with this simple medium was also higher than the mutant strain (0.56 g-ethanol/g-glycerol) (Nwachukwu *et al.*, 2013). However, mutant strains were able to grow at higher glycerol concentrations (50 g/L), resulting in a high ethanol concentration (25.4 g/L) as well. The ethanol production from this study was also higher than that from *E. aerogenes* ACTC 29007 produced under the optimized medium with crude glycerol as a carbon source (5.29 g/L) during continuous fermentation (Lee *et al.*, 2017).

Despite having yield higher than its theoritical value, the global ethanol yield from crude glycerol used in this study was compartively lower than several 2nd generation carbon sources. The global ethanol yield per mass of crude

glycerol used in this study was approximately equal to 15.24 g-ethanol/ 100 g-crude glycerol (0.62 g-ethanol/g-glycerol). Meanwhile, the highest ethanol yied for sugarcane bagasse was reported at 34 g-ethanol/ 100 g-sugarcane baggase using *Saccharomyces cerevisae* PE-2 (de Araujo Guilherme *et al*., 2019). The yield is comparable with the performance of *Scheffersomyces stipitis* NRRL-Y7124 at 15 g-ethanol/ 100 g-sugarcane baggase (Hilares *et al*., 2017), but higher than the result from *S. cerevisae* using sorghum stem at 7.0 g-ethanol/100 g-sorghum stem (Yu et al., 2010). Process configuration must be appropriately selected to increase substrate utilization especially for the wild strain that is less tolerant at high glycerol concentration. Fed-batch fermentation may be suitable for further investigation.

3.2 Improve biotransformation of crude glycerol to ethanol of *E. aerogenes* by two-stage redox potential fed-batch process under microaerobic environment

In the present study, various aeration methods during microaerobic fermentation were carried out to compare its effectiveness in increasing ethanol productivity, i.e. continuous aeration at 0.5 vvm (B_C); aeration by ORP controller at -350 mV (B_{350}) and -400 mV (B_{400}); and without aeration (B_0). Fermentation was carried out in a 5-L CSTR with a working volume of 3 L integrated with DO sensor and ORP controller connected to the aerator. In controlled aeration, aerator was activated only when the ORP value fell below the reference point (-

350 and -400 mV). Meanwhile, during the non-aerated condition, only oxygen in the 2-L reactor headspace was consumed with the help of 60-rpm agitation.

The profile of redox potential normally shows in the form of a bathtub curve with three zones namely declining, basin, and uprising (Lin *et al.*, 2010). All fermentations were initiated at the ORP value of -190 to -197 mV or equivalent to 3.5% DO concentration. The oxygen concentration fell close by to 0% defining two distinctive phases: an aerobic phase characterized by decreasing DO concentration and followed by microaerobic phase indicated as zero dissolved oxygen concentration. The microaerobic phase was achieved at different times depending on the aeration strategy: B_0 (6 h), B_{350} and B_{400} (9 h) and the longest at B_C (12 hours).

In this study, the full bathtub curves observed only when continuous aeration (B_C) took place. The basin zone was observed in all aeration strategy approximately after 12 h fermentation. The average range of ORP achieved by B_C was -323±8.1 mV and dropped to -448±14 mV during non-aeration counterparts. Uncontrolled ORP system such as B_C and B_0 tends to have basin zone with fluctuating ORP value, which also found by Liu *et al.* (2015). However, controlling the input of aeration by ORP could result in a steadier basin zone as seen in B_{350} and B_{400}. The ORP profile was actually the net redox in the extracellular environment. If cell activity is high, the ORP value tends to be more negative due to the accumulation of reducing equivalents and the decrease in ORP could be countered by adding oxygen through aeration. Fluctuation is a

sign of viable cell activity; thus, the length of the basin zone determines the duration of ethanol production for which the longer it is, the better the fermentation will be (Liu *et al.*, 2011). The termination of the basin zone could be influenced by many factors, such as substrate depletion or product inhibition as both could disturb cell viability (Thani *et al.*, 2016). In this study, B_C had the shortest basin zone. A shorter basin zone in B_C was possibly due to early substrate depletion and/or acetic acid accumulation, but not because of ethanol inhibition. This was confirmed by comparing the result with B_{350}, B_{400}, and B_0, where cells were exposed to higher ethanol concentrations (than in B_C) but the cell remain viable by actively consumed glycerol or produced ethanol as long as glycerol had not run out; indicates that the decrease in cell activity was not due to inhibition of ethanol. Termination of the basin zone due to run out of substrate was also observed by Liu *et al.* (2015) using glucose as a substrate. This condition could be utilized as a sign to start the next batch cycle for the repeated batch process.

In the continuous aeration (B_C), after the basin zone, the ORP value began to re-rise (the uprising zone) at 42 h of fermentation. This zone was the period where cell activity started to slow down as indicated by a low oxygen dissimilation (DO rose from 0% to 3.35%) followed by the cessation of ethanol production. This was also observed at B_{350} and B_{400}, but the uprising of ORP value was not as significant as in B_C. This happened because the aeration of B_C was held continuously, whereas B_{350} and B_{400} were not. The inactivation of

aeration by the end of fermentation time in B_{350} and B_{400} occurred because the ORP no longer fall below the reference value as the production of reducing power decreased (the cell began to be inactive). Inactivation of aerator had caused the ORP value in both processes to increase more slowly than the B_C since oxygen was sourced only from the 2-L reactor headspace. However, if the cell remained active this zone may not appear, causing an extension of the basin zone, such as in B_0 (Liu *et al.*, 2015).

The reliability of the DO sensor to detect oxygen at low concentration (~ 0 %) required for the microaerobic condition led to the need of more sensitive measurement, in this case, the redox potential (ORP) sensor (Zeng *et al.*, 1994). Unlike the DO sensor, that specifically measures the presence of oxygen; the ORP sensor measures the net result of the redox reaction between the reducing and the oxidizing agent in the fermentation broth (Liu *et al.*, 2011). Although not as specific as the DO sensor, the success of the ORP sensor in monitoring fermentation at low oxygen concentrations is motivated by a high reduction potential (E0 = +820 mV) of oxygen (Liu *et al.*, 2013). So that, even when oxygen is at a trace level, the ORP sensor will remain sensitive to it as the net redox of the fermentation broth will become more positive. Here, the ORP sensor successfully monitored and controlled the oxygen concentration throughout the fermentation. This has opened up opportunities to explore more characteristics of the microaerobic process, which were initially difficult to do with the DO sensor (Liu *et al.*, 2017).

In general, the increase in aeration intensity increased ethanol productivity (g/L/h), the specific growth rate and cell concentration. However, higher aeration intensity reduced ethanol production (g/L) and the conversion yield of ethanol ($Y_{P/S}$). The highest ethanol concentration and yield were actually achieved during the non-aeration system (B_0) as much as 18.78 g/L and ethanol yield of 0.94 g/g, 88% higher than the theoretical yield. This also occurred in our previous study since tuna condensate itself contains high organic carbon indicated by its high COD value (Sunarno et al., 2019; Boukaew et al., 2018). This excess of organic carbon was likely to be assimilated by *E. aerogenes* as an additional carbon and energy source resulted in an extra ethanol synthesis. However, the specific ethanol production rate (qp) of B_0 (0.120 g-ethanol/g-cell/h) was only second to the controlled aeration B_{400} (0.133 g-ethanol/g-cell/h), indicating that the optimum condition for the cell to produce ethanol might actually take place at -400 mV. Besides, the specific glycerol consumption rate (qs) of B_{400} was not significantly different from -350 mV (B_{350}) (0.259 and 0.253 g-glycerol/g-cell/h, respectively).

The effect of substrate concentration on specific cell growth rates at various aeration methods was evaluated. The result implied that when the aeration was limited (a more reductive external environment), the concentration of the substrate appeared to be the sole limiting factor for cell growth. In contrast, when the aeration was held continuously, the growth could be improved and

maintained until the glycerol was 50% consumed (approximately 10 g/L glycerol), before it finally decreased following the substrate depletion.

In the presence of electron acceptor (aerobic condition), the incoming glycerol is captured by glycerol kinase (GK) to produce glycerol-3-phosphate (G3P) with one mole of ATP consumption. G3P is further converted to Dihydroxyacetone phosphate (DHAP) by aerobic glycerol-3-phosphate dehydrogenase (ae-G3PDH). From here, DHAP enters the glycolysis pathway to pyruvate where only one mole of NADH and two moles of ATP are produced resulting in one mole of net ATP. Meanwhile, in the absence of electron acceptor (anaerobic condition), glycerol phosphorylation was performed by two successive enzymatic reactions catalyzed by glycerol dehydrogenase (glyDH) and dihydroxyacetone kinase (DHAK) to produce DHAP (Ruch *et al.*, 1974). This phosphorylation consumes one mole of ATP and produces one mole of NADH resulting in one mole of net ATP and two moles of NADH acquired by the cell at the end of glycolysis.

The tendency of utilizing carbon for cell growth rather than ethanol production could be the signal of a more dominant glycerol kinase **(**GK**)** activity, as this pathway tends to accumulate ATP and lack of NADH per glycerol consumed**.** The presence of ATP has a more positive influence on the initial velocity of GK than the glycerol concentration itself **(**Applebee *et al.*, 2011; Holtman *et al.*, 2001**).** Thus, cell growth could be less sensitive toward glycerol depletion when ATP is in excess due to the stability of GK activity**.** This might

explain that why at the beginning of the B_C process, the cell growth rate was increase or maintained at 100**%** before its concentration fell under 10 g/L**.** The journey from 20 g/L to 10 g/L glycerol consumption in B_C was actually occurred in the aerobic phase**.** It is likely that the intracellular compartment was high in ATP concentration and GK activity is also high as observed by Durnin *et al.* **(**2009**).** After that, the cell entered the microaerobic phase (less ATP production) and cell growth started to decelerate along with the decrease in substrate concentration. Despite fast cell growth, the ethanol production during aerobic phase would not be exaggerated as shown by carbon recovery for ethanol production that is decrease when a more intensive aeration was applied. Ethanol production of *E. aerogenes* is carried out by two NADH-linked enzyme including alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH). Meanwhile, only 1 NADH per molecule of glycerol is available after pyruvate is formed requiring the cell to break down 1 more glycerol molecule. This condition is certainly less energy-efficient than converting NADH directly to ATP through the electron transport chain (ETC). Thereby, ethanol production under more intensive aerobic condition would be lower.

In absence of terminal electron acceptor (anaerobic condition), the intracellular NAD+ will gradually diminish as it is used as the cofactor of glyDH at the initial pathway of glycerol dissimilation. To avoid the shortage of NAD+, it must be regenerated so the substrate uptake could be sustained. With the absence of oxygen, cells can only regenerate NAD+ through ADH and ALDH.

The tendency to produce more ethanol at limited oxygen concentration could be the reflection of higher ADH and ALDH activity in order to recalibrate the intracellular ratio of NADH/NAD+. Glycerol dissimilation for cell maintenance can be sustained, even though the progression of the fermentation became slower. Durnin *et al.* (2009) stated that during microaerobic fermentation, the aerobic and anaerobic pathway were active but the activity was changing dynamically according to the level of oxygen concentration. The more oxygen is presence, the activity of GK and ae-G3PDH would be more dominant than the other enzymes. Meanwhile, the less oxygen presence the activity of glyDH and DHAK would be more dominant.

On the other hand, byproducts formation such as acetic acid was prominent at B_C and B_{350}. It was also confirmed in the previous report that high oxygen supplies decreased ethanol production, but increased acetic acid and lactic acid products along with faster cell propagation (Choi *et al.*, 2011; Nwachukwu *et al.*, 2012, 2013). Meanwhile, acetic acid did not appear in two other aeration strategies (B_{400} and B_0). In *E. coli*, the species in the same family of Enterobacteriaceae, NADH has been reported to be one of the inhibitors of phosphotransacetylase, the enzyme that catalyzes the conversion from pyruvate to acetyl-phosphate (a precursor of acetic acid) (Campos-Bermudez *et al.*, 2010). This accumulation of NADH during oxygen limitation can possibly be the cause of acetic acid product suppression. Increased production of acetic acid can be a

problem for ethanol production, considering *E. aerogenes* is more susceptible to acetic acid than ethanol itself (Zeng and Deckwer, 1991).

The intensity of aeration has been shown to regulate the rate of fermentation kinetics associated with the level of oxygen supply. Controlling oxygen concentration during the microaerobic fermentation process is, therefore, important to ensure the cell physiology favors ethanol production. Oxygen is an excellent electron acceptor due to its high reduction potential. Oxygen will be utilized by the cells to convert the vast reducing equivalents (NADH) to ATP via electron transport chain (ETC), leading to a more progressive cell growth but lower ethanol production. In contrast, minimum oxygen concentration rendered slower growth kinetic behavior but higher ethanol production.

Carbon recovery (CR) was evaluated from biomass, ethanol and acetic acid concentration (end-products) divided by the total glycerol consumption (substrate) (Ellis et al., 2012). According to the analysis of carbon recovery (Table 3), reducing aeration intensity (from B_C to B_0) showed increased in carbon recovery to ethanol which supported the finding that oxygen deprivation led to more dominant ethanol production. In contrast, predisposition of utilizing carbon for cell growth under more intensive aeration was also observed by CR analysis. Results of this study shows overall carbon recovery (CR) values higher than one, suggesting that the medium contained a vast of readily assimilable carbon sources beside glycerol. This was mentioned in our previous study as the benefit of using tuna condensate (Sunarno *et al.*, 2019).

Looking at its total carbon recovery alone, the non-aeration process (B_0) seems to have slightly better carbon utilization than the rest of the process.

Previously, the batch process had been tested using two-stage aeration where the first stage was micro-aerated to stimulate cell growth and after the cell reached the maximum, fermentation continued using anaerobic processes (Saisaard *et al.*, 2011). This process produced 24.5 g/L ethanol from 43.75 g/L crude glycerol for 32 h. Meanwhile, the ethanol concentration in this study was still low given that the optimum concentration of glycerol used was also low (~ 20 g/L), so a fed-batch process was proposed to boost substrate utilization. Information about the effects of aeration on batch reactors would be used as a means for consideration.

Fed-batch fermentation was carried out to increase ethanol production by maintaining high cell growth and improving substrate utilization. In this study, the fed-batch with intermittent feeding was chosen due to its ease of operation (Asenjo, 1994). All fed-batch fermentation in this study began at an initial glycerol concentration of ~20 g/L. When glycerol consumption had reached 50% (about 10 g/L); fresh crude glycerol was fed into the reactor to restore glycerol concentration to 20 g/L to maintain the specific cell growth rate (μ). This lower limit was decided because the cell's specific growth rate had decreased significantly when glycerol falls below ~10 g/L). Tuna condensate was not included to simplify the process medium. Then, various aerations strategies were evaluated to achieve optimal ethanol concentration including

single-stage aeration and two-stage aeration. Single-stage aeration was performed using continuous aeration of 0.5 vvm (FB-1). Two-stage aeration consisted of: a) Continuous and controlled aeration at ORP -350 mV (FB-2), b) Continuous and controlled aeration at ORP -400 mV (FB-3), and c) Aeration controlled at ORP -350 mV and -400 mV (FB-4).

The first configuration (FB-1) was chosen considering that cell growth and glycerol consumption occurred most rapidly under continuous aeration. Despite low ethanol production, fed-batch cultivation was expected to increase ethanol concentration by increasing substrate utilization. The cultivation lasts for 84 h with 15 mL of samples withdrawn at every 3 h. The volume had changed during the course of fermentation due to sampling and replenishment. It was initiated with 3000 mL of medium at 20 g/L of glycerol. At the first 12 h of fermentation, glycerol concentration had fell to 10 g/L at which the volume had also decreased to 2925 mL due to sampling. The crude glycerol was replenished back to 20 g/L by adding 125 mL of fresh crude glycerol and the volume increased to 3050 mL. From here, it took 21 h (or 33 h from initial cultivation) for glycerol concentration to decrease to 10 g/L with the remaining volume of 2930 mL (due to sampling). Glycerol concentration was again replenished by adding 125 mL fresh crude glycerol and the volume increased to 3055 mL. By the end of fermentation (84 h), the remaining volume was 2785 mL. FB-1 produced up to 25.95 g/L ethanol or approximately twice that of its batch counterparts (12.33 g/L). With twice-fresh glycerol feeding (2-

folds higher glycerol loading), the consumption of glycerol also increased 2-fold which indicates that the performance of cells in consuming glycerol and producing ethanol was not much different in the two reactor configurations (fed-batch and batch). This could also be confirmed from the yield of the two processes, which were relatively the same. However, to achieve two-fold of ethanol production and glycerol consumption, it required a minimum of 72 h, longer than twice the time required by a batch process, leading to a slight decrease in ethanol productivity to 0.344 g/L/h. The slowdown in ethanol production could be triggered by the slowdown of cell growth when fermentation had already run halfway (after 33 hours). Potential reduction values of the ORP sensor also notified the decreasing of cell activity, noting that the value became more positive exactly when cell growth began to slow down. In addition, from Table 4, the carbon predisposition to the cell was lower in comparison with its batch process (B_C) counterparts confirming that the cell growth in FB-1 was not as good as in the batch process (B_C). An increase in acetic acid, methanol, and ethanol could also be the reasons for this phenomenon. The cell growth slowed, yet the cell concentration still increases and had not completely stopped. Cells appeared to enter the decline phase just after 72 hours. However, the ethanol concentration no longer increases significantly. This suggested that maintaining aeration at the end of the fed-batch fermentation benefit to neither cell growth nor ethanol production. This led to the next process configuration, two-stage aeration. Due to plausible inhibition aforementioned previously, sustaining high

cell growth rate would be difficult. Thus, the purpose of the final stage was changed to produce ethanol as a priority. Here, the aeration on the final stage of the fed-batch process, which turned out to be less effective using continuous aeration, was now controlled by the reduction potential (FB-2 and FB-3). Considering that under oxygen deficiency, the conversion of glycerol to ethanol was higher than the continuous aeration process.

The redox potential-driven aeration implemented at the final stage of the fed-batch was successful in increasing ethanol concentration to 28.21 g/L and 30.21 g/L, respectively for FB-2 and FB-3. These concentrations were achieved with a minimum fermentation time of 75 h. In terms of glycerol consumption, FB-1 and FB-2 were both achieved complete substrate utilization, except that FB-2 required more time. However, FB-3 only consumed 38.72 g/L of glycerol up to 84 h fermentation or decreased by 8% from total glycerol consumption in FB-1 and FB-2. This indicates that, indeed, the consumption of glycerol was lower, but glycerol could be converted more towards ethanol by means of aeration control. Another advantage of using two-stage aeration is the concentration of acetic acid drops by 50%. Production of acetic acid is the side effect in a continuous aeration system. The production of acetic acid was suppressed when aeration was controlled, so its concentration was barely increased. This condition is very beneficial for *E. aerogenes*. This decrease also has an impact on the selectivity of ethanol, where the selectivity increases above 6.0 g-ethanol/g-byproducts in FB-2 and FB-3.

Aeration control in microaerobic fermentation using redox potential provides flexibility in process optimization, especially in ethanol production. It was previously explained (in section 3.2) that continues aeration was beneficial for accelerating cell growth, but ethanol production was ruled out, and the opposite was the case with no aeration. That is, by controlling aeration using redox potential, we can find the optimal point between cell growth and ethanol production. This then became the rationale for testing the last configuration, FB-4. In this configuration, the aeration at the first stage was controlled at -350 mV and then to -400 mV in the next stage. Although in this condition cell growth would not be as fast as continuous aeration; the yield of the ethanol conversion, specific ethanol production rate and glycerol consumption rate were higher than in continuous aeration. Similar with the FB-1, FB-2 and FB-3, the initial working volume was set to 3000 mL with initial glycerol of 20 g/L. The results showed that it took 24 h to complete the first stage with an ethanol concentration of 11.51 g/L and the volume decreased to 2865 mL. Then, fermentation continued to the second stage by adding 125 mL fresh crude glycerol (working volume increased to 2990 mL) and the aeration was controlled at -400 mV, a condition where ethanol production was more prioritized. At the end of fermentation, the highest ethanol concentration that can be achieved was ~30 g/L, equivalent to the FB-3 process. This concentration was achieved by consuming less substrate, which is 35.37 g/L, making the yield of glycerol to ethanol the highest compared to other process configurations. More

importantly in this process, the production of acetic acid could be fully suppressed. Then, a one-time addition of glycerol was also beneficial because methanol (an inhibitor) will not be accumulated. By this, cell viability could be secured and thereby, improve glycerol conversion to ethanol.

Controlling the microaerobic process in different ORP value, we found a significantly different result in terms of ethanol production, substrate consumption, cell growth, and even by-product production. The best result achieved in this study was 30 g/L. Aeration of two stages at -350 mV and -400 mV was considered more effective because it consumed less substrate and used a moderate aeration requirement so that operating costs could be reduced. The carbon recovery analysis of fed-batch fermentation also showed that FB-4 resulted in a better overall carbon recovery, particularly to ethanol. Furthermore, the inhibitor concentration such as acetic acid and methanol from the impurities of crude glycerol could also be lowered. The ethanol concentration in this experiment was the highest for wild species *E. aerogenes* and was achieved using the simplest medium (Saisaard *et al.*, 2011; Yuwa-amornpitak and Chookietwatana, 2016). The ethanol concentration of wild *E. aerogenes* in this study also offset the mutant species, but the mutant had a higher productivity (Oh *et al.*, 2011). This strain also has a higher ethanol productivity than the yeast strain of *Pachysolen tannophilus* CBS404 (Liu *et al.*, 2012). *E. aerogenes* mutant strain can produce up to 38.32 g/L ethanol by deleting lactic acid pathways and increasing the expression of alcohol dehydrogenase (ADH) (Thapa *et al.*, 2015).

This opens up opportunities in the future for further exploration particularly to increase ethanol concentration. Increased ethanol concentration above 40 g/L will be preferred because at this concentration the downstream process can run efficiently (Huang and Percival Zhang, 2011). Microaerobic fermentation using two-stage ORP controlled aeration could also be implemented for another metabolite product, especially acid production such as lactic acid and succinic acid.

4. Concluding remark

The production of ethanol from crude glycerol and tuna condensate using *Enterobacter aerogenes* TISTR 1468 was optimized. The optimum crude glycerol concentration was ~20 g/L and the ethanol production was 6.72 g/L. The replacement of complex nutrient by crude glycerol and tuna condensate (GT medium) resulted in a comparable ethanol production. The addition of inorganic salts and trace element into GT medium could further increase the ethanol production to 12.73 g/L. Among the components of the inorganic salts, phosphate buffer played a very important role (77.6% contribution) but it could be replaced by controlling pH at 7.0 (adjusted with 3N NaOH). Increase in ethanol production (12.33 g/L), ethanol yield (0.62 g-ethanol/g-glycerol) and selectivity (4.31 g-ethanol/g-byproducts) as well as cell concentration (2.51 g/L) was achieved under the optimum condition.

Aeration greatly influenced the performance of the microaerobic process and the use of ORP sensors was proven to facilitate control. In batch process cultivation, increasing the intensity of aeration could increase specific cell growth rate and fermentation time. Under continuous aeration conditions, cell growth was not only influenced by the concentration of substrates, but also by oxygen concentration. Decreasing substrate concentration was not followed by a decrease in cell growth provided the substrate concentration was still above 10 g/L. However, the higher the intensity of aeration could reduce the final concentration of ethanol. The yield and selectivity of ethanol also decreased

followed by an increase in cell concentration and acetic acid (byproducts). The use of tuna condensate as a substitute for complex nutrients had been shown to increase ethanol concentration and yield. The highest specific ethanol production rate was achieved by controlling aeration at -400 mV, while at -350 mV, faster specific glycerol uptake rate was obtained. Meanwhile, the highest ethanol concentration in the batch process was 18.78 g/L with a yield of 0.94 g-ethanol/g-glycerol, in conditions without aeration.

Ethanol concentration was then increased using a fed-batch process. Variation in aeration had also been shown to influence the performance of the fed-batch process. The best aeration variation was achieved using two-stage aeration, i.e. FB-3 and FB-4 processes, both of which reached ethanol concentrations up to 30 g/L. However, the performance of FB-4 was considered better because higher ethanol yields and production of acetic acid could be reduced. In fact, this concentration was achieved only by doing one addition of crude glycerol, so that it could avoid the accumulation of methanol in the reactor. The results of this experiment could be used as an alternative to the utilization of crude glycerol. Ethanol concentration can be further improved using microorganisms that have a higher ethanol production capacity and are more tolerant to inhibitors that exist during the process. This two-stage aeration strategy was also useful for the production of other metabolites produced under microaerobic conditions.

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APPENDIX

☙ PAPER I ❧

Biodiesel derived crude glycerol and tuna condensate waste as an alternative low-cost fermentation medium for ethanol production by *Enterobacter aerogenes*

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Biodiesel derived crude glycerol and tuna condensate as an alternative low-cost fermentation medium for ethanol production by *Enterobacter aerogenes*

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ABSTRACT

In this work, waste-based raw materials; crude glycerol from biodiesel plant and tuna condensate from tuna canning factory, were used to substitute the expensive complex medium for ethanol production from *Enterobacter aeroegenes* TISTR 1468. The optimum crude glycerol concentration was 21.43 g/L (~20 g/L), giving 6.72 g/L ethanol. Using tuna condensate as a nitrogen source for studying the effect of initial C/N ratio (115–365 g/g) revealed the insignificant difference on ethanol production in every C/N ratio tested with a noticeable increase of byproducts (2,3-butanediols) at the high C/N ratio. The addition of inorganic salts and trace element into crude glycerol-tuna condensate (GT) medium resulted in 2-fold increase of ethanol production (12.73 g/L), complete substrate consumption and reduced the formation of byproducts. Optimization studies based on Plackett-Burman design indicated that the most influential parameter amongst the inorganic salt components was phosphate buffer with 77.6% contribution. However, phosphate buffer could be replaced by keeping the pH constant at 7.0 using 3 N NaOH. Under the optimum condition; 20 g/L crude glycerol, initial C/N ratio of 115 g/g and the pH maintained at 7, the ethanol production was 12.33 g/L with 24% higher than the theoretical yield. The selectivity of ethanol production increased upto 3-fold larger than the expensive complex medium.

1. Introduction

Biodiesel, a renewable fuel made to substitute petroleum diesel, is increasingly popular nowadays along with the expansion of the transportation sector. Biodiesel is produced from lipids/oil such as vegetable oils, waste vegetable oils, and animal fats through trans-esterification reaction with alcohol (methanol and/or ethanol), generating biodiesel and glycerol. Biodiesel is considered advantageous economically because it can reduce crude oil imports which prices have been volatile lately. On the environmental aspect, biodiesel combustion releases lower harmful materials such as particulate, carbon monoxide, unburnt hydrocarbons, sulfur oxide as much as 40, 44, 68 and 100%, respectively (Talebian-Kiakalaieh et al., 2013).

Currently, methanol is the main source of alcohol because it is cheap and easy for separation (simple process) (Meher et al., 2006). However, methanol is produced unsustainably from fossil sources offsetting the environmental benefits of biodiesel as a renewable fuel. In addition, methanol can cause serious health impact on human (Jahan et al., 2015). As a result, interest in using ethanol to replace methanol for biodiesel feedstock has begun to emerge lately (Bikou et al., 1999; Da Silva et al., 2011; Dias et al., 2014; Farobie et al., 2015; Komintarachat et al., 2015; Muppaneni et al., 2012; Velez et al., 2012; Virgínio e Silva et al., 2018). Ethanol exposure to human is harmless and environmentally more desirable as it is easily produced from renewable sources.Biodiesel from ethanol can accelerate local economic growth, considering that the two raw materials can be produced domestically without the needs of imports. Besides, ethanol also has higher energy content than methanol suggesting biodiesel with higher energy content.

Glycerol can be directly converted into ethanol via fermentative routes using *Enterobacter aerogenes* (Saisaard et al., 2011), *Kluyvera cryocrescens* (Choi et al., 2011), *Pachysolen tannophilus* (Stepanov and Efremenko, 2017), *Klebsiella pneumoniae* (Oh et al., 2011) and *Escherichia coli* (Cofré et al., 2016).In principle, ethanol production from glycerol is intriguing the fact that the biodiesel industry itself produced glycerol as byproducts. However, as glycerol obtained from biodiesel industry is contaminated by various substances such as methanol, salts and fatty acid, sufficient nutrient in the medium is important to maintain high cell performances following plausible inhibition caused

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by impurities (Venkataramanan et al., 2012). Thus, the production of ethanol from glycerol is always associated with a complex medium (Ito et al., 2005), medium containing various inorganic salts, organic nitrogen, and trace elements. This complexity could restrain the development of this kind into the commercial stage due to a foreseeable high production cost. Waste-based nutritional sources lately gain much attention as a substitution for a complex substrate due to its abundance and lower price. Tuna condensate is the waste of tuna canning industry that is rich in biogenic substance essential for cell growth. The waste is considered underutilized and its abundance could complicate its handling and management, so valorization of this waste into the value-added product could become an alternative profitable solution. The utilization of tuna condensate as a growth supplement for microbial fermentation has been successfully investigated such as for *Rhodocyclus gelatinosus* (Prasertsan et al., 1997), *Streptomyces philanthi* RL-1-178 (Boukaew et al., 2018), *Candida rogusa* 8YB and *Lactobacillus futsaii* CS3 (Sanchart et al., 2018) but not yet for growth and ethanol production from *Enterobacter aerogenes*.

The production of ethanol from biodiesel derived crude glycerol has been associated with a complex medium to render high cell performance effectuating high cost production. Therefore, the objectives of this study were to develop a minimal crude-glycerol based medium forethanol production using *E. aerogenes* TISTR 1468 supplemented by a cheap nutrient substrate available (tuna condensate). The specific objectives were as follows: 1) optimizing crude glycerol concentration, 2) optimizing tuna condensate concentration by employing initial C/N ratio as a parameter, 3) screening of inorganic salts and 4) studying the effect of pH control.

2. Materials and methods

2.1. Microorganism

E. aerogenes TISTR 1468 was purchased at Thailand Institute of Scientific and Technological Research (Pathum Thani, Thailand) and preserved at −20 °C in 15% pure glycerol. For a routine application, the bacteria were re-streaked on a nutrient agar plate containing 3% pure glycerol which was the result of our preliminary study and kept in the 4 °C room. The culture was subcultured once a month.

2.2. Feedstock

The crude glycerol was obtained from Fatty Acid Methyl Ester Pilot Plant, Faculty of Engineering, Prince of Songkla University, Thailand. The biodiesel was produced from waste cooking oil and methanol with potassium hydroxide as the catalyst. The crude glycerol was stored in the 20-L container at room temperature. The tuna condensate was obtained from Songkla Canning Co. Ltd., Thai Union Group (Songkhla, Thailand) and stored in the 1.5-L plastic bottle at the -20 °C room.

2.3. Medium composition

The complex medium contained organic nitrogen sources, inorganic salts compounds, and trace element solution.The organic nitrogen consisted of 5.0 g/L yeast extract and 5.0 g/L tryptone. The inorganic salts compounds used were 7.0 g/L K_2HPO_4, 5.5 g/L KH_2PO_4, 1.0 g/L $(NH_4)_2SO_4$, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 0.021 g/L $CaCl_2 \cdot 2H_2O$, 0.12 g/L $Na_2MoO_4 \cdot 2H_2O$, 2.0 mg/L nicotinic acid and 0.02 mg/L $NiCl_2$. Trace element solution used in the medium as much as 10 ml/L containing 0.5 g/L $MnCl_2 \cdot 4H_2O$, 0.1 g/L H_3BO_4, 0.01 g/L $AlK(SO_4)_2 \cdot H_2O$, 0.001 g/L $CuCl_2 \cdot 2H_2O$ and 0.5 g/L Na_2EDTA (Ito et al., 2005). Crude glycerol medium (CG) was prepared by adding crude glycerol at the desired concentration in the complex medium described above. Meanwhile, crude glycerol and tuna condensate medium (GT) was only consisted of both constituents.

2.4. Analytical method

The concentrations of glycerol, ethanol, acetic acid and 2,3-butanediol were determined by High-Performance Liquid Chromatography (HPLC) (Agilent Technologies 1200 series, USA) equipped with the Aminex HPX-87H ion exclusion column (300 mm x 7.8 mm) (Bio-Rad, USA). The column temperature was kept at 80 °C and the sample volume was 10 µL. The mobile phase was H_2SO_4 (5 mM) at a flow rate of 0.6 mL/min.

For determination of dry cell weight (DCW), a 2-mL of fermentation broth was centrifuged at 10,000 rpm (6708 × g) for 5 min. The pellet was washed twice with 10 mM phosphate buffer (pH 7.4), centrifuged between each washing to remove the supernatant and dried at 105 °C for 24 h and weighed after cooling in the desiccator (Jo et al., 2008).

The total Kjeldahl nitrogen (TKN) of the feedstock was determined according to AOAC Official Method 981.10 (AOAC International, 2016). COD was determined using Spectroquant® COD Cell test kits (Merck KGaA, Germany) using the standard procedure given. Total organic carbon (TOC) was then estimated using a linear correlation between COD and TOC for the influent wastewaters (Dubber and Gray, 2010).

$$COD = 49.2 + 3.00 \times TOC \quad (1)$$

2.5. Parameter determination

The total glycerol consumption (%) is calculated from the initial glycerol concentration - the final glycerol concentration and divided by the initial glycerol concentration, then multiply by 100. The ethanol yield (g/g) was calculated from final ethanol concentration divided by the total amount of glycerol consumed. In addition, the term selectivity was used to evaluate the production of the desired product in multiple reaction system, which possibly produced a significant amount of by-products. A higher value means the system produced a more desired product than a byproduct. In this study, the selectivity of ethanol (g/g) was calculated from its concentration divided by total byproducts formed by the end of fermentation (Yang et al., 2013).

2.6. The effect of initial crude glycerol concentration

The preparation of seed culture was initiated by inoculating a loopful of *E. aerogenes* TISTR 1468 into a tube containing 10 mL CG medium (10 g/L glycerol) and incubated at 37 °C for 12 h. This seed was subsequently re-inoculated into fresh CG medium (100 mL) incubated on a shaker (150 rpm) at 37°C for 24 h.

The CG medium used to investigate the optimum crude glycerol concentration was prepared by adding crude glycerol into the complex medium (section 2.3) to the desired concentrations (10, 20, 30, 40, 50 and 60 g/L). The pH was adjusted to 8.0 using either 6.0 N NaOH or HCl prior to the sterilization procedure (Saisaard et al., 2011). The fermentation took place by inoculating 10% (v/v) of the seed culture into 100 mL of CG or GT medium and cultivation on a shaker (150 rpm) at 37 °C for 72 h. Samples were taken for determination of pH, glycerol concentration, biomass, metabolite products such as ethanol, acetic acid and 2,3-butanediol (2,3-BDO), then the calculation for glycerol utilization (consumption) and ethanol yield.

2.7. The effect of initial C/N ratio

The influence of carbon to nitrogen (C/N) ratio was investigated in term of total organic carbon (TOC) to total Kjeldahl nitrogen (TKN) ratio. The crude glycerol and tuna condensate medium (GT medium) was prepared by using the optimum crude glycerol concentration (20 g/L) and varying the concentration of tuna condensate to obtain the final C/N ratio of 115, 200, 257 and 365. Likewise, the initial pH of the GT

medium was also adjusted to 8.0. The experiment procedure was as described above (Section 2.6).

2.8. The effects of inorganic salts, phosphate buffer and pH control

The influence of inorganic salts and trace elements was studied by adding them into the GT medium with various C/N ratio. The effect of phosphate buffer was studied by adding phosphate buffer (80 mM; pH 8.0) instead of inorganic salts and trace elements. The pH of the GT medium was controlled at pH 7.0 and the results with the use of phosphate buffer was compared. The experiment was as described above (Section 2.7).

2.9. Statistical analysis

The significance of each set of experiment was analyzed by ANOVA with Tuckey pairwise method using statistical software Minitab 17 (Minitab Inc.) at a confidence level of 95% ($p = 0.05$).

The screening of the essential inorganic salts compounds was performed using Placket-Burman statistical design (PBD) with Design-Expert® software (version 10) (Stat-Ease, USA). All nine factors (inorganic salts and trace solution) chosen in this study were tested at two levels (i.e. "-1"represented the absence of compounds and "+1" represented the presence of the compounds in the substrate), based on the Plackett-Burman matrix design (see Table 5). The main effect was calculated as a difference between the average measurements of each variable made at a high level (+1) and a low level (-1). This design screened variable based on a first-order model:

$$Y = \beta_0 + \Sigma \beta_i \mid X_i \quad (2)$$

Where Y was the response (ethanol concentration), β_0 was the model intercept and β_i was the variable estimated. The percent contribution of each individual factorwas obtained by summing all the term sum of squares (SS) and then taking each individual SS and dividing by the total SS and multiplying by 100. The percent contribution in the present study was used to determine which inorganic salt compounds gave larger contribution in improving cell performance.

2.10. The Morris method of sensitivity analysis

The sensitivity analysis of input parameter was performed using the Morris method (Morris, 1991; Sun et al., 2012). The input parameters were defined as:

1) CG was the sensitivity of various crude glycerol concentration of CG medium on the ethanol production.
2) GT was the sensitivity of various C/N ratio of GT medium on the ethanol production.
3) GT_{IOSTE} was the sensitivity of various C/N ratio of GT medium with inorganic salts and trace elements.
4) GT_{Buffer} was the sensitivity of various C/N ratio of GT medium with buffer
5) GT_{pH-C} was the sensitivity of various C/N ratio of GT medium with pH controlled at pH 7.0.

The method is based on elementary effect (d_i) for the *i*th input (C/N ratio, glycerol concentration) on the output (ethanol production) and is defined as followed:

$$d_i(x) = [y(x_1,\ldots,x_{i-1}, x_i + \Delta, x_{i+1},\ldots,x_k)\text{-}y(x)] / \Delta \quad (3)$$

Where, y is the output variable and Δ is the difference amongst two input variable in consideration. The method takes sensitivity measures ($\hat{\mu}$) and the standard deviation (σ) to detect input factors with an important overall influence on the output parameter. Both were calculated as follow:

Table 1
Characteristic of crude glycerol and tuna condensate waste used in the present study.

Parameter	Unit	Crude glycerol	Tuna condensate waste
pH		9.57	5.67
Density	g/L	1055	1077
Glycerol	mg/L	259,460	N/A*
Methanol	mg/L	102,953	N/A
Chemical oxygen demand (COD)	mg/L	1,175,000	79,000
Total Kjeldahl Nitrogen (TKN)	mg/L	1120	6527
Ammonium-Nitrogen (NH_4-N)	mg/L	0	400
Total phosphorous	mg/L	129	665
Magnesium	mg/L	574	264
Iron	mg/L	15.4	2.4
Chloride	mg/L	37,250	7500
Total solids (TS)	mg/L	4589	53,560
Suspended Solids (SS)	mg/L	4290	4311

* N/A: Not applicable.

$$\mu^* = \sum_{i=1}^{r} \frac{d_i}{r} \quad (4)$$

$$\sigma = \sqrt{\sum_{i=1}^{r} \frac{(d\text{-}\mu)^2}{r}} \quad (5)$$

3. Results and discussion

3.1. Characteristics of biodiesel derived crude glycerol and tuna condensate

The characteristics of crude glycerol and tuna condensate are summarized in Table 1. They are wastes from different industries; non-food (biodiesel) and food (canned tuna) plants, respectively, hence varied in composition. Crude glycerol had alkaline pH (9.57) and density of 1055 g/L. It contained about 70% of the total mass (259,460 mg/L glycerol) with the residual methanol at 28% (102,953 mg/L). The crude glycerol had very high COD (1,175,000 mg/L) but very low total nitrogen (TKN) (1120 mg/L) without ammonium-nitrogen (NH_4-N) and giving the C/N ratio of 1049:1. Other chemicals were phosphorous (129 mg/L), iron (15.4 g/L) and magnesium (574 mg/L).

The tuna condensate contained lower COD (79,000 mg/L) but higher total nitrogen (6527 mg/L) with 6.1% of ammonium-nitrogen (400 mg/L) than those of the crude glycerol. The C/N ratio of tuna condensate was 12:1 which indicated the more suitable for being substrates for microbial growth. Unlike crude glycerol, it had an acidic pH (5.67) and contained higher phosphorous (665 mg/L) but lower magnesium (264 mg/L) and iron (2.4 mg/L).

3.2. The effect of initial crude glycerol concentration

The effect of initial crude glycerol concentration in the range of 9.32 g/L to 59.77 g/L (about 10–60 g/L) was evaluated (Table 2). Three metabolites were produced with ethanol being the major compounds (2.04–6.72 g/L) followed by acetic acid (1.01–3.09 g/L) and 2,3-butanediol (0.63-0.74 g/L). The optimum concentration of the crude glycerol was 21.43 g/L, giving the highest ethanol concentration of 6.72 ± 0.59 g/L. The ethanol yield (0.51 g-ethanol/g-glycerol) and selectivity (1.42 g-ethanol/g-byproducts) observed at this concentration were also the highest amongst the other counterparts. This optimum concentration of crude glycerol and the ethanol yield from this study were similar to those previously reported (20 g/L glycerol and ˜6 g/L ethanol, respectively) (Chantoom et al., 2014; Lee et al., 2012).

Increasing initial crude glycerol concentration caused an inhibition in cell growth indicated by decreasing cell concentration. As a result,

Table 2
The effect of initial crude glycerol concentration on the performance of *E. aerogenes* TISTR 1468.

Initial crude glycerol (g/L)	Methanol[1] (g/L)	Final pH	Glycerol utilization (%)	Biomass (g/L)	Products (g/L)			Ethanol Yield (g/g)	Selectivity (g/g)
					Ethanol	Acetic acid	2,3-Butanediol		
9.32	1.91	5.58	100	1.93 ± 0.14	4.63 ± 0.30	3.09 ± 0.07	0.74 ± 0.02	0.50	0.80
21.43	3.46	5.66	62	1.69 ± 0.30	6.72 ± 0.59	2.32 ± 0.04	0.73 ± 0.01	0.51	1.42
31.44	5.01	5.74	42	1.63 ± 0.12	5.84 ± 0.06	1.89 ± 0.04	0.73 ± 0.01	0.45	1.38
42.73	7.64	5.70	30	1.61 ± 0.08	4.55 ± 0.14	1.59 ± 0.03	0.72 ± 0.01	0.36	1.16
51.06	9.21	5.76	20	1.34 ± 0.02	3.76 ± 0.51	1.04 ± 0.04	0.64 ± 0.01	0.36	1.24
59.77	11.24	5.74	14	1.13 ± 0.10	2.04 ± 0.36	1.01 ± 0.01	0.63 ± 0.01	0.25	0.74

CG medium at initial pH of 8.0 incubated at 150 rpm and 37^{0}C for 72 h. The data were the average of triplication experiment.
[1] The methanol composition in the crude glycerol medium after sterilization at 121^{0}C for 15 min.

glycerol consumption and ethanol production, which were influenced by the cell growth, they were also adversely affected. A complete crude glycerol consumption, as well as fast glycerol uptake rate, was occurred at the lowest concentration supplied and decreased thereafter. Likewise, the ratio of glycerol conversion to ethanol (ethanol yield) dropped below the theoretical value at crude glycerol 31.44 g/L and higher. Reduced performance at increasing glycerol concentration has been associated with feedback inhibition of glycerol kinase which in charges in glycerol assimilation (Zwaig and Lin, 1966). The inhibition of glycerol kinase is characterized by slow glycerol uptake rate and a lower tendency toward cell production as shown in the result of this study. Fed-batch fermentation used to reduce the impact of substrate inhibition could be an alternative for the conventional batch fermentation (Oh et al., 2011).

Furthermore, the impurities contained in the crude glycerol such as inorganic salts (NaCl and KCl) (37,250 mg/L chloride) or methanol (102,953 mg/L) might also influence on the inhibition during the application of crude glycerol (Venkataramanan et al., 2012). The cell inhibition occurred at the inorganic salts higher than 1% wt. (± 10 g/L) was reported (Lee et al., 2012). However, after the dilution to the expected concentration, the initial salts concentration dropped in the range of 1.34–8.58 g/L, which was below the maximum threshold reported. So hypothetically, inorganic salts inhibition might not occur during the fermentation.

Methanol is one of the inhibitors in the crude glycerol. Our preliminary studies indicated that methanol concentration from 1% to 3% decreased the ethanol production as much as 7% to 20%, respectively (Sunarno and Prasertsan, 2018). Methanol higher than 2.3 g/L had been reported to be inhibitive for *E. aerogenes* by reducing substrate utilization (Sarma et al., 2013). After dilution of the crude glycerol to the initial concentration of 21.43 g/L, the methanol content was still higher than the reported threshold (3.46 g/L). Consequently, the total consumption of glycerol also decreased following the increase of methanol concentration (Table 2). Thus, methanol was also likely to contribute to the inhibition when the glycerol concentration increased. Though longer chain alcohol (such as ethanol which is the product in this study) could vent more dangerous impact on the cell, the fact that methanol encounters the cell earlier at the beginning of the fermentation allowing it to control the progression of the fermentation (Patra et al., 2006). Sarma et al. (2013) stated that if the effect of methanol was unavoidable by dissolving it, then purification of glycerol by heating at a temperature of 65 ^{0}C was permissible. The removal of methanol may improve the performance of fermentation. But in certain cases, the cell performance in pure glycerol and crude glycerol medium did not show a significant difference, it could even produce higher ethanol (Chanthoom et al., 2016). This happens if the inhibition due to glycerol is more dominant than the inhibitor. So that even though the impurities have been eliminated, there will be no visible increase in performance. Thus, the purification of crude glycerol must be decided by knowing information about the critical concentration of each inhibitor. However, since crude glycerol purification is cost intensive, the purification of crude glycerol will not be performed in this study to keep the production cost as minimum as possible. Alternatively, the potential of using crude glycerol without pretreatment was further investigated by optimizing the nutrient composition only.

3.3. The effect of the initial C/N ratio

Previous works published so far have been relying on expensive organic supplements such as yeast extract or tryptone as in the complex medium (Ito et al., 2005). This step aimed for simplifying the complex medium using only crude glycerol and tuna condensate (referred to as GT medium). However, due to the heterogeneous nature of tuna condensate composition, the experiment was focused solely on its total nitrogen content (TKN) and the initial carbon to nitrogen (C/N) ratio was chosen as a control variable. The initial crude glycerol concentration was maintained at its optimum level ˜20 g/L (the actual concentration was 23.33 ± 0.48 g/L).

The results on the effect of C/N ratio of the GT medium are shown in Table 3 and Fig. 1 with the control experiment representing the fermentation using the complex medium. Ethanol was the major product alongside with two others byproduct during the fermentation with GT medium and the highest ethanol concentration was observed at the initial C/N ratio of 257 g/g. The overall difference between the control and GT medium was not significant ($p > 0.05$) as the control experiment was statistically classified into both groups, C and D, by Tuckey's pairwise test. This finding suggested that the complex composition (organic nitrogen, inorganic salts, and trace elements) of the complex medium could be replaced by GT medium without causing a significant impact on the ethanol production.

Meanwhile, cell growth and byproduct formation were affected by the initial C/N ratio. The highest cell concentration of 1.65 ± 0.02 g/L was obtained at the optimum C/N ratio of 115 g/g. Nutrient sufficiency is vital for cell growth and affects its continuation. As the cell grew prosperously from high to low C/N ratio (low to high tuna condensate concentration), evidentially, tuna condensate is rich ingrowth promoting substances. This finding is also observed by Prasertsan, et al. (1997) on the growth of *Rhodocyclus gelatinosus* where repeated addition of fresh tuna condensate increased the cell concentration up to four-folds. Contrarily, when the C/N ratio increased (decreased tuna condensate concentration), the cell concentration dropped and the assimilated carbon was moved toward the formation of the metabolite such as 2,3-butanediol (2,3-BDO; $C_4H_{10}O_2$). The advent of 2,3-BDO was eminent at the C/N ratio of 200–365 where up to 5.71 ± 0.15 g/L of 2,3-BDO was acquired along with the decrease in cell concentration.

Since ethanol is a growth-associated product, enhancing cell growth is important. According to Table 3, the complex medium (control) accumulated 18% higher cell concentration than GT medium (C/N ratio of 115 g/g) suggesting that the former was a better medium for growth. Possibly, some components in the complex medium such as inorganic salts and trace elements are complementary, not substitutive to GT medium. Hence, to elaborate the function of inorganic salts and trace

Table 3
The effect of C/N ratio and the addition of inorganic salts on the growth and metabolite production of *E. aerogenes* TISTR 1468.

C/N ratio(g/g)	Final pH	Total glycerol consumption (%)	Biomass (g/L)	Metabolite (g/L)			Ethanol yield (g/g)	Ethanol selectivity (g/g)
				Ethanol	Acetic acid	2,3-BDO		
Control	6.11	64%	2.00 ± 0.05	6.45 ± 0.12^{C,D}	2.66 ± 0.03	0.73 ± 0.01	0.45	1.22
GT medium								
115	5.87	51%	1.65 ± 0.02	6.11 ± 0.33^{D}	3.06 ± 0.19	1.81 ± 0.01	0.51	0.95
200	5.74	78%	1.00 ± 0.04	6.73 ± 0.28^{C,D}	1.45 ± 0.14	5.71 ± 0.15	0.37	0.84
257	5.72	66%	0.40 ± 0.03	7.25 ± 0.31^{B,C,D}	1.47 ± 0.09	5.13 ± 0.17	0.47	1.05
365	5.62	64%	0.12 ± 0.01	6.23 ± 0.03^{D}	1.50 ± 0.06	4.83 ± 0.02	0.42	0.98
GT medium + inorganic salts + trace elements								
115	5.53	100%	2.03 ± 0.02	12.73 ± 0.28^{A}	3.14 ± 0.14	0.62 ± 0.03	0.55	2.24
200	5.67	89%	1.72 ± 0.05	11.54 ± 0.30^{A}	2.25 ± 0.13	0.64 ± 0.01	0.55	2.57
257	5.75	79%	1.48 ± 0.01	8.50 ± 0.29^{B}	2.17 ± 0.03	1.69 ± 0.05	0.46	1.62
365	5.82	79%	1.28 ± 0.06	7.69 ± 0.18^{B,C}	2.09 ± 0.08	2.10 ± 0.04	0.42	1.44
GT medium + phosphate buffer								
115	5.65	100%	2.08 ± 0.03	12.24 ± 0.34^{A}	3.13 ± 0.09	0.67 ± 0.01	0.50	2.08
200	5.73	92%	1.96 ± 0.01	11.73 ± 0.11^{A}	2.44 ± 0.15	0.69 ± 0.03	0.52	2.31
257	5.74	76%	1.86 ± 0.03	8.99 ± 0.23^{B}	2.39 ± 0.04	1.50 ± 0.14	0.48	1.57
365	5.67	79%	1.78 ± 0.01	8.35 ± 0.17^{B}	2.32 ± 0.14	1.81 ± 0.03	0.43	1.42
GT medium with pH controlled (pH ~ 7)								
115	7.18	100%	2.51 ± 0.03	12.33 ± 0.52^{A}	0.35 ± 0.07	0	0.62	4.31
200	7.20	94%	2.37 ± 0.02	11.32 ± 0.39^{A}	0.31 ± 0.13	0	0.61	4.22
257	7.32	87%	2.01 ± 0.03	8.88 ± 0.31^{B}	0.27 ± 0.04	0	0.52	3.89
365	7.15	80%	1.98 ± 0.01	7.98 ± 0.09^{B}	0.11 ± 0.01	0	0.50	3.82

$^{A,\ B,C,D}$Different letters indicate significant statistical difference in ethanol production ($p < 0.05$, Tuckey's test).

elements further, the fermentation on the GT medium supplemented with both components was performed and the result is presented in Table 3. The addition of inorganic salts and trace elements could increase the production of ethanol (1.2–2.1 folds) and acetic acid (1.0–1.5 folds) but reduced the 2,3-BDO (to only about 1/3). Therefore, the ethanol production increased by about 2-fold as compared with the control and GT medium only. The rise of ethanol production was possible following the reduction of 2,3-BDO production. Considering it is a 4-carbon compound, the advent of 2,3-BDO was actually a loss of carbon flux toward ethanol. Reducing 2,3-BDO synthesis is, therefore, important as for each molecule of 2,3-BDO not produced, 2 ethanol molecules will be produced. Besides, during the biosynthesis of 2,3-BDO, the cell used up 1 out of 2 NADH molecules obtained during the catabolism of glycerol while at the same time, ethanol synthesis required 2 NADH molecules (Um et al., 2017). By the addition of inorganic salts and trace elements into the GT medium, the carbon predisposition toward ethanol pathway (the main product) increased and resulted in lower byproduct formation than using sole GT medium (1.5–3 times higher ethanol selectivity).

The effect of the initial C/N ratio on cell growth with the addition of inorganic salts exhibited the same trend. The lower the C/N ratio the higher the cell concentration. The highest cell concentration of 2.03 ± 0.02 g/L was obtained at the C/N ratio of 115 g/g and this result was comparable to the control (complex medium). It proved that one or more components of inorganic salts and trace elements were supporting cell growth. Furthermore, the addition of salts and trace elements also improved the glycerol consumption from 51 to 78% (control) to 79%–100% at the C/N ratio of 115 g/g. From this result, it is justifiable to conclude that the correlation of inorganic salts and tuna condensate was more toward complementarity than replacing each other as what might initially be perceived.

3.4. Screening of inorganic salts

The addition of inorganic salts and trace elements into the GT medium had improved the performance of *E.aerogenes* TISTR 1468. In this way, it is important to select only the components with the most contribution instead of using all of them. Plackett-Burman experimental

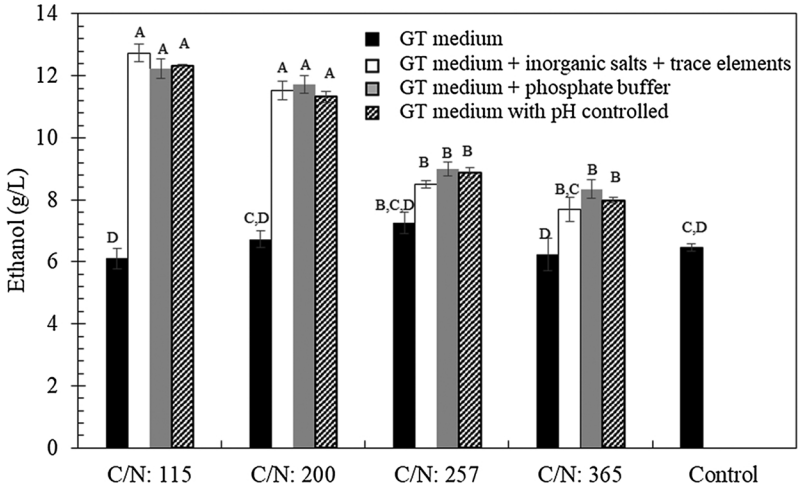

Fig. 1. Ethanol production at various initial C/N ratio with different medium composition.

Table 4
Levels of the variables and statistical analysis of Plackett–Burman design.

Code	Variable (g/L)	Low level (-1)	High level (+1)	Effects (Ex_i)	F-Values	Prob > F[a]	% Contribution
X_1	K_2HPO_4	0	7	1.645	150.7	0.007	56.981
X_2	KH_2PO_4	0	5.5	0.990	54.59	0.018	20.639
X_3	$(NH_4)SO_4$	0	1	0.684	26.03	0.036	9.843
X_4	$MgSO_4$	0	0.25	−0.216	2.597	0.248	0.982
X_5	$CaCl_2$	0	0.021	0.615	21.05	0.044	7.961
X_6	Na_2MoO_4	0	0.12	0.211	2.475	0.273	0.849
X_7	Nicotinic acid (mg/L)	0	2	0.201	2.245	0.293	0.754
X_8	$NiCl_2$ (mg/L)	0	0.02	−0.189	1.994	0.256	0.936
X_9	Trace elements (ml/L)	0	10	−0.224	2.791	0.237	1.055

[a] Values of Prob > F less than 0.05 indicate model terms are significant.

Table 5
Plackett–Burman design matrix for evaluating factors influencing the ethanol production of *E. aerogenes* TISTR 1468.

Run	X1	X2	X3	X4	X5	X6	X7	X8	X9	P_s[a] (g/L)
1	1	−1	1	1	−1	1	1	1	−1	11.59
2	1	1	1	−1	−1	−1	−1	1	1	12.00
3	−1	−1	1	−1	1	1	1	−1	1	10.58
4	1	1	−1	−1	−1	1	1	−1	1	11.98
5	−1	1	1	−1	1	−1	1	1	−1	11.46
6	−1	1	1	1	−1	1	−1	−1	−1	10.66
7	−1	−1	−1	−1	−1	−1	−1	−1	−1	9.25
8	1	1	−1	1	1	−1	1	−1	−1	12.33
9	−1	−1	−1	1	−1	−1	1	1	1	8.57
10	1	−1	−1	−1	1	1	−1	1	−1	11.27
11	−1	1	−1	1	1	1	−1	1	1	10.44
12	1	−1	1	1	1	−1	−1	−1	1	11.66

[a] P_s represented for the average of ethanol production in triplicate experiments.

design accompanied by an analysis of variance (ANOVA) of Design Expert software (version 10) was performed to screen the significant factor upon ethanol production (P_s) and evaluated according to its standardized effects (Ex_i), p-value < 0.05 and the percentage of contribution. All the factors considered in this screening and result of ANOVA are summarized in Table 4. The experimental design matrix is presented in Table 5.

K_2HPO_4 was the most significant constituents with more than 50% contribution (56.9%) for ethanol production while KH_2PO_4 came second with 20.6% contribution. Both combinations made up the phosphate buffer with a total contribution of 77.6%. The confirmation experiment was performed using GT medium at the C/N ratio 115 g/g with the addition of phosphate buffer prescribed as in CG medium. Using only phosphate buffer, the difference in ethanol production was insignificant with full inorganic salts and trace element addition and both were classified into one group (A) by Tuckey's test. Complete substrate consumption was also achieved. In fermentation, phosphate buffer is used to maintain the medium pH from fluctuation or substantial drop due to acid generation from microbial activity. Being the most significant factor has emphasized the fact that *E. aerogenes* is a pH-sensitive strain and ethanol production may occur in merely narrow pH range. $(NH_4)_2SO_4$ and $CaCl_2$ were also significant variables with 9.843% and 7.961% contribution, respectively. Whereas, Na_2MoO_4 and nicotinic acid were insignificant with positive effects for ethanol production. However, to maintain the simplicity of the medium, these four later constituents were ignored because their contribution was far below the phosphate buffer.

3.5. The effect of pH control

As phosphate buffer is barely cost-effective for the industry, the GT medium without phosphate buffer fermented under pH controlling scheme was performed. The pH of the medium was controlled at ˜ 7 by the addition of 3 N NaOH for 72 h of fermentation (Lee et al., 2012). Result in Table 3 showed in an improved performance after the pH controlling scheme. The difference in ethanol production was insignificant as it also belongs to group A according to the result of Tuckey's pairwise. This result confirmed that the phosphate buffer could be eliminated and alternatively, the pH would be controlled by the addition of sodium hydroxide only. At particular pH of 7, the cell was able to convert most of the carbon available into ethanol either glycerol or another carbon. This resulted in higher ethanol yield (0.62 g-ethanol/g-glycerol) than other treatment done before (0.37 -0.55 g-ethanol/g-glycerol), and even 24% higher than the theoretical yield of glycerol to ethanol. Due to the complexity of tuna condensate and crude glycerol, they may contain another carbon besides glycerol indicated by high COD content. This extra carbon could be assimilated by *E. aerogenes* to produce ethanol or to maintain cell viability resulting in a yield higher than the theoretical yield (Nwachukwu et al., 2012). The increase in yield during pH control indicated that pH control was very effective to increase glycerol consumption and ethanol production. Besides, the cell concentration was also increased by 20%. A wide pH gradient between inside and outside the cell requires more ATP for the extrusion or intrusion of a proton to maintain pH homeostasis (Zeng et al., 1990). This causes the depletion on the amount of ATP for cell growth. Controlling the pH near neutral zone hereby helps the cell to conserve more energy so it can be allocated for growth as well as ethanol since it is the growth-associated product (Zeng et al., 1990). This could explain why the growth was better under pH controlling scheme. Furthermore, it was also able to decrease the formation of acetic acid and 2,3-BDO as much as 88.9% and 100%, respectively. It might be because the two by-products were not produced predominantly at pH 7. For example, the optimal production of 2,3-BDO was reported in the pH range of 5.5–6.5, above which lower production rate was observed (Um et al., 2017; Zeng et al., 1990). This led to an increase in ethanol selectivity up to 2-folds higher than using phosphate buffer (at the initial C/N ratio of 115 g/g).

The sensitivity analysis in Fig. 2 showed that crude glycerol had the most significant impact on the ethanol production in compar to the effect of C/N ratio itself. The effect of C/N ratio became significant after the addition of inorganic salts constituent, which lead to the discovery that buffer phosphate was the main contributor. In fact, the effect of C/N ratio at controlled pH showed that it was better than the result of phosphate buffer supplementation. The performance of cell at constant pH showed how important pH is to the metabolism pathway of *E. aerogenes*, signifying the importance of pH control scheme. Initially, phosphate buffer had failed to maintain the pH in all previous experiments that could be seen from the final pH of fermentation which all were below 7. This infers that buffer must be supplemented excessively into the medium which could result in a more expensive production cost (Ito et al., 2005). The pH controlling scheme is alternatively more cost-friendly than phosphate buffer for industrial application. The sensitivity analysis in Fig. 2 showed that the pH controlling was more effective than just adding phosphate buffer and thereby, the fermentation medium can be further simplified consisting only crude glycerol

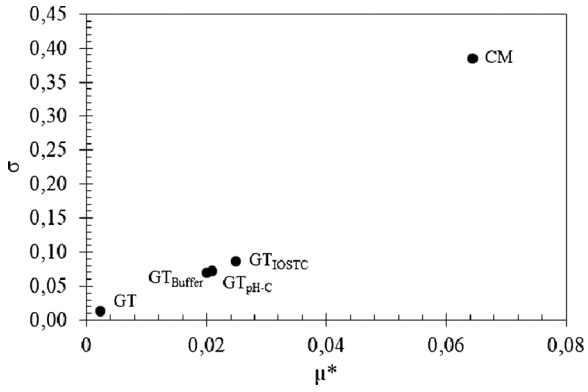

Fig. 2. Graph displaying the Morris sensitivity measures (μ^*) and standard deviation (σ) for input factors (CG: effect of crude glycerol concentration; GT: effect of C/N ratio with GT medium only; GT_{IOSTE}: GT medium with inorganic salts and trace elements; GT_{Buffer}: GT medium with buffer; and $GT_{pH\text{-}C}$: GT medium with pH controlled). Higher σ and μ^* indicates that the change over the factors has a higher sensitivity to the output variable.

and tuna condensate. The simple medium in the previous study produced ethanol as much as 4.96 g/L with a yield of 0.415 g ethanol/g-glycerol (Lee et al., 2017). Nevertheless, this medium still used peptone and citrate buffer. The results of this study produced 2.5 times more ethanol (12.33 g/L) with higher yields (0.62 g-ethanol/g-glycerol). The yield achieved with this simple medium was also higher than the mutant strain (0.56 g-ethanol/g-glycerol) (Nwachukwu et al., 2013). However, mutant strains were able to grow at higher glycerol concentrations (50 g/L), resulting in a high ethanol concentration (25.4 g/L) as well. The ethanol production from this study was also higher than that from *E. aerogenes* ATTC 29007 produced under the optimized medium with crude glycerol as a carbon source (5.29 g/L) during continuous fermentation (Lee et al., 2017).

Process configuration must be appropriately selected to increase substrate utilization especially for the wild strain that is less tolerant at high glycerol concentration. Fed-batch fermentation is then suitable for further investigation.

4. Conclusion

The production of ethanol from crude glycerol and tuna condensate using *Enterobacter aerogenes* TISTR 1468 was optimized. The optimum crude glycerol concentration was ˜20 g/L and gave ethanol production of6.72 g/L.The replacement of complex nutrient by crude glycerol and tuna condensate (GT medium) showed comparable ethanol production. The addition of inorganic salts and trace element into GT medium could further increase the ethanol production to 12.73 g/L. Among the components of the inorganic salts, phosphate buffer played a very important role (77.6% contribution) but it could be replaced by controlling pH at 7 (adjusted with 3 N NaOH). Increase in ethanol production (12.33 g/L), ethanol yield (0.62 g-ethanol/g-glycerol) and selectivity (4.31 g-ethanol/g-byproducts) as well as cell concentration (2.51 g/L) was achieved under the optimum condition.

Acknowledgments

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☙ PAPER II ❧

Improve biotransformation of crude glycerol to ethanol of *Enterobacter aerogenes* by two-stage redox potential fed-batch process under microaerobic environment

(Submitted to Industrial Crops and Products**)**

Improve biotransformation of crude glycerol to ethanol of *Enterobacter aerogenes* by two-stage redox potential fed-batch process under microaerobic environment

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ABSTRACT

Ethanol production from crude glycerol under microaerobic condition was optimized. The aeration rate in the reactor was controlled using redox potential (ORP) as a replacement of a conventional oxygen sensor that became insensitive at low oxygen concentration. The performance of *Enterobacter aerogenes* TISTR1468 in a batch reactor using a simple medium (crude glycerol and tuna condensate medium) was evaluated on various aeration strategies: continuous aeration at 0.5 vvm; controlled aeration at -350 mV and -400 mV ORP; and no

aeration. The 72-h batch fermentation showed that a more reductive environment (lower aeration and ORP value) increased ethanol concentration and yield, but decreased the ethanol productivity. No-aeration fermentation with the lowest ORP value achieved the highest ethanol production (18.78 g/L) and yield (0.94 g-ethanol/g-glycerol). A more oxidative environment (higher aeration and ORP value) fasten the specific growth rate (μ), but ethanol concentration and yield decreased. The optimum specific ethanol production rate (q_p) and glycerol consumption rate occurred at ORP of -400 mV and -350 mV, respectively. When the ORP was very low, the μ decreased following substrate depletion, but this phenomenon was not observed at higher ORP value, an evident that adequate oxygen supply could maintain cell viability. Fed-batch with two-stage aeration strategy (-350 mV and -400 mV ORP) acquired higher ethanol production (30.31 g/L) than fed-batch with single-stage aeration (25.95 g/L) and batch process (12.33 g/L). This study has revived the potential of crude glycerol biotransformation to ethanol and opened up opportunities for optimization of related microaerobic systems other than ethanol.

Keywords: Microaerobic fermentation; Ethanol fermentation; Redox potential; Crude glycerol; Fed-batch fermentation; *Enterobacter aerogenes*.

1. Introduction

For every 10 kg biodiesel produced, 1 kg of glycerol will be generated (Zhou *et al*., 2018). Glycerol derived from biodiesel industry contains various impurities for which selling it directly will be less profitable(Rodrigues

et al., 2019). In addition, with slower growth of downstream industries, crude glycerol utilization has been barely increasing. This potentially burdens the biodiesel industry due to the handling cost associated with it. Consequently, the diversification of crude glycerol to various products could plausibly solve the overflowing of crude glycerol in the market while opens up an opportunity to reap additional incomes.

The diversification of crude glycerol attracts a lot of attention across multiple products, one of which is ethanol. Bio-based ethanol is the largest biofuel in terms of production volume and hence, the largest renewable fuel in the global transport sector. In addition, glycerol-based ethanol has 40% lower production cost than corn-based ethanol because of simpler process configuration and cheaper feedstock's price (Yazdani and Gonzalez, 2007). Ethanol is also the basic building block of various future bio-based chemicals whose commercialization is being promoted (Posada *et al*., 2013). Ethanol as alcohol can also be used as a complement to methanol (co-utilization) in biodiesel production, thereby reducing the dependency on methanol, which is a fossil-based chemical (Bolonio *et al*., 2019; Fadhil and Ahmed, 2018).

Species of Enterobacteriaceae was the most efficient producer in the production of ethanol from glycerol; for examples *E. aerogenes* (0.39 g/g) *Klebsiella pneumoniae* (~ 0.25 g/g), *Escherichia coli* (0.33 g/g), and the newly isolated *Kluyvera cryocresans* (0.30 g/g (Choi *et al*., 2011; Cofré *et al*., 2016; Oh *et al*., 2011; Saisaard *et al*., 2011). Classified as heterofermentative bacteria,

the species of Enterobacteriaceae produce not only ethanol but also hydrogen, succinic acid, lactic acid, acetic acid, propionic acid, formic acid, 1.3-propanediol and 2.3-butanediol (Oh *et al*., 2011). Meanwhile, *E. Aerogenes* produces ethanol as the main metabolite with fewer byproducts when glycerol used as the sole carbon source (Ito *et al*., 2005; Nwachukwu *et al*., 2012). It was also a fast-growing microorganism and easily cultured with various types of substrate, making it one of the most versatile bacteria to date (Zhang *et al*., 2011). *E. Aerogenes* also has its full genome-sequenced, providing a wider opportunity to improve its performance by genetic engineering (Shin *et al*., 2012).

E. aerogenes growing at anoxic environment produced higher ethanol yield than at full aeration (Lee *et al*., 2012). However, the oxygen present at a limited concentration (microaerobic) has been reported to improve ethanol productivity by a couple of folds (Choi *et al*., 2011; Oh *et al*., 2011). In practice, microaerobic fermentation is operated under dissolved oxygen tension (DOT) close to zero (Zeng *et al*., 1994). However, in practice, determining oxygen at trace level concentration has been an arduous task since the conventional oxygen probes would become insensitive under this condition (Liu *et al*., 2017)**.** Hence, strategies have been developed dedicated to maintaining the progression of microaerobic fermentation, including cell respiration control (Franzén, 2003; Zeng *et al*., 1994), constant aeration rate (Oh *et al*., 2011; Saisaard *et al*., 2011), and redox potential control (Liu *et al*., 2013). Cell respiration was controlled by means of keeping the respiratory quotient (RQ) value at constant. RQ is the

molar ratio between carbon dioxide and oxygen by which both profiles were the reflection of the cell's dynamic metabolism. Ethanol production is directly proportional to carbon dioxide release under fermentation route; meanwhile, excess of oxygen supply directs the cell to the respirative pathway where cell growth would be much appreciated than ethanol production. A higher RQ value is preferable, but oxygen must remain sufficient for cell growth. Thus, the aeration is controlled to maintain the RQ at optimum, so the expected metabolite production could be maximized (Zeng *et al*., 1994). Although the metabolite production was successfully upgraded, this type of control seems luxurious for industrial scale, due to the need for robust control mechanism and two indicators are required for oxygen and carbon dioxide. In contrast, supplying a low rate of oxygen is indeed simpler and cheaper, as neither controller nor sensor is needed. The microaerobic process could be achieved conveniently by constant aeration at 0.5 vvm (Oh *et al*., 2011; Saisaard *et al*., 2011). However, this strategy ignores the fact that cell metabolism at each phase of cell growth shall have different oxygen demand, leading to non-stable and uncontrollable process performance. In addition, depending merely on the aeration rate will also complicate the scale-up process because the difference in reactor geometry renders non-identical process behavior due to the difference in the reactor hydrodynamics (such as oxygen transfer rate, mixing time, mass transfer, etc.) (Zeng *et al*., 1994).

The main purpose of limiting oxygen supply in microaerobic fermentation is actually to control the net balance of intracellular reducing

equivalents in favor of ethanol production. Since direct intracellular redox measurement is barely feasible for an industrial scale, control of intracellular redox is then carried out indirectly by adjusting the extracellular redox potential. The correlation between extracellular and intracellular redox potential occurs indirectly through the cytomembrane barrier and regulation of metabolism (Li *et al*., 2019). For microaerobic fermentation, regulating extracellular redox potential had been successfully implemented for high gravity ethanol production using *Saccharomyces cerevisiae* (Liu *et al*., 2016). The advantage of redox potential to regulate the aeration rate is interesting to be explored, particularly on crude glycerol to ethanol biotransformation using *E. aerogenes*, considering it is also carried out under microaerobic conditions. This method of control for ethanol production using *E. aerogenes* has never been done before, so it is worth to investigate.

2. Materials and methods

2.1. Microorganism and pre-cultivation medium

E. aerogenes TISTR1468 purchased from Thailand Institute of Scientific and Technological Research (TISTR) (Pathum Thani, Thailand). The cell was cultivated in a 250 mL flask containing 100 ml of pre-culture medium. It was consisted of crude glycerol (20 g/L), yeast extract (5 g/L), tryptone (5 g/L), $(NH_4)_2SO_4$ (1.0 g/L), $MgSO_4 \cdot 7H_2O$ (0.25 g/L), KH_2PO_4 (5.5 g/L), K_2HPO_4 (7 g/L), $CaCl_2 \cdot 2H_2O$ (0.021 g/L), $Na_2MoO_4 \cdot 2H_2O$ (0.12 g/L), nicotinic acid (2.0 mg/L), $NiCl_2$ (0.02 mg/L) and trace elements (10 mL/L). Trace element solution

used in the medium contained 0.5 g/L $MnCl_2 \cdot 4H_2O$, 0.1 g/L H_3BO_4, 0.01 g/L $AlK(SO_4(_2 \cdot H_2O$, 0.001 g/L $CuCl_2 \cdot 2H_2O$ and 0.5 g/L Na_2EDTA) (Ito *et al*., 2005). The pH of the medium was adjusted to 8.0 prior to sterilization. Inoculum size of 10% (v/v) was used and the flask was incubated at 37 ºC on a rotary shaker at 150 rpm for 24 h.

2.2. The ethanol production medium

The crude glycerol was obtained from Fatty Acid Methyl Ester Pilot Plant, Faculty of Engineering, Prince of Songkla University (Songkhla, Thailand). Tuna condensate was collected from Songkla Canning Co. Ltd., Thai Union Group (Songkhla, Thailand).In our previous study (Sunarno *et al*., 2019), the simple medium so-called crude glycerol and tuna condensate (GT) medium was developed to be an alternative cheap nutrient to replace the expensive complex medium. The GT medium was consisted of (v/v) 9 % of crude glycerol, 14% of tuna condensate, and 77% of deionized water to achieve final glycerol concentration of approximately 20 g/L. The medium was sterilized at 121 ºC for 15 min.

2.3. Batch cultivation

The fermentation was performed in a 5-L desktop continuous stirred tank reactor (CSTR) (B.E. Marubishi model MDL) equipped with dissolved oxygen (DO)sensor and an oxidation-reduction potential (ORP) controller connected directly to the aerator pump (Figure 1). Before entering the reactor, the air was pre-filtered by an autoclavable 0.22 μm nylon filter. The 2.7

L sterilized GT medium was prepared and the pH was pre-adjusted in the range of 7.0 – 8.0 by adding NaOH and/or HCl (6.0 N). Then, the medium was inoculated with 300 mL of inoculum (10% v/v). The fermentation was carried out at 37 ºC for 72 h at the constant pH and agitation of 7.0 and 60 rpm, respectively. The intensity of aeration was varied from constant aeration at 0.5 vvm (B_C), partial aeration controlled at ORP value of -350 mV (B_{350}) and -400 mV (B_{400}), and without aeration (B_0). In controlled aeration, aerator was activated only when the ORP value fell below the reference point (-350 and -400 mV). Meanwhile, during the non-aerated condition, only oxygen in the 2-L reactor headspace was consumed assisted by 60-rpm agitation. During the course of fermentation, a sample of 15 ml was withdrawn every 3 h for determination of dry cell weight (DCW), glycerol and metabolites concentration.

2.4. Fed-batch cultivation

The preparation of fed-batch fermentation was similar to the batch fermentation described previously. The cultivation time lasted for 84 h with 15 mL of samples withdrawn every 3 h. The fed-batch fermentation was initiated at a 3000 mL medium with an initial glycerol concentration of 20 g/L. The incubation was conducted at a constant pH, temperature, and agitation rate (7.0, 37 ºC and 60 rpm) for 84 h cultivation. Various aeration strategy was studied. Single-stage aeration (FB-1) was conducted by maintaining constant aeration at 0.5 vvm throughout fermentation. Meanwhile, two-stage aeration was performed by combining two different aeration strategies sequentially. The aeration for FB-

2 and FB-3 process was constant at 0.5 vvm up to 36 h of fermentation. Then, the aeration was switched to controlled aeration at ORP value of -350 mV (FB-2) and -400 mV (FB-3), respectively up to the end of fermentation. The fed-batch was aimed to maintain specific cell growth rate by controlling glycerol concentration. The replenishment of crude glycerol was done when the concentration had dropped to 10 g/L. In such a way, as much as 125 mL of sterilized fresh crude glycerol (undiluted) was introduced twice into the reactor at 12 h and 36 h of fermentation to replenish glycerol concentration back to 20 g/L.

2.5. Method of analysis

Of the 15 mL samples taken, 5 mL of the sample was used to determine the DCW in three replications. The culture broth was centrifuged (6708×*g*; 10 min) to remove cells. The supernatant was transferred to another tube and the precipitate (cell mass) was rinsed with phosphate buffer (10 mM; pH 7.0). The precipitate was dried at105 ºC for 24 h), cooled in the desiccator to room temperature for 6 h and weighed to determine DCW (Jo *et al*., 2008). The supernatant was filtered using nylon filter (0.22 µm) into a vial (three replications). These vials were used to determine substrate and metabolite concentrations using high-performance liquid chromatography (HPLC) (Agilent Technologies 1200 series, USA). The HPLC was equipped with an Aminex HPX-87H ion exclusion column (300 mm x 7.8 mm) (Bio-Rad, USA). The column temperature was kept at 80°C and the sample volume was 10 µL. The

mobile phase was H_2SO_4 (5 mM) at a flow rate of 0.6 mL/min (Saisaard *et al*., 2011).

2.6. Kinetic parameters

The specific growth rate (μ) was determined in the exponential growth phase by plotting ln (DCW) vs time (h) and μ (h^{-1}) is the gradient of the curvature (Equation 1). The specific rate of ethanol formation (q_p) was the average ethanol production per hour for each gram of cell (g-ethanol/g-DCW/h) (Equation 2). The specific rate of glycerol consumption (q_s) was the average glycerol consumption per hour for each gram of cell (g-ethanol/g-DCW/h) (Equation 3). The estimation of μ, q_s, and q_p was performed using non-linear curve fitting of MATLAB 2017a (Jin et al., 2012).

[1] $$\mu = \frac{1}{X}\frac{dX}{dt}$$

[2] $$q_P = \frac{1}{X}\frac{dP}{dt}$$

[3] $$q_S = \frac{1}{X}\frac{dS}{dt}$$

Glycerol to ethanol yield ($Y_{P/S}$) was defined as the ratio between maximum ethanol productions to total glycerol consumption (g-ethanol/g-glycerol). The selectivity of ethanol was defined as the ratio of ethanol to the byproduct (acetic acid and cells) (g-ethanol/g-byproducts). The volumetric rate

of ethanol production or productivity (g/L/h) was determined as the total production of ethanol divided by the total fermentation time (Yang et al., 2013).

2.7. Carbon recovery

Carbon recovery was calculated according to the net reaction of mass conservation corresponding to the formation of each end-product (Barbirato et al., 1995).

[4] Ethanol : $C_3H_8O_3 + ADP \rightarrow C_2H_6O + HCOOH + ATP$

[5] Acetate : $C_3H_8O_3 + H_2O + 2\ NAD^+ + 2\ ADP \rightarrow C_2H_4O_2 + HCOOH + 2(NADH + H^+) + 2\ ATP$

The chemical formula of $C_4H_7O_2N$ was assumed as the biomass of *E. aerogenes* according to the chemical composition of various aerobic bacteria with a molecular weight of 156.64 g/mol (Bauer and Ziv, 1976).

[6] Biomass : $4/3\ C_3H_8O_3 + NH_3 + Energy \rightarrow C_4H_7O_2N + 2\ H_2O + 8/6\ (NADH + H^+)$

For each of ethanol and acetic acid produced, a mole of formate is generated and each of biomass contained four moles of carbon. Hence, the calculation of carbon recover (CR) was performed using the equation:

[7] $$CR = \frac{3 \times ([EtOH] + [Ac.\,Acid]) + 4 \times [Biomass]}{3 \times [Glycerol]}$$

3. Results and discussion

3.1. Effect of the aeration condition and ORP profiles during ethanol production using batch process

Various aeration methods during microaerobic fermentation were carried out to compare the effectiveness in increasing ethanol productivity, i.e. continuous aeration at 0.5 vvm (B_C); aeration by ORP controller at -350 mV(B_{350}) and -400 mV(B_{400}); and without aeration (B_0). The ORP profiles of redox potential normally shown in the form of a bathtub curve with three zones namely declining, basin, and uprising (Lin *et al*., 2010).All fermentations were initiated at the ORP value of -190 to -197 mV or equivalent to 3.5% DO concentration(Figure 2). In DO profile (Figure 2A), the oxygen concentration fell close to 0% defining two distinctive phases: an aerobic phase characterized by decreasing DO concentration and followed by microaerobic phase indicated as zero dissolved oxygen concentration. The time to reach the microaerobic phase was varied depending on the aeration strategy such as 6 h for B_0, 9 h for B_{350} and B_{400} and 12 h for B_C.

In the ORP profile, the full bathtub curves only observed when continuous aeration (B_C) took place (Figure 2.A). The basin zone was observed in all aeration strategies approximately after 12 h fermentation. The average range of ORP achieved by B_C was -323±8.1 mV and dropped to -448±14 mV from non-aeration condition (B_0). Uncontrolled ORP system such as B_C and B_0 tends to have a basin zone with fluctuating ORP value, which also found by Liu

et al. (2015). However, controlling the input of aeration by ORP could result in a steadier basin zone as seen in B_{350} and B_{400}. The ORP profile was actually the net redox in the extracellular environment. If cell activity is high, the ORP value tends to be more negative due to the accumulation of reducing equivalents and the decrease in ORP could be countered by adding oxygen through aeration. Fluctuation is a sign of viable cell activity; thus, the length of the basin zone determines the duration of ethanol production for which the longer it is, the better the fermentation will be (Liu *et al*., 2011). The termination of the basin zone could be influenced by many factors, such as substrate depletion or product inhibition as both could disturb cell viability (Thani *et al*., 2016). The B_C process had the shortest basin zone which was possibly due to early substrate depletion and/or acetic acid accumulation, but not because of ethanol inhibition. This was confirmed by comparing the result with B_{350}, B_{400}, and B_0 (see Figure 3), where cells were exposed to higher ethanol concentrations (than in B_C) but the cell remained viable by actively consumed glycerol or produced ethanol as long as glycerol had not run out. This indicated that the decrease in cell activity was not due to inhibition of ethanol. Termination of the basin zone resulted from substrate depletion was also observed by Liu *et al*. (2015) using glucose as a substrate. This condition could be utilized as a sign to start the next batch cycle for the repeated batch process.

In the continuous aeration (B_C), after the basin zone, the ORP value began to re-rise (the uprising zone) at 42 h of fermentation. This zone was the

period where cell activity started to slow down as indicated by a low oxygen dissimilation (DO rose from 0% to 3.35%) followed by the cessation of ethanol production (Figure 3.A). This was also observed at B_{350} and B_{400} except that the uprising of ORP was not as significant as in B_C. This happened because the aeration in B_C was held continuously, whereas B_{350} and B_{400} were not. The inactivation of aeration by the end of fermentation in B_{350} and B_{400} occurred because the ORP no longer fell below the reference value as the production of reducing power decreased (the cell began to be inactive). Inactivation of aerator had caused the ORP value in both processes to increase more slowly than the B_C since oxygen was sourced only from the 2-L reactor headspace. However, if the cell remained active, this zone may not appear, causing an extension of the basin zone, such as in B_0 (Liu *et al*., 2015).

The reliability of the DO sensor to detect oxygen at low concentration (~ 0 %) required for the microaerobic condition led to the need of more sensitive measurement, in this case, the redox potential (ORP) sensor (Zeng *et al*., 1994). Unlike the DO sensor that specifically measures the presence of oxygen, the ORP sensor measures the net result of the redox reaction between the reducing and the oxidizing agent in the fermentation broth (Liu *et al*., 2011). Although not as specific as the DO sensor, the success of the ORP sensor in monitoring fermentation at low oxygen concentrations is motivated by a high reduction potential (E^0 = +820 mV) of oxygen (Liu *et al*., 2013). So that, even when oxygen is at a trace level, the ORP sensor will remain sensitive to it as the

net redox of the fermentation broth will become more positive. Here, the ORP sensor successfully monitored and controlled the oxygen concentration throughout the fermentation. This has opened up opportunities to explore more characteristics of the microaerobic process, which were initially difficult to do with the DO sensor (Liu *et al*., 2017).

3.2. The effect of aeration strategy during batch ethanol production in a 5L CSTR

Various aeration strategies in microaerobic conditions (B_C, B_{350}, B_{400}, and B_0) were studied and the results are summarized in Figure 3 and Table 1. In general, the increase in aeration intensity increased ethanol productivity (g/L/h), the specific growth rate and cell concentration. However, higher aeration intensity reduced ethanol production (g/L) and the conversion yield of ethanol ($Y_{P/S}$). The highest ethanol concentration and yield were actually achieved during the non-aeration system (B_0) as much as 18.78 g/L and ethanol yield of 0.94 g/g, 88% higher than the theoretical yield. This also occurred in our previous study since tuna condensate itself contains high organic carbon indicated by its high COD value (Sunarno *et al*., 2019; Boukaew *et al*., 2018). This excess of organic carbon was likely to be assimilated by *E. aerogenes* as an additional carbon and energy source resulted in an extra ethanol synthesis. However, the specific ethanol production rate (q_p) of B_0 (0.120 g-ethanol/g-cell/h) was only second to the controlled aeration B_{400} (0.133 g-ethanol/g-cell/h), indicating that the optimum condition for the cell to produce ethanol might

actually take place at -400 mV. Besides, the specific glycerol consumption rate (qs) of B_{400} was not significantly different from -350 mV (B_{350}) (0.259 and 0.253 g-glycerol/g-cell/h, respectively).

Figure 4 shows the effect of glycerol consumption on percentage relative specific growth rates (μ) at various aeration methods. The percentage relative of μ (%) is the change of μ relative to its initial rate (μ_0) due to substrate depletion. This value was calculated by dividing the μ during the progression of fermentation with its initial μ_0 when the concentration of glycerol was still 20 g/L. The figure implied that when the aeration was limited (a more reductive external environment), the concentration of the substrate appeared to be the sole limiting factor for cell growth. It was confirmed by B_{350}, B_{400}, and B_0 where the cell growth rate linearly decreased relative to the initial rate as the substrate was consumed, which resulted in a lower biomass concentration. In contrast, when the aeration was held continuously, such as in B_C (a more oxidative external environment), the growth could be improved (as discussed above) and maintained until the glycerol was 50% consumed (approximately 10 g/L glycerol) before it finally decreased following the substrate depletion.

In the presence of electron acceptor (aerobic condition), the incoming glycerol is captured by glycerol kinase (GK) to produce glycerol-3-phosphate (G3P) with one mole of ATP consumption. G3P is further converted to dihydroxyacetone phosphate (DHAP) by aerobic glycerol-3-phosphate dehydrogenase (ae-G3PDH). From here, DHAP enters the glycolysis pathway to

pyruvate where only one mole of NADH and two moles of ATP are produced resulting in one mole of net ATP. Meanwhile, in the absence of electron acceptor (anaerobic condition), glycerol phosphorylation was performed by two successive enzymatic reactions catalyzed by glycerol dehydrogenase (glyDH) and dihydroxyacetone kinase (DHAK) to produce DHAP (Ruch *et al*., 1974). This phosphorylation consumes one mole of ATP and produces one mole of NADH resulting in one mole of net ATP and two moles of NADH by the end of glycolysis.

The tendency of utilizing carbon for cell growth rather than ethanol production could be the signal of a more dominant glycerol kinase (GK) activity, as this pathway tends to accumulate ATP and lack of NADH per glycerol consumed. The presence of ATP has a more positive influence on the initial velocity of GK than the glycerol concentration itself (Applebee *et al.*, 2011; Holtman *et al.*, 2001). Thus, cell growth could be less sensitive toward glycerol depletion when ATP is in excess due to the stability of GK activity. This might explain why at the beginning of the B_C process (Figure 4), the cell growth rate could increase by over 100%. As confirmed through Figure 2.B and 3.A, the glycerol consumption from 20 g/L to 10 g/L in B_C actually occurred in the aerobic phase. It is likely that the intracellular compartment was high in both ATP concentration and GK activity as likely observed by Durnin *et al.* (2009). After that, the cell entered the microaerobic phase (less ATP production) and cell growth started to decelerate along with the decrease in substrate concentration.

Despite fast cell growth, ethanol production during the aerobic phase would not be exaggerated as shown by the decrease of ethanol yield that is decreased when more intensive aeration was applied (Table 2). Ethanol production of *E. aerogenes* is carried out by two NADH-linked enzymes including alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH). Meanwhile, only 1 NADH per molecule of glycerol is available after pyruvate is formed requiring the cell to break 1 more glycerol molecule. This pathway is certainly less energy-efficient than converting NADH directly to ATP through the electron transport chain (ETC). Thereby, ethanol production under more intensive aerobic condition would be lower.

In absence of terminal electron acceptor (anaerobic condition), the intracellular NAD+ will gradually diminish as it is used as the cofactor of glyDH at the initial pathway of glycerol dissimilation. To avoid the shortage of NAD+, it must be regenerated so the substrate uptake could be sustained. With the absence of oxygen, cells can only regenerate NAD+ through ADH and ALDH. The tendency to produce more ethanol at limited oxygen concentration could be the reflection of higher ADH and ALDH activity in order to recalibrate the intracellular ratio of NADH/NAD+. Glycerol dissimilation for cell maintenance can be sustained, even though the progression of the fermentation became slower. Durnin *et al*. (2009) stated that during microaerobic fermentation, the aerobic and anaerobic pathway were active but the enzyme activity responsible in both processes were changing dynamically according to the level of oxygen

concentration. The more oxygen is present, the activity of GK and ae-G3PDH would be more dominant than the other enzymes. Meanwhile, the less oxygen presence the activity of glyDH and DHAK would be more dominant.

On the other hand, by-product such as acetic acid was prominent at B_C and B_{350}. The previous report also confirmed that higher oxygen supplies decreased ethanol production, but increased acetic acid and lactic acid products along with faster cell propagation (Choi *et al*., 2011; Nwachukwu *et al*., 2012). Meanwhile, acetic acid did not appear in two other aeration strategies (B_{400} and B_0). In *E. coli*, the species in the same family of Enterobacteriaceae, NADH has been reported to be one of the inhibitors of phosphotransacetylase, the enzyme that catalyzes the conversion from pyruvate to acetyl-phosphate (a precursor of acetic acid) (Campos-Bermudez *et al*., 2010). This accumulation of NADH during oxygen limitation can possibly be the cause of the suppression of acetic acid production. Reduced acetic acid production is beneficial, considering *E. aerogenes* is more susceptible to acetic acid than ethanol itself (Zeng and Deckwer, 1991).

The intensity of aeration has been shown to regulate the rate of fermentation kinetics associated with the level of oxygen supply. Controlling oxygen concentration during the microaerobic fermentation process is, therefore, important to ensure the cell metabolism favors ethanol production. Oxygen is an excellent electron acceptor due to its high reduction potential. Oxygen will be utilized by the cells to convert the vast reducing equivalents (NADH) to ATP via

electron transport chain (ETC), leading to more progressive cell growth but lower ethanol production. In contrast, minimum oxygen concentration rendered slower growth kinetic behavior but higher ethanol production.

Carbon recovery (CR) was evaluated from biomass, ethanol and acetic acid concentration (end-products) divided by the total glycerol consumption (substrate) (Ellis et al., 2012). According to the analysis of carbon recovery (Table 3), reducing aeration intensity (from B_C to B_0) showed increased in carbon recovery to ethanol which supported the finding that oxygen deprivation led to more dominant ethanol production. In contrast, predisposition of utilizing carbon for cell growth under more intensive aeration was also observed by CR analysis. Results of this study show overall carbon recovery (CR) values higher than one, suggesting that the medium contained a vast of readily assimilable carbon sources beside glycerol. This was mentioned in our previous study as the benefit of using tuna condensate (Sunarno *et al.*, 2019). Looking at its total carbon recovery alone, the non-aeration process (B_0) seems to have slightly better carbon utilization than the rest of the process.

Previously, the batch process had been tested using two-stage aeration where the first stage was micro-aerated to stimulate cell growth and after the cell reached the maximum, fermentation continued using anaerobic processes (Saisaard *et al*., 2011). This process produced 24.5 g/L ethanol from 43.75 g/L crude glycerol for 32 h. Meanwhile, the ethanol concentration in this study was still low given that the optimum concentration of glycerol used was also low (~

20 g/L), so a fed-batch process was proposed to boost substrate utilization. Information about the effect of aeration on batch reactors would be used as a means for consideration.

3.3. Single-stage and two-stage aeration on ethanol production using fed-batch process

Fed-batch fermentation was carried out to increase ethanol production by maintaining high cell growth and improving substrate utilization. In this study, the fed-batch with intermittent feeding was chosen due to its ease of operation (Asenjo, 1994). All fed-batch fermentation began at an initial glycerol concentration of ~ 20 g/L. When glycerol consumption had reached 50% (about 10 g/L); fresh crude glycerol was fed into the reactor to restore glycerol concentration to 20 g/L to maintain the specific growth rate (μ). This lower limit was decided because the cell's specific growth rate had decreased significantly when glycerol fell below ~10 g/L (as seen in Figure 3). Tuna condensate was not included to simplify the process medium. Then, various aerations strategies were combined to achieve optimal ethanol concentration including single-stage aeration and two-stage aeration. Single-stage aeration was performed using continuous aeration of 0.5 vvm (FB-1). Two-stage aeration consisted of: a) continuous and controlled aeration at ORP -350 mV (FB-2), b) continuous and controlled aeration at ORP -400 mV (FB-3), and c) aeration controlled at ORP -350 mV and -400 mV (FB-4). The results are shown in Figure 5.

The first configuration (FB-1) was chosen considering that cell growth and glycerol consumption occurred most rapidly under continuous aeration. Despite low ethanol production, fed-batch cultivation was expected to increase ethanol concentration by increasing substrate utilization. The glycerol fell to 10 g/L after 12 h of fermentation and the working volume had decreased to 2925 mL due to sample withdrawal at every 3 h. The crude glycerol in the reactor was replenished back to 20 g/L by adding 125 mL of fresh crude glycerol and the volume increased to 3050 mL. Glycerol concentration was decreased to 10 g/L after 21 h fermentation (or 33 h from the initial time of cultivation) and the remaining medium after sample withdrawal was 2930 mL. Here, glycerol concentration was replenished by adding 125 mL fresh crude glycerol and led the volume increased to 3055 mL. By the end of fermentation (84 h), the remaining volume was 2785 mL. The FB-1 could produce up to 25.95 g/L ethanol or approximately twice that of its batch counterparts (12.33 g/L) (Figure5.A). In addition, the consumption of glycerol in FB-1 also increased 2-fold which indicated that the performance of cells in consuming glycerol and producing ethanol was not much different in the two reactor configurations. This can be confirmed from the yield of the two processes in Table 1 and Table 2, which were relatively the same. However, to achieve two-fold of ethanol production and glycerol consumption, it required a minimum of 72 h, twice longer than the time required by a batch process, leading to a slight decrease in ethanol productivity to 0.344 g/L/h. The slowdown in ethanol production could

be triggered by the slowdown of cell growth when fermentation had already run halfway (after 33 h). Potential reduction values of the ORP sensor also notified the decreasing of cell activity, noting that the value became more positive exactly when cell growth began to slow down. In addition, from Table 3, the carbon predisposition to the cell was lower in comparison with the batch process (B_C) counterparts confirming that the cell growth in FB-1 was not as good as in the batch process (B_C). An increase in acetic acid, methanol, and ethanol could also be the reasons for this phenomenon. Although cell growth slowed down, cell concentration still increased and had not completely stopped. Cells appeared to enter the decline phase just after 72 h. This suggested that maintaining aeration at the end of the fed-batch fermentation benefit to neither cell growth nor ethanol production. This led to the next process configuration, two-stage aeration. Due to plausible inhibition aforementioned previously, sustaining high cell growth rate would be difficult. Thus, the goal of the final stage was changed to produce ethanol as a priority. Here, the aeration on the final stage of the fed-batch process, which turned out to be less effective using continuous aeration, was now controlled by the ORP (FB-2 and FB-3). Considering that under oxygen deficiency, the conversion of glycerol to ethanol was higher than the continuous aeration process. The redox potential-driven aeration implemented at the final stage of the fed-batch was successful in increasing the ethanol concentration to 28.21 g/L and 30.21 g/L for FB-2 and FB-3, respectively. These concentrations were achieved with a minimum fermentation time of 75 h. In term of glycerol

consumption, FB-1 and FB-2 were not much different, except that FB-2 required more time. However, FB-3 only consumed 38.72 g/L of glycerol up to 84 h fermentation or decreased by 8% from total glycerol consumption in FB-1. This indicated that, indeed, the consumption of glycerol was slower, but glycerol could be converted more towards ethanol by means of aeration control. It can be seen in Table 2 and Table 3 that the yields and carbon recovery to ethanol of FB-2 and FB-3 were higher than FB-1. Another advantage of using two-stage aeration was the 50% decrease in acetic acid concentration. Acetic acid production is the main side effect in a continuous aeration system and was suppressed when aeration was being limited as explained previously, so its concentration was barely increased. This condition was very beneficial for *E. aerogenes*. This decrease also had an impact on the increase of ethanol selectivity above 6.0 g-ethanol/g-byproducts in FB-2 and FB-3.

Aeration control in microaerobic fermentation using redox potential provides flexibility in process optimization, especially in ethanol production. It was previously explained (in section 3.2) that continuous aeration was beneficial for accelerating cell growth, but not ethanol production and the opposite was the case with no aeration. That illustrated that by controlling aeration using redox potential, the optimal point between cell growth and ethanol production could be achieved. This then became the rationale for testing the two-stage aeration (FB-4). In this configuration, the aeration at the first stage was controlled at -350 mV and then to -400 mV in the next stage. Although in this

condition cell growth would not be as fast as continuous aeration; the yield of the ethanol conversion, specific ethanol production rate and glycerol consumption rate were higher than in continuous aeration. Similar to the FB-1, FB-2, and FB-3, the initial working volume was set to 3000 mL with initial glycerol concentration of 20 g/L. The results (Figure 5.D) showed that it took 24 h to complete the first stage with an ethanol concentration of 11.51 g/L and the volume decreased to 2865 mL. Then, fermentation continued to the second stage by adding 125 mL fresh crude glycerol (working volume increased to 2990 mL) and the aeration was controlled at -400 mV, a condition where the specific ethanol production rate was optimized. At the end of fermentation, the highest ethanol concentration that could be achieved was ~30 g/L, equivalent to the FB-3 process. This concentration was achieved by consuming less substrate, which was 35.37 g/L, making the highest yield of glycerol to ethanol compared to the other process configurations. More importantly in this process, the production of acetic acid could be fully suppressed. Then, a one-time addition of glycerol was also beneficial because methanol (an inhibitor) did not accumulate. By this, cell viability could be secured and thereby, improved glycerol conversion to ethanol.

Controlling the microaerobic process in different ORP values resulted in a significantly different result in terms of ethanol production, substrate consumption, cell growth, and even by-product production. Not only aeration was important, but the concentration of inhibitors must also be suppressed as it could further improve the ethanol production by securing cell

viability. The best result achieved in this study was 30 g/L. Two-stage aeration at -350 mV and -400 mV (FB-4) was considered more effective because it consumed less substrate and used a moderate aeration requirement so that operating cost could be reduced. The carbon recovery analysis of fed-batch fermentation also showed that FB-4 resulted in a better overall carbon recovery, particularly to ethanol. Furthermore, the concentration of inhibitors such as acetic acid and methanol from the impurities of crude glycerol could also be lowered.

The ethanol concentration in this experiment (~30 g/L) was the highest for wild species of *E. aerogenes* using the simplest medium (Saisaard *et al*., 2011; Yuwa-amornpitak and Chookietwatana, 2016). In addition, ethanol concentrations of wild species *E. aerogenes* also offset the species of mutant, but the mutant species had higher productivity (Oh *et al*., 2011). *E. aerogenes* TISTR1468 had higher ethanol productivity than the yeast strain of *Pachysolen tannophilus* CBS404 (Liu *et al*., 2012). *E. aerogenes* mutant strain could produce up to 38.32 g/L ethanol by deleting the lactic acid pathway and increasing the expression of alcohol dehydrogenase (ADH) (Thapa *et al*., 2015). This opened up opportunities in the future for further exploration particularly to increase ethanol concentration. Increased ethanol concentration above 40 g/L would be preferred because at this concentration the downstream process can run efficiently (Huang and Zhang, 2011). Microaerobic fermentation using two-

stage ORP controlled aeration could also be implemented for another metabolite product, especially acid production such as lactic acid and succinic acid.

Conclusion

Aeration greatly influences the performance of the microaerobic process and the use of ORP sensor was proven to facilitate the control. In batch process cultivation, increasing the intensity of aeration could increase the cell growth and volumetric ethanol productivity (g/L/h). Under continuous aeration conditions, cell growth was not only influenced by the concentration of substrates, but also by oxygen concentration. Decreasing substrate concentration was not followed by a decrease in cell growth provided the substrate concentration was still above 10 g/L. However, the higher intensity of aeration could reduce the final concentration and yield of ethanol and the optimum specific ethanol production rate was occurred at -400 mV ORP. The highest ethanol concentration in the batch process was 18.78 g/L with a yield of 0.94 g/g, in conditions without aeration.

Ethanol concentration was then increased using a fed-batch process. Variation in aeration also influenced the performance of the fed-batch process. The best aeration strategy was two-stage aeration, i.e. FB-3 and FB-4 processes in which ethanol concentrations up to 30 g/L was achieved. However, the performance of FB-4 was considered better because of its higher ethanol yields, reduction of acetic acid production, and only one-time addition of crude glycerol. This can avoid the accumulation of methanol in the reactor. This two-

stage aeration strategy is also useful for the production of other metabolites produced under microaerobic condition**.**

Acknowledgments

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Table 1. Result of batch cultivation of ethanol production using crude glycerol under various aeration strategy

Parameter	Continuous aeration (B_C)	Controlled aeration at -350 mV (B_{350})	Controlled aeration at -400 mV (B_{400})	No aeration (B_0)
Max. cell (g/L)	2.83	2.58	2.51	2.28
Max. ethanol (g/L)	12.23	14.37	16.48	18.78
Max. acetic acid (g/L)	0.73	0.45	0	0
Specific cell growth rate (μ) (h^{-1})	0.098	0.066	0.053	0.032
Specific ethanol production rate (q_p) (g-ethanol/g-DCW/h)	0.067	0.116	0.133	0.120
Specific glycerol consumption rate (q_s) (g-glycerol/g-DCW/h)	0.195	0.259	0.253	0.147
Ethanol yield (g-ethanol/g-glycerol)	0.62	0.65	0.72	0.94
Ethanol selectivity (g-ethanol/g-byproducts)	3.46	4.74	6.57	8.22
Volumetric ethanol productivity (g/L/h)	0.363	0.342	0.343	0.261
Fermentation time (h)	34	42	48	72

Table 2. Result of fed-batch cultivation of ethanol production using crude glycerol under various aeration strategy

	Single-stage aeration	Two-stage aeration			
	FB-1	Stage	FB-2	FB-3	FB-4
Aeration	0.5 vvm.	I	0.5 vvm.	0.5 vvm.	-350 mV
		II	-350 mV	-400 mV	-400 mV
Max. ethanol concentration (g/L)	25.95		28.21	30.21	30.31
Max. acetic acid concentration (g/L)	1.31		0.70	0.63	0
Total glycerol consumption (g/L)	42.21		41.88	38.72	35.37
Volumetric ethanol productivity (g/L/h)	0.344		0.342	0.374	0.409
Ethanol yield (g-ethanol/g-glycerol)	0.61		0.67	0.78	0.86
Ethanol selectivity (g-ethanol/g-byproducts)	4.54		6.24	6.78	7.05
Total fermentation time (h)	72		75 - 84	75 - 84	72 - 84

Table 3. Carbon recovery (CR) of batch and fed-batch process at various aeration strategy

Process	Glycerol cons. (mmole)	Biomass (mmole)	Products (mmole)		CR of glycerol to biomass	CR of glycerol to ethanol	Overall carbon recovery
			Ethanol	Acetic acid			
Batch							
B_C	600.01	59.93	734.66	33.37	0.13	1.22	1.41
B_{350}	684.66	52.22	856.21	20.57	0.10	1.25	1.38
B_{400}	689.43	43.99	981.93	0.00	0.09	1.42	1.51
B_0	598.22	39.96	1099.31	0.00	0.09	1.84	1.93
Fed-Batch							
FB-1	1350.39	75.25	1683.54	61.30	0.07	1.25	1.37
FB-2	1339.81	65.23	1883.29	34.40	0.06	1.41	1.50
FB-3	1239.05	75.91	1952.41	25.55	0.08	1.58	1.68
FB-4	1101.31	81.68	1899.63	0.00	0.10	1.72	1.82

* The elemental composition of *E. aerogenes* was $C_4H_7O_2N$ with molecular weight of 156.64 g/mol

Table 4. Performance comparison of ethanol production using fed-batch cultivation with two-stage aeration

Microorganism	Method	Aeration condition	Total ethanol production (g/L)	Final ethanol yield (g/g)	volumetric ethanol productivity (g/L/h)	References
E. aerogenes TISTR1468	Fed-batch (two-stage aeration)	Micro aerobic	30.31	0.86	0.41	Present study
E. aerogenes G2WG	Fed-batch	Micro aerobic	13.07	0.35	0.36	Yuwa-amornpitak and Chookietwatana (2016)
E. aerogenes TISTR1468	Batch (two-stage aeration)	Micro aerobic-anaerobic	24.50	0.54	0.51	Saisaard *et al.* (2011)
Enterobacter aerogenes SUMI2008 (mutant strain)	Batch	Micro aerobic	38.32	0.48	0.49	Thapa *et al.* (2015)
Escherichia coli MG1655	Fed-batch	Anaerobic	7.58	0.33	0.05	Cofré *et al.* (2016)
Klebsiella pneumoniae (mutant strain)	Fed-batch	Micro aerobic	31.0	0.45	1.20	Oh *et al.* (2011)
Pachysolen tannophilus CBS404	Fed-batch	Micro aerobic	28.1	0.13	0.11	Liu *et al.* (2012)

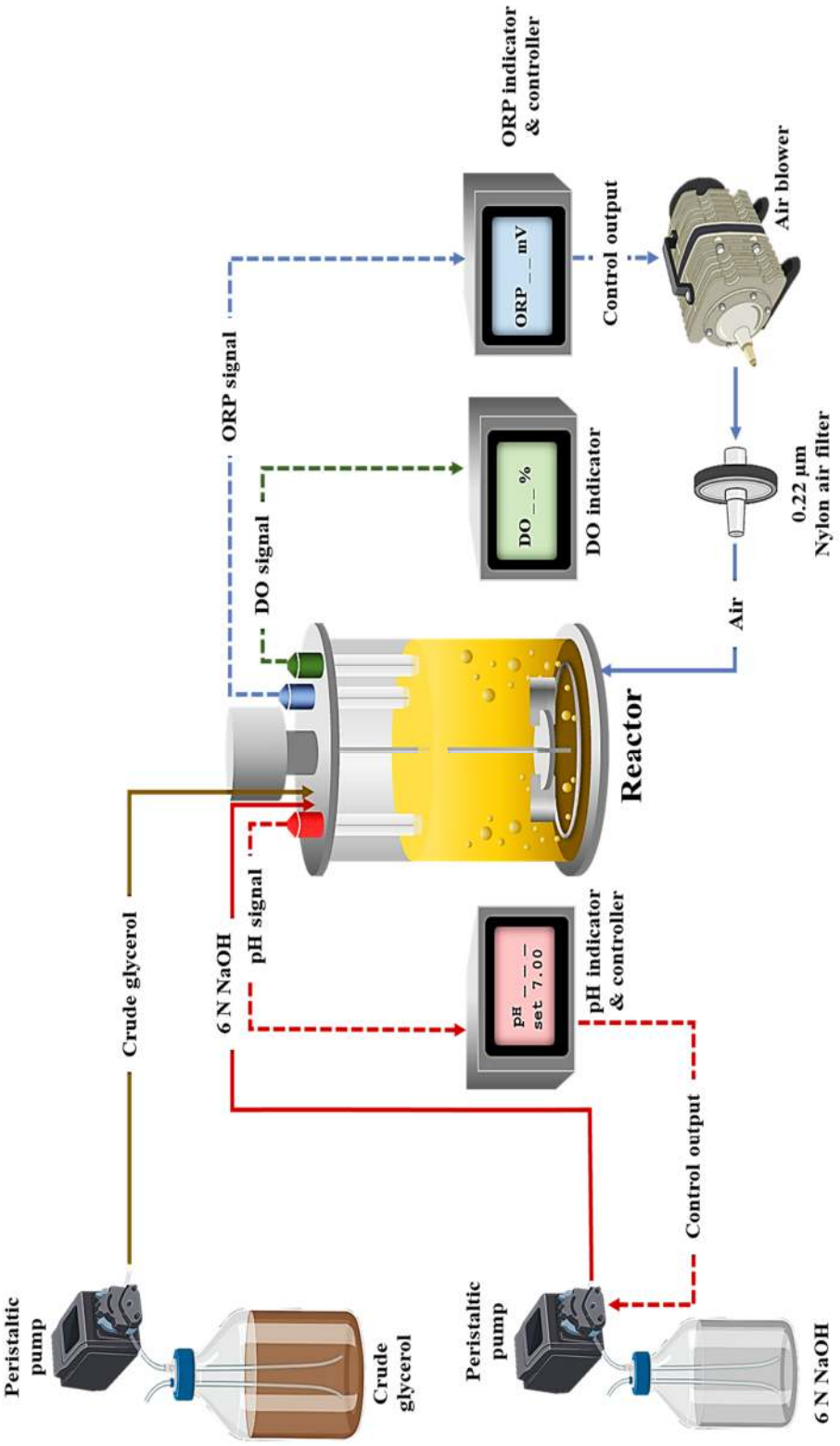
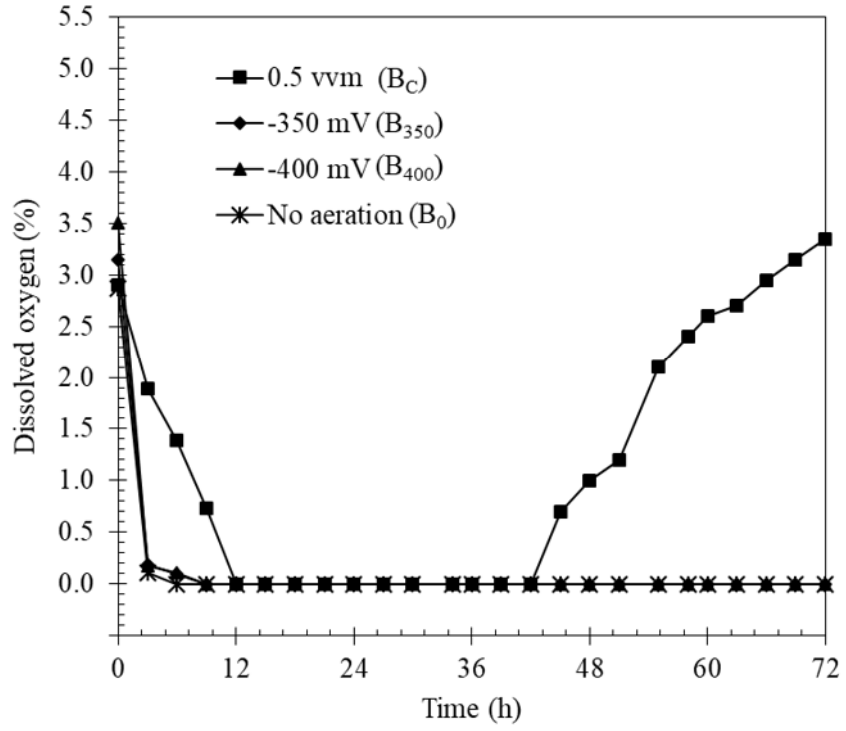
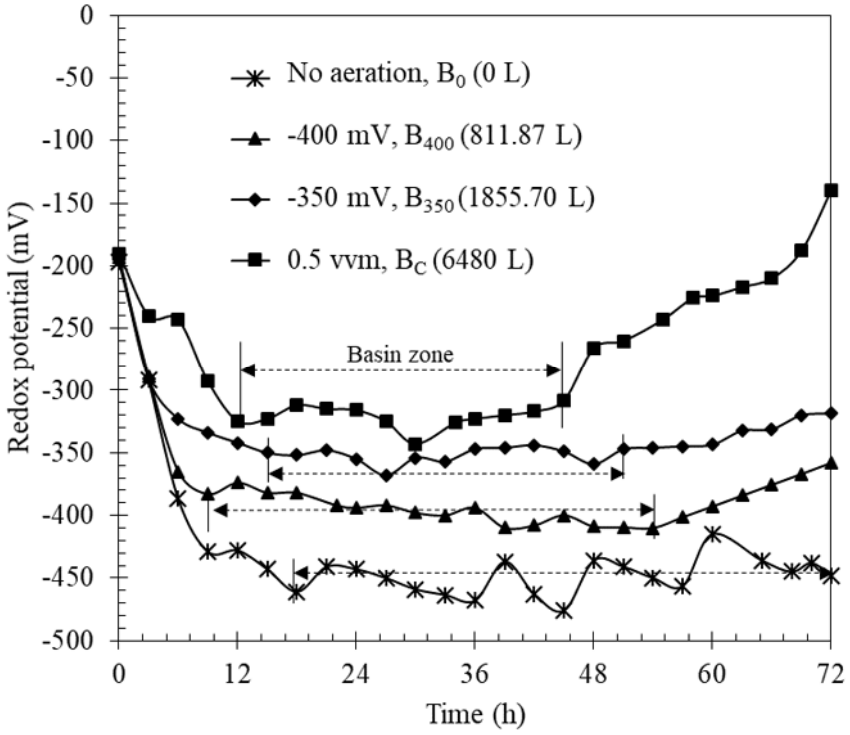


Figure 1. Schematic diagram of batch and fed-batch cultivation for ethanol production from crude glycerol using *Enterobacter aerogenes* TISTR1468



A.



B.

Figure 2**.** Profile of redox potential (A) and dissolved oxygen (B) during batch cultivation under various aeration condition

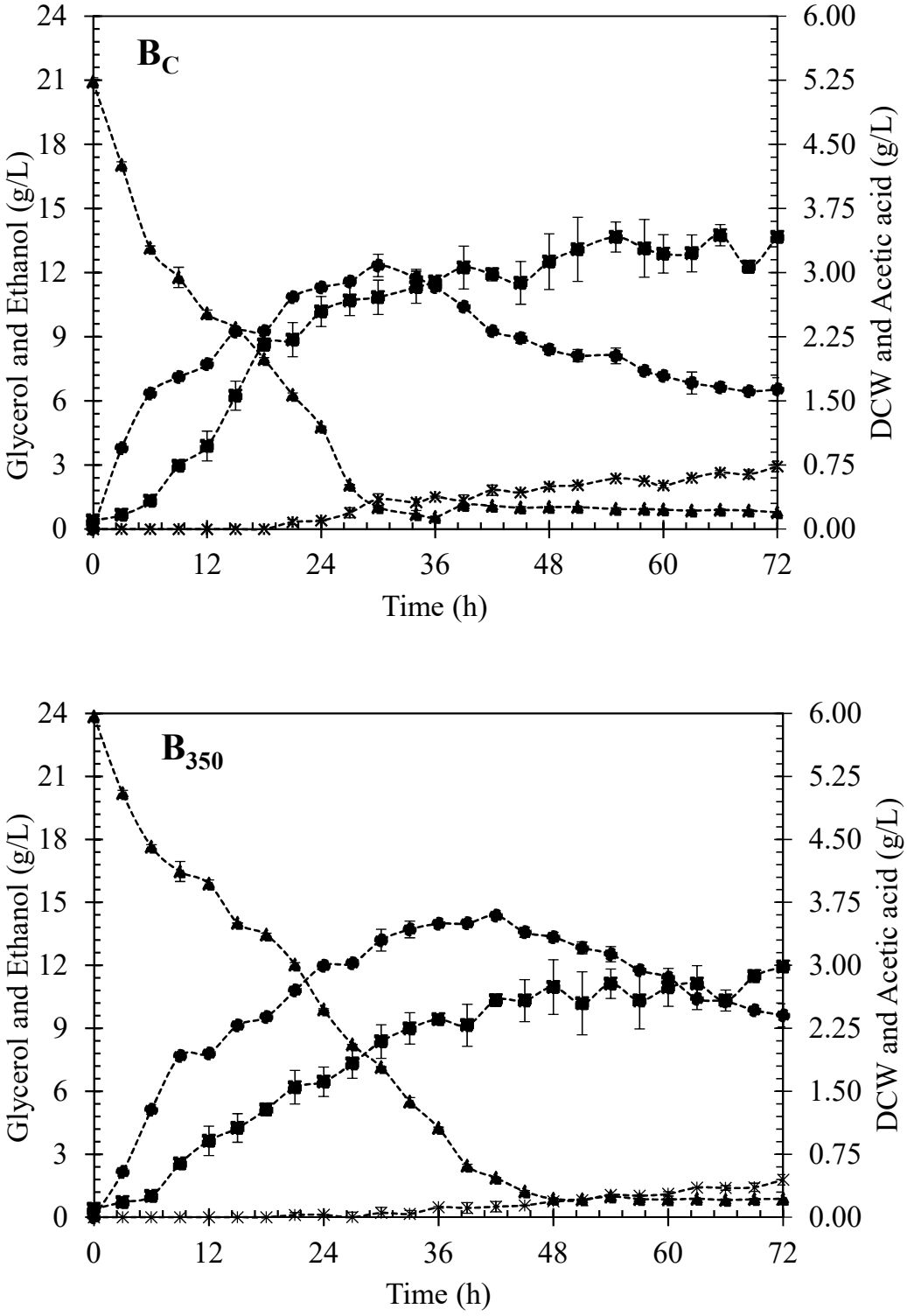


Figure 3**.** Time-course of ethanol production in batch reactor under various aeration condition**. (**Glycerol, ▲; Ethanol, ●; Acetic acid, x; DCW, ■**)**

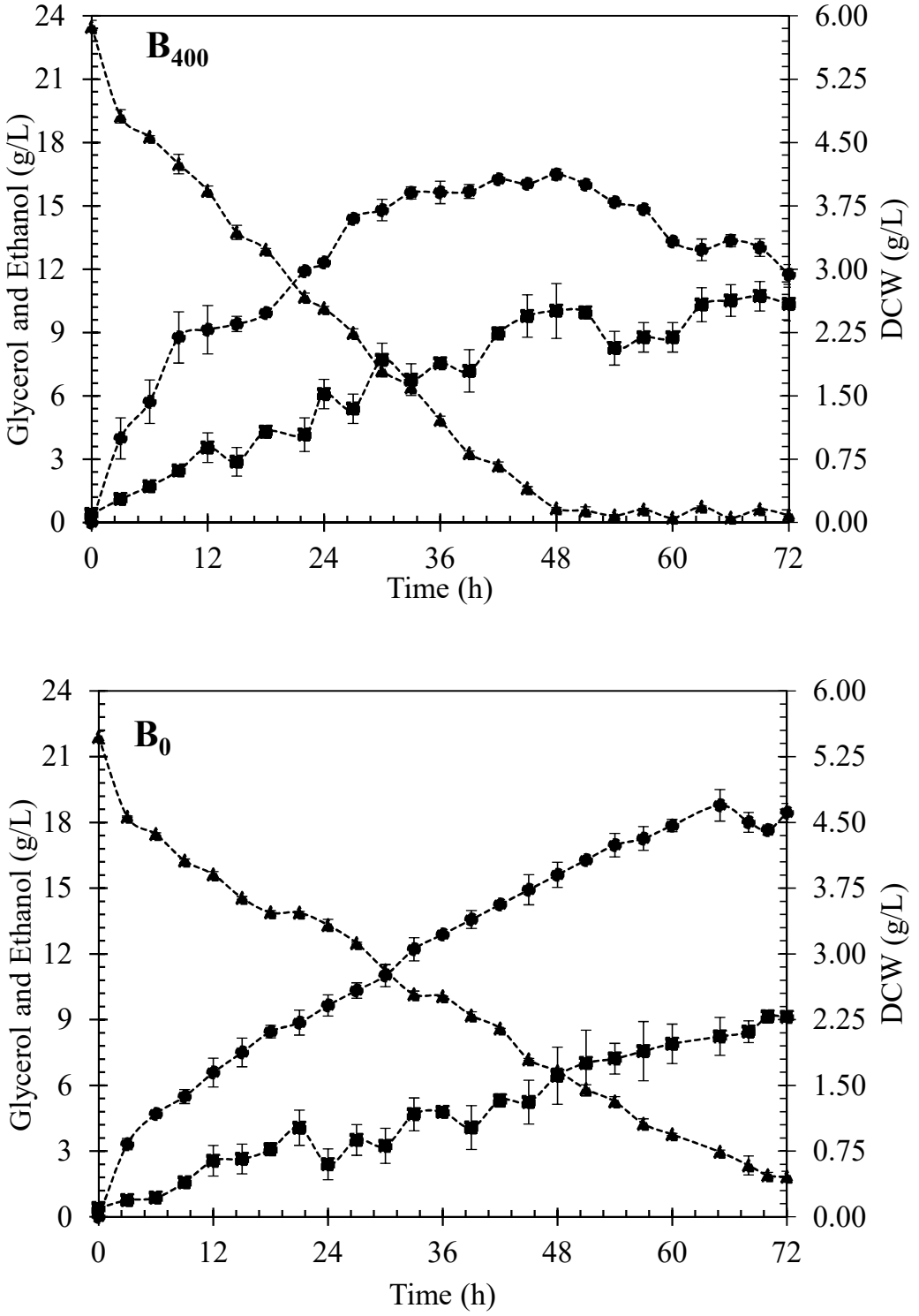


Figure 3. Time-course of ethanol production in batch reactor under various aeration condition. (Glycerol, ▲; Ethanol, ●; Acetic acid, x; DCW, ■)

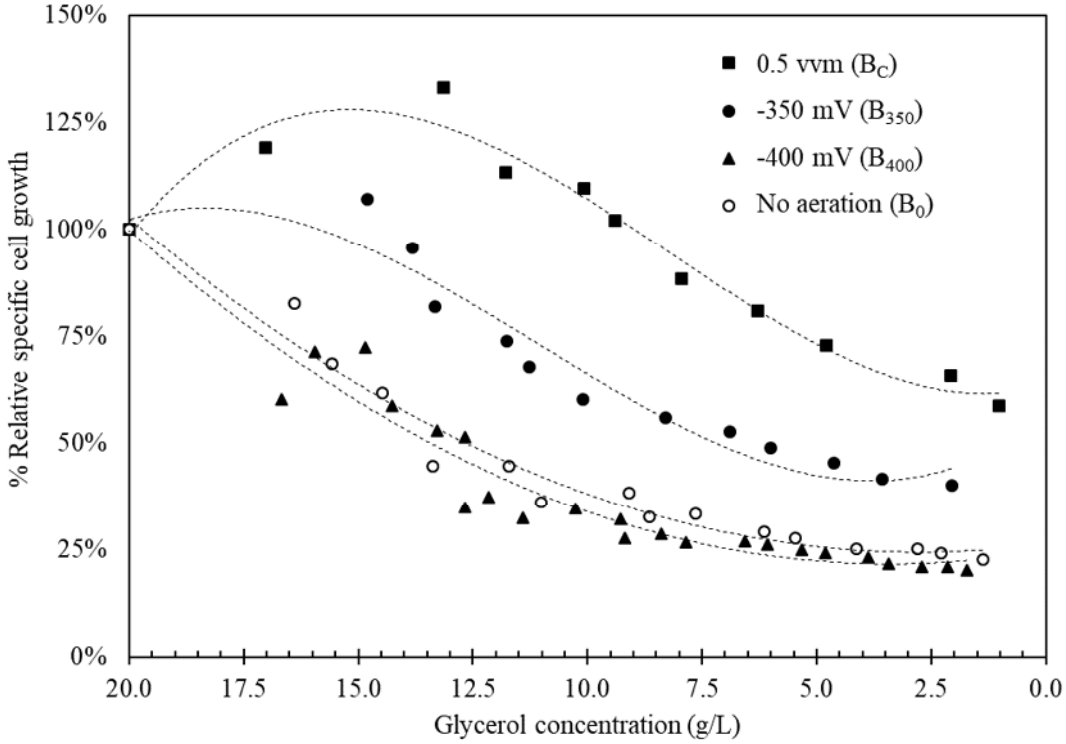


Figure 4. The correlation between glycerol concentration and % relative specific growth rate during batch cultivation in respect to various aeration condition

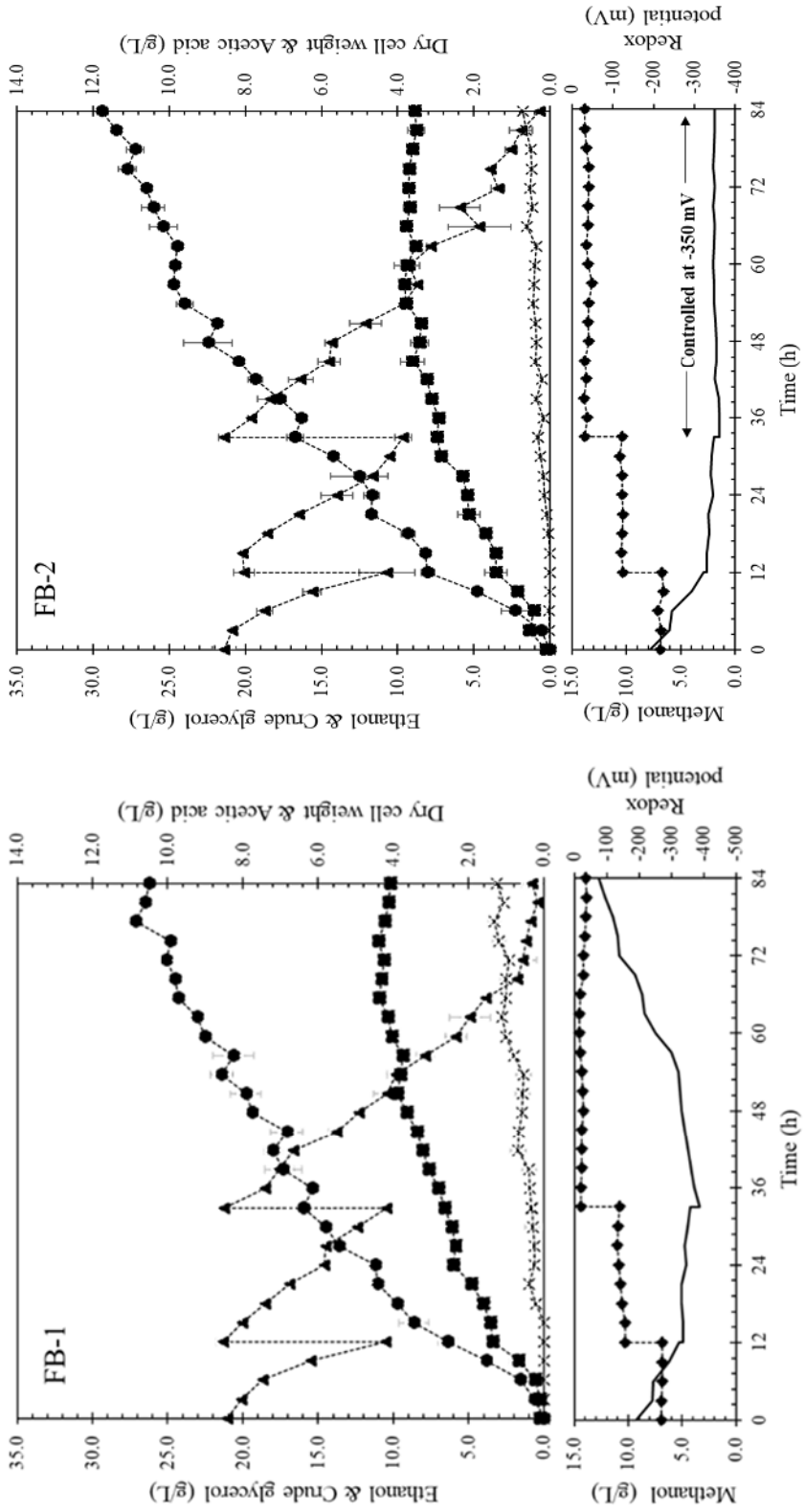


Figure 5. Time-course during fed-batch cultivation of ethanol production with various aeration strategy. (Glycerol, ▲; Ethanol, ●; Acetic acid, x; DCW, ■)

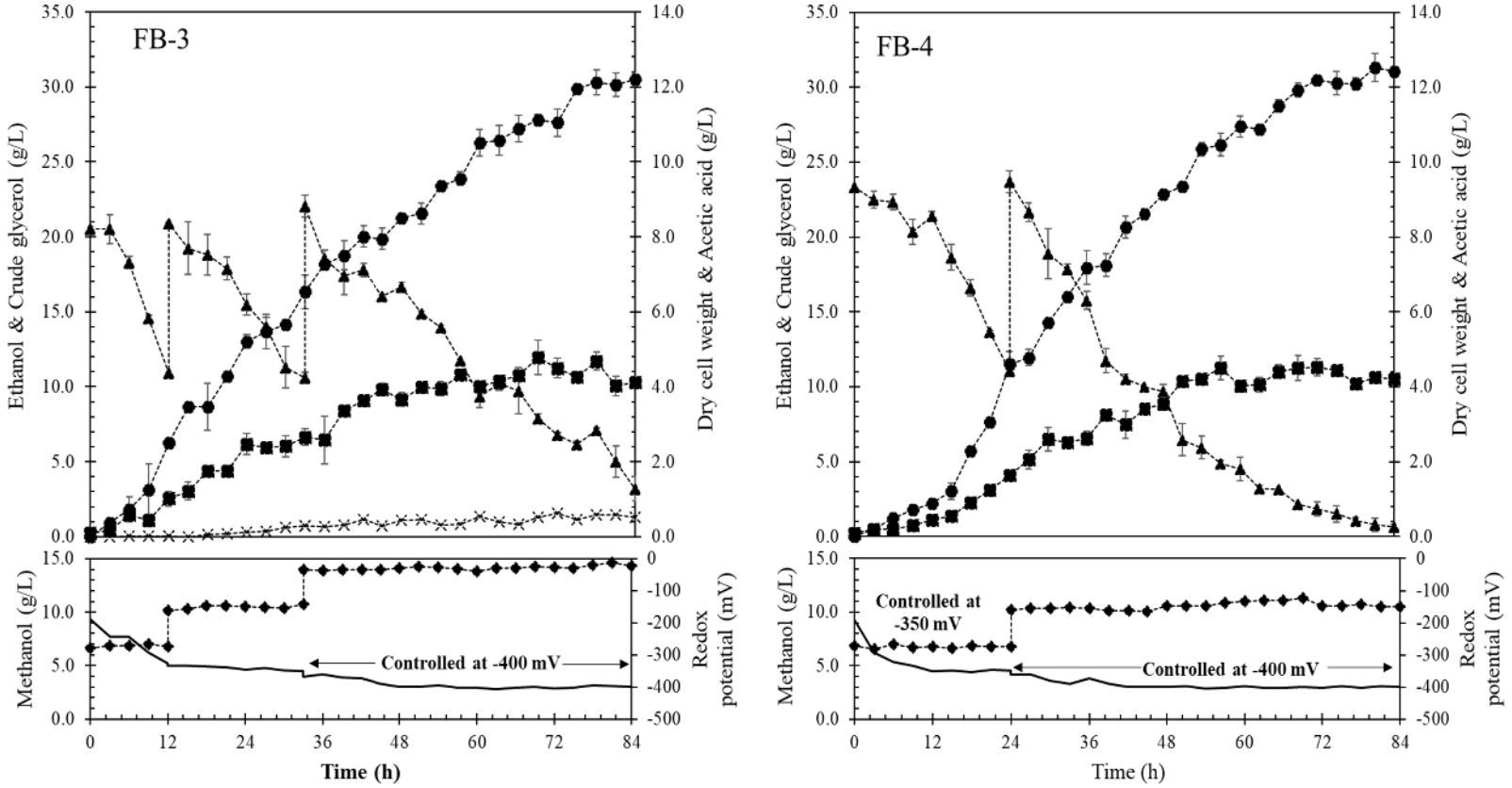


Figure 5. Time-course during fed-batch cultivation of ethanol production with various aeration strategy. (Glycerol, ▲; Ethanol, ●; Acetic acid, x; DCW, ■)

APPENDIX A

Table 1. The data of pH-controlled scheme at various initial C/N ratio using 100 mL GT medium

1. Initial C/N ratio 115

Time (h)	pH	pH correction	Vol. 3 N NaOH (mL)
0	8.00	-	-
6	6.80	8.30	0.5
12	7.11	7.97	0.3
18	6.79	8.29	0.5
24	7.07	7.93	0.3
30	6.75	8.26	0.5
36	7.05	8.00	0.3
42	6.78	8.32	0.5
48	7.13	8.04	0.3
54	6.83	8.31	0.5
60	7.10	7.94	0.3
66	6.77	8.26	0.5
72	7.14	-	-
		Total	4.5

2. Initial C/N ratio 200

Time (h)	pH	pH correction	Vol. 3 N NaOH (mL)
0	8.00	-	-
6	6.82	8.35	0.5
12	7.17	8.16	0.3
18	6.99	8.44	0.5
24	7.29	8.33	0.3
30	7.18	8.15	0.3
36	7.01	8.44	0.5
42	7.30	8.37	0.3
48	7.22	8.21	0.3
54	7.04	8.54	0.5
60	7.39	8.28	0.3
66	7.13	8.11	0.3
72	7.29	-	-
		Total	4.1

3. Initial C/N ratio 257

Time (h)	pH	pH correction	Vol. 3 N NaOH (mL)
0	8.0		
6	7.21	7.95	0.3
12	6.80	8.32	0.5
18	7.23	8.15	0.3
24	7.04	8.55	0.5
30	7.43	8.41	0.3
36	7.30	8.35	0.3
42	7.22	8.37	0.3
48	7.22	8.28	0.3
54	7.15	8.11	0.3
60	7.00	8.50	0.5
66	7.40	8.41	0.3
72	7.2		
	Total		3.90

4. Initial C/N ratio 365

Time (h)	pH	pH correction	Vol. 3 N NaOH (mL)
0	8		
6	7.27	8.39	0.3
12	7.43	7.43	0
18	6.32	7.84	0.5
24	7.06	8.10	0.3
30	7.14	8.02	0.3
36	7.10	8.17	0.3
42	7.23	8.19	0.3
48	7.07	8.00	0.3
54	7.14	8.09	0.3
60	6.99	8.01	0.3
66	6.90	7.93	0.3
72	7.15		
	Total		3.2

APPENDIX B

1. MATLAB Script to determine specific cell growth rate (μ) using curve-fitting method.

Determining qp using *fminsearch*	`clear all; clc; close all;`
	`global tspan xdata`
	`%Experiment`
	`tspan=[…];`
	`xdata=[…];`
	`%Nonlinear minimization using fminsearch`
	`init=6;`
	`[eval]=fminsearch(@minspecgrowth,init);`

Data evaluation and graphic visualization	`%Graph visualization`
	`m=size(tspan)-1;`
	`miu=eval`
	`xhit=0;`
	`for a=1:m(2)`
	`xdata(1)=0.10;`
	`xhit(a+1)=xdata(a)+(miu.*(tspan(a+1)-tspan(a)));`
	`end`
	`hold on`
	`plot(tspan,xdata,'*',tspan,xhit(1,:)')`
	`hold off`
	`title('Curve-fitting of cell concentration');`
	`xlabel('Time (h)');`
	`ylabel('Biomass concentration (g/L)')`
	`legend({'Experiment data','Prediction'},'Location','southeast')`

Minimization function for determining the qp	`function [e]=minspecgrowth(miu)`
	`global tspan xdata`
	`m=size(tspan)-1;`
	`xhit=0;`
	`for a=1:m(2)`
	`xdata(1)=0.10;`
	`xhit(a+1)=xdata(a)+(miu.*(tspan(a+1)-tspan(a)));`
	`end`
	`e=sum((xhit(1,:)'-xdata(1,:)').^2);`
	`end`

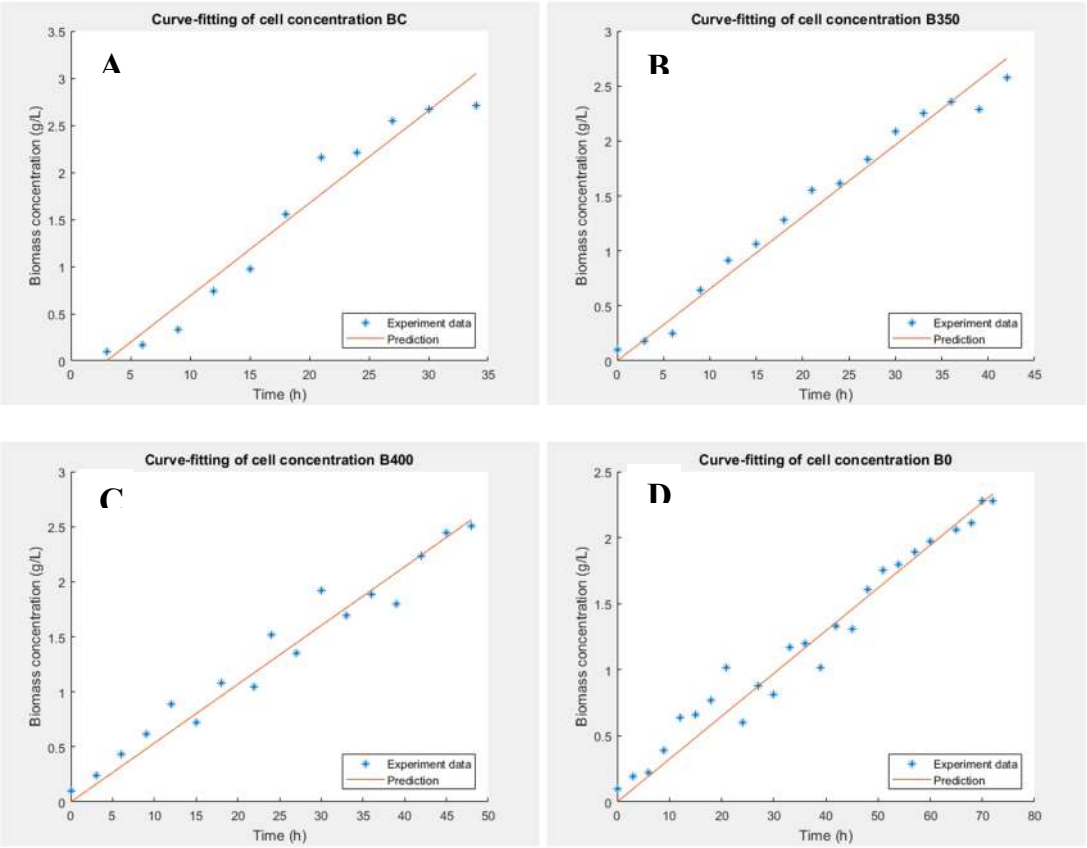


Figure 1. The result of curve-fitting for determining of specific cell growth rate (μ) of various aeration strategy (A) continuous aeration, B_C; (B) controlled aeration at -350 mV, B_{350}; (C) controlled aeration at -400 mV, B_{400} and (D) non-aeration process (B_0).

2. MATLAB Script to determine specific ethanol production rate (qp) using curve-fitting method.

Determining qp using *fminsearch*	`clear all;close all; clc`
	`%Nonlinear minimization using fminsearch`
	`init=6;`
	`[eval]=fminsearch(@ethanolprod,init);`

Data evaluation and graphic visualization	`%Graph visualization`
	`biomass=[…];`
	`tspan=[…];`
	`pdata=[…];`
	`m=size(biomass)-1;`
	`Qp=eval`
	`for a=1:m(2)`
	`P(a+1)=pdata(a)+(biomass(a).*Qp.*(tspan(a+1)-tspan(a)))`
	`end`
	`hold on`
	`plot(tspan,pdata,'*',tspan,P(1,:)')`
	`hold off`
	`title('Curve-fitting of ethanol production');`
	`xlabel('Time (h)');`
	`ylabel('Ethanol concentration (g/L)')`
	`legend({'Experiment data','Prediction'},'Location','southeast')`

Minimization function for determining the qp	`%Minimization function`
	`function [e]=ethanolprod(k)`
	`biomass=[…];`
	`tspan=[…];`
	`pdata=[…];`
	`%%numerical diff. using Euler method`
	`m=size(biomass)-1;`
	`P=0;`
	`Qp=k;`
	`for a=1:m(2)`
	`P(a+1)=pdata(a)+((biomass(a).*Qp*(tspan(a+1)-tspan(a))));`
	`end`
	`%%sum square of error`
	`e=sum((P(1,:)'-pdata(1,:)').^2);`
	`end`

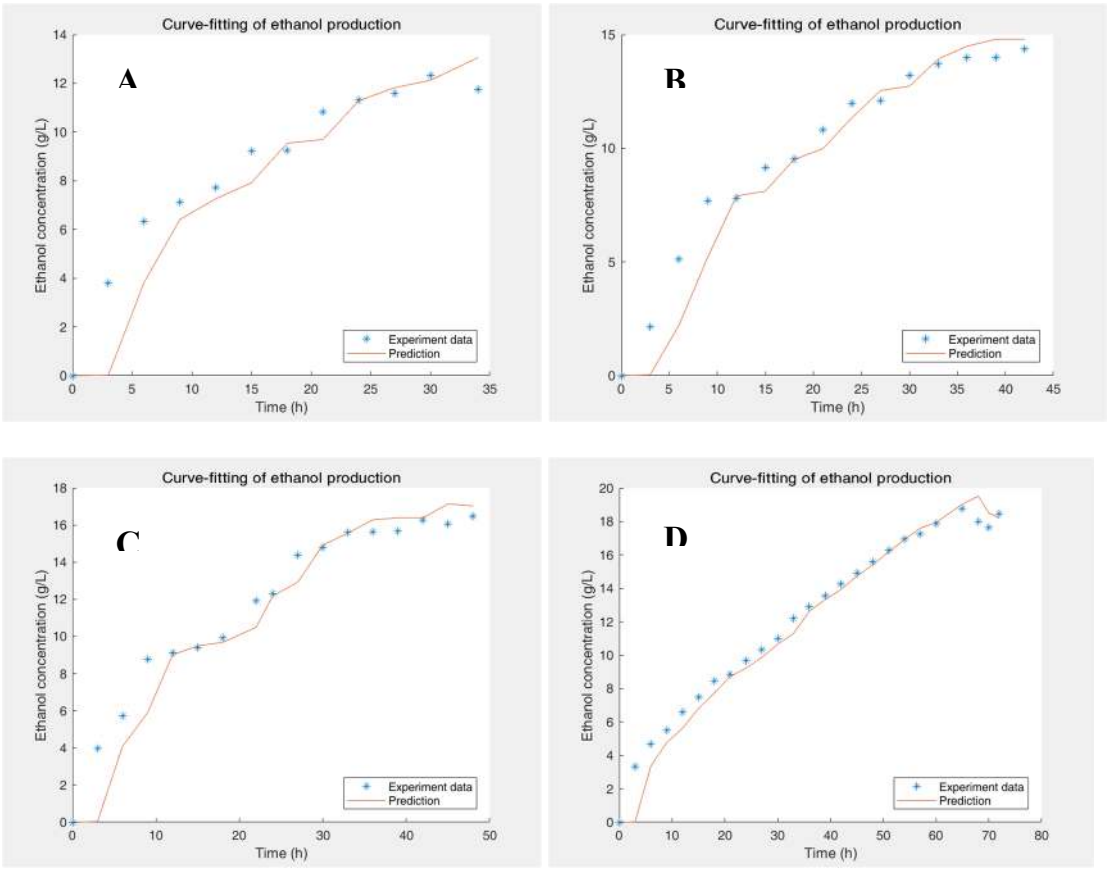


Figure 2. The result of curve-fitting for determining of specific ethanol production rate (qp) of various aeration strategy (A) continuous aeration, B_C; (B) controlled aeration at -350 mV, B_{350}; (C) controlled aeration at -400 mV, B_{400} and (D) non-aeration process (B_0).

3. MATLAB Script to determine specific glycerol consumption rate (qs) using curve-fitting method.

Determining qp using *fminsearch*	`clear all;close all; clc`
	`%Nonlinear minimization using fminsearch`
	`init=6;`
	`[eval]=fminsearch(@glycerolcons,init);`

Data evaluation and graphic visualization	`%Graph visualization`
	`biomass=[…];`
	`tspan=[…];`
	`sdata=[…];`
	`m=size(biomass)-1;`
	`Qs=eval;`
	`for a=1:m(2)`
	`S(1)=sdata(1)`
	`P(a+1)=sdata(a)-`
	`(biomass(a).*Qs.*(tspan(a+1)-tspan(a)))`
	`end`
	`hold on`
	`plot(tspan,pdata,'*',tspan,P(1,:)')`
	`hold off`
	`title('Curve-fitting of ethanol production');`
	`xlabel('Time (h)');`
	`ylabel('Ethanol concentration (g/L)')`
	`legend({'Experiment data','Prediction'},'Location','southeast')`

Minimization function for determining the qs	`%Minimization function`
	`function [e]=glycerolcons(k)`
	`biomass=[…];`
	`tspan=[…];`
	`sdata=[…];`
	`%%numerical diff using Euler method`
	`m=size(biomass)-1;`
	`Qs=k;`
	`for a=1:m(2)`
	`S(1)=sdata(1)`
	`S(a+1)=sdata(a)-`
	`((biomass(a).*Qs*(tspan(a+1)-tspan(a))));`
	`end`
	`%%sum square of error`
	`e=sum((P(1,:)'-sdata(1,:)').^2);`
	`end`

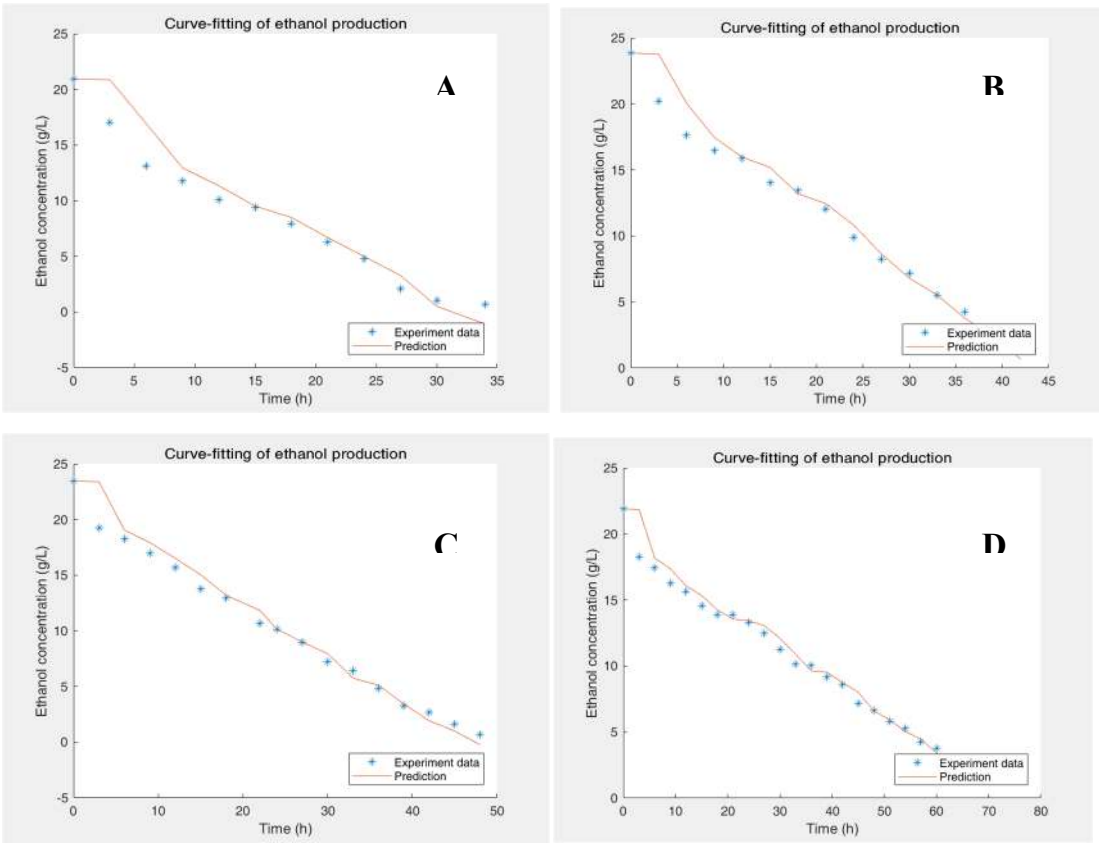


Figure 3. The result of curve-fitting for determining of specific glycerol consumption rate (qs) of various aeration strategy (A) continuous aeration, B_C; (B) controlled aeration at -350 mV, B_{350}; (C) controlled aeration at -400 mV, B_{400} and (D) non-aeration process (B_0).

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List of Publications and Proceedings

Publications

Sunarno, J.N., Prasertsan, P., Duangsuwan, W., Cheirsilp, B., and Sangkharak, K. 2019. Biodiesel derived crude glycerol and tuna condensate as an alternative low-cost fermentation medium for ethanol production by *Enterobacter aerogenes*. Ind. Crops Prod. Vol. 138; 111451. (*https://doi.org/10.1016/j.indcrop.2019.06.014*)

Sunarno, J.N., Prasertsan, P., Duangsuwan, W. Cheirsilp, B., and Sangkharak, K. 2019. Improve biotransformation of crude glycerol to ethanol of *Enterobacter aerogenes* by two-stage redox potential fed-batch process under microaerobic environment. (*Submitted to*. *Ind*. *Crops Prod.)*

Proceeding

Wibowo, W.A., Pranolo, S.H., Sunarno, J.N., and Purwadi, D. 2014. Effect of biomass feed size and airflow rate on the pressure drop of gasification reactor. J. Teknol. 68. doi:10.11113/jt.v68.2962.

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Sunarno, J.N., and Prasertsan, P. 2018. Kinetics of glycerol and methanol inhibition on growth and ethanol production of *Enterobacter aerogenes* TISTR 1468. Oral presentation at The International Conference on Food and Applied Bioscience 2018. 1-2 February, 2018, Chiang Mai, Thailand.

Wibowo, W.A., Pranolo, S.H., Sunarno, J.N., and Purwadi, D. 2014. Effect of biomass feed size and airflow rate on the pressure drop of gasification reactor. The Internatioal Conference on Current and Emerging Trends in Science and Engineering (ICCETSE). 14 March, 2014, Batam, Indonesia.