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Fermentation of Glycerol from Biodiesel Waste to 1,3-Propanediol by *Enterobacter Aerogenes*

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ABSTRACT

One of the most valuable products in chemical industry is 1,3-Propanediol (1,3-PD) because it has numerous applications for the production of polymers, cosmetics, foods, medicines, and lubricants. 1,3-PD production can be synthesized with chemical and fermentation method. Chemical method requires high pressure and temperature as well as catalysts are expensive. 1,3-PD production with fermentation method from crude glycerol as by-product of the biodiesel is promising, because it will increase the economic value of the crude glycerol beside the process is less expensive and eco friendly. In this study, crude glycerol was used as a raw material and *Enterobacter aerogenes* as inoculum in the fermentation process. First crude glycerol was purified using H_3PO_4 into pH 2 and then neutralized with NaOH. It was continued with bleaching process using activated carbon and water evaporation to obtained glycerol with high purity. Various fermentation process variables such fermentation time, temperature of fermentation, and amount of inoculum were studied. Crude glycerol, purified glycerol, and fermentation results were analyzed by Gas Chromatography (GC) to obtained the purity. Glycerol from purification result had achieved commercial glycerol standard. The best fermentation result was obtained at 1 day of fermentation with purity of 1,3-PD was 46.0685% ; temperature of 25 ^oC with purity of 1,3-PD was 51.2461% ; and 7% volume of inoculum with purity of 1,3-PD was 31.5919%.

Keyword: crude glycerol, *enterobacter aerogenes*, 1,3-propanediol

1. INTRODUCTION

Glycerol represents 10% of the by-products generated in the biodiesel production. Due to the fast growth of the biodiesel industry in the last decades, disposal of these massive amount of glycerol became a complex and expensive process [1]. Beside that, as the supply of glycerol increases, the price of glycerol becomes considerably lower. It is therefore of great interest to convert glycerol into other value-added products. One of the most interesting compounds that could be produced from glycerol is 1,3-Propanediol (1,3-PD) [2].

1,3-Propanediol ($CH_2(CH_2OH)_2$), a typical product of glycerol fermentation, is one of the most interesting raw materials for chemical industries due to its wide use in the different field [3]. 1,3-Propanediol can be formulated into composites, adhesives, laminates, powder an UV-cured coatings, mouldings, novel aliphatic polyesters, co-polyesters, solvents, and anti-freeze. One of the most successful applications has been in the formulation of corterra polymers [4]. Molecular mass of 1,3-Propanediol is 76,09 g x mol^{-1}, the boiling point is 210-212℃ and melting point is -28℃ [5].

1,3-Propanediol is a linear aliphatic glycol with two functional groups, which can be synthesized by chemical and biotechnological methods [6]. Two major routes for chemical synthesis are the conversion of acrolein to 1,3-Propanediol via 3-hydroxypropionaldehyde or the conversion of ethylene oxide to 1,3-Propanediol via 3-hydroxipropionaldehyde. Disadvantage of these production routes are

toxic intermediates and the dependence on the feedstock based on fossil resources [7]. Beside that, chemical synthesis requires a high pressure, high temperature, and expensive catalysts. Consequently, chemical synthesis is expensive, and, thus, 1,3-Propanediol still has a low market volume. Because of the environmental benefits and use of renewable feedstock, the biotechnological synthesis of 1,3-Propanediol appears to be an attractive alternative to chemical synthesis [8].

The microbial conversion of glycerol to 1,3-Propanediol was first described by August Freund (1981). Since then, a multitude of microorganism naturally capable of 1,3-Propanediol production were isolated and caharacterized. Known 1,3-Propanediol producers are on the one hand facultative anaerob *Enterobactericeae* from the genera *Klebsiella*, *Citrobacter*, and *Enterobacter* and on the other hand obligate anaerob *Clostridia*. Some *Lactobacilli* strains are also able to produce 1,3-Propanediol from glycerol as an external hydrogen acceptor [9]. The most common mechanism of glycerol fermentation involves a reductive pathway and oxidative pathway. In the reductive pathway, a vitamin B_{12}-dependent glycerol dehydratasae catalyses glycerol conversion to 3-hydroxypropionaldehyde, and this compound is further reduced to 1,3-Propanediol by 1,3-propanediol oxidoreductase. In the oxidative pathway, glycerol is dehydrogenated to dihydroxyacetone by a NAD^{+-} linked glycerol dehydrogenase, which is then converted to dihydroxyacetone phosphate by an ATP-dependent dihydroxyacetone kinase. Dihydroxyacetone phosphate is an intermediate of the glycolysis that can be further converted to acetate and to other products, depending on the type of bacterium [10].

Enterobacter aerogenes (*E. aerogenes*) belongs to a class *Enterobacteriaceae*, a facultative anaerobic bacteria with the ability to produce H_2. This bacteria have characteristic of bacil with width of 0.6 to 1.0 and length of 1.2-3.0, gram negative, producing colonies with a smooth shape with flagella and can move [11].

Besides 1,3-Propanediol some alcohols, acids, and other compounds are also produced, and the separation of 1,3-Propanediol from the aqueous system of fermentation broth therefore, becomes a big challenge. Several separation methods have been used for separation of 1,3-Propanediol from aqueous fermentation broth, such as distillation, liquid extraction, and pervaporation [2]. The conventional evaporation and distillation techniques normally used in the removal of water and purification of 1,3-Propanediol suffer from the problem of high energy consumption [12].

The objective of this study are to produce 1,3-Propanediol through fermentation of crude glycerol as a by-product of biodiesel by *Enterobacter aerogenes* and get the kinetic data in order to know the best condition in produce 1,3-Propanediol. The effect of various fermentation process variables such fermentation time, volume of inoculum, and fermentation temperature are investigated.

2. METHODS

2.1 Crude glycerol

Crude glycerol was obtained from one of Biodiesel Plant at Dumai, Riau, Indonesia. Crude Palm Oil (CPO) was used as a raw material and the process employed an alkali-catalyzed transesterification reaction.

2.2 Microorganism

Enterobacter aerogenes was obtained from Microbiology Laboratorium at Badan Pengawasan Obat dan Makanan (BPOM), North Sumatera, Indonesia. *Enterobacter aerogenes* was cultured and kept in an agar slant.

2.3 Purification of Crude Glycerol

150 ml crude glycerol was prepared in glass beaker, then heated at 60 ℃ for 1 hour to evaporated all the alcohol contents in crude glycerol. Then, 50 ml H3PO4 5% was added into it to the desired pH (pH=2), formed two separate layers, where the top layer is fatty acid phase, and the bottom layer is glycerol rich phase. Glycerol had been obtained from separation was added with NaOH solution until the pH of glycerol was neutral (pH=7). And then, glycerol was heated to formed Na_3PO_4 salts and was filtered to separated salts with glycerol. Glycerol was added with activated carbon as much 2% of total weight of glycerol, to removed the colour of glycerol. The process was heated at 80 ℃ and left for 12 hours. After that, glycerol was filtered to separated activated carbon from it. Glycerol form purification still contained water which affected the purity of glycerol. Therefore, the evaporation was carried out at 110 ℃ for 2 hours in oven, to get glycerol with a higher purity.

2.4 Stock culture preparation

Macro elements: 20 gr $FeCl_3.6\ H_2O$; 10 gr $CaCl_2\ H_2O$; 0.03 gr $CuSO_4.5\ H_2O$; 0.05 gr $MnSO_4.4\ H_2O$; 0.1 gr $ZnSO_4.7H_2O$ were dissolved into 1 L distilled water in erlenmeyer. Micro elements such 0.5 gr $(NH_4)_2SO_4$; 0.4 gr $MgSO_4.7H_2O$; 9.65 gr Na_2SO_4; 2.65 gr KH_2PO_4 were added into the solution. Extra nutritions such 3 gr peptone; 3 gr margarine; 10 gr glucose were added too. The pH of solution was measured by indicator pH. The pH of solution controlled in the range of 6.4-7.4. If the solution was very acid, NaOH solution was added until the desired pH. If the solution was very base, HCl solution was added until the desired pH. Solution was heated until it boiled and left for several minutes. After that, the solution in erlenmeyer was closed by cotton and wrapped by paper and bounded by rubber. Then it was sterilized into autoclave at 121 ℃ for 20 minutes. After sterilization process in autoclave, the process was continued with sterilization with UV light for 30 minutes. Stock culture was carried out with culture of *Enterobacter aerogenes* was added into the erlenmeyer. Then it was incubated in stated incubator for 3 days at 37 ℃ . After that, the stock culture could be used for fermentation.

2.5 Fermentation of glycerol

50 ml glycerol was added into fermentor, and then stock cultures of *Enterobacter aerogenes* as much 5, 7, and 10% from total of glycerol were added. After that, the fermentor was closed and fermented at 37 ℃ and 25 ℃ for 1, 2, and 3 days.

2.6 Separation of 1,3-Propanediol

The results from fermentation were distilled by atmospheric distillation method for separated another compounds beside 1,3-Propanediol in order to obtained 1,3-Propanediol with high purity.

2.7 Analysis of 1,3-Propanediol

1,3-Propanediol from separation process was analyzed using Gas Chromatography to obtained the purity. The analysis was performed on QP2010 Shimadzu GC equipped with automatic sampling system and DB 5 HT column. 100% dimethyl polysiloxane was used as filler material. Distilled water was used as solvent and helium was used as a carrier gas. Temperature of oven and injection were set at 60 ℃ and 370 ℃. Temperature of ion source and surface were set at 370 ℃ and 360 ℃. The flow rate and column rate were set at 125.1 ml/min and 2.42 mil/min. The pressure was set at 100 kPa.

2.5 Other analysis

The physical properties of crude glycerol and purified glycerol such colour, density, free faty acids (FFA), water content, ash content, glycerol content were analyzed in this study.

3. RESULTS

3.1 Purification of Glycerol

The results of physical properties between crude glycerol and purified glycerol is presented in Table 1.

Table 1. Characteristics of crude glycerol and purified glycerol

Physical Properties	*Crude* glycerol	Purified Glycerol
Colour	Reddish Brown	Clear
Density	1.24	1.2777
FFA	26.22	7.83
Water (%)	2.64	1.298
Ash (%)	11	6
Glycerol (%)	29.306	83.2572

3.2 Effect of fermentation time on the purity of 1,3-Propanediol

Fermentation was carried out at 37 ℃ and volume of inoculum was 10% from total of glycerol that was fermented. Figure 1 shows the purity of 1,3-Propanediol decreased when fermentation time increased. At the first day of fermentation the purity of 1,3-Propanediol was 46.0685%. And it decreased at the second day of fermentation into 32.6744%. At the third day of fermentation the purity of 1,3-Propanediol was more decreased into 27.7948%. Glycerol was not found at the first day of fermentation because it had been completely converted.

Fermentation of glycerol did not only produced 1,3-Propanediol, but it also produced an unknown compound such A, B, C, and D. Therefore, these compounds were classified based on retention time on Gas Chromatography analysis. Retention time for 1,3-Propanediol was 4.915.

In fermentation process, the time of fermentation also really affects the works of microorganism. The longer the time is, the higher the activity of microorganism to utilize substratum is, so that influences the results of fermentation [13]. The result of this study showed the high purity of 1,3-Propanediol formed at the first day of fermentation. It can be concluded that the purity of 1,3-Propanediol was inversely related to enhancement of fermentation time. Increasing time does not mean producing more secondary metabolites, it may produce more toxins to inhibit the production of antimicrobial metabolites [14]. Beside that, 1,3-Propanediol that has been formed can be converted back into another compounds such ethanol, 1,2-Propanediol, succinic acid, and so on [15]. Degradation in the purity of 1,3-Propanediol from the first day until third day of fermentation might be due to the activity of *Enterobacter aerogenes* that converted back 1,3-Propanediol into A, B, C, and D compound.

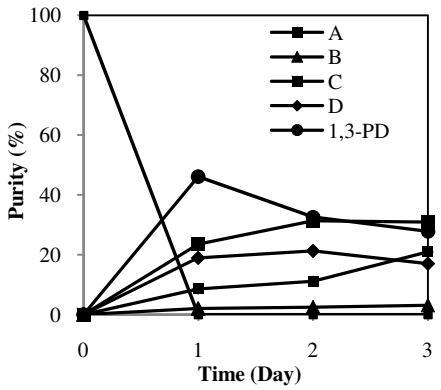

Fig. 1. Effect of Fermentation Time on The Purity of 1,3-PD (Fermentation Temperature: 37 °C and Inoculum Volume: 10%)

3.3 Effect of Inoculum Volume on the purity of 1,3-Propanediol

Fermentation was carried out at 37 ℃ and lasted for 3 days. Figure 2 shows the purity of 1,3-Propanediol fluctuated when the volume of inoculum increased. At inoculum volume was 5%, the purity of 1,3-Propanediol was 29.5197%. At 7% volume, the purity of 1,3-Propanediol increased to 31.5919%. But at 10% volume, the purity of 1,3-Propanediol decreased to 27.7948%.

A lower inoculum may reduce product formation, whereas a higher inoculums may lead to the poor product formation, especially the large accumulation of toxic substances [14]. Bacterial or inoculums growth were divided into four phase such lag phase, log phase, stationary phase, and death phase [16]. Enhancement in the purity of 1,3-Propanediol from 5% into 7% inoculum volume was classified in log phase, where substrate split at the large scale for inoculums growth to produce optimum product [16]. After that, the nutritions decreased when the volume of inoculum increased to 10% so there was a competition between the inoculum and finally it reached death phase and affected the purity of 1,3-Propanediol.

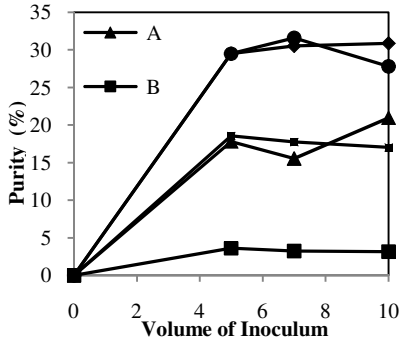

Fig. 2. Effect of Inoculum Volume on The Purity of 1,3-PD (Fermentation Temperature: 37 °C and Fermentation Time: 3 days)

3.4 Effect of fermentation temperature on the purity of 1,3-Propanediol

Fermentation was carried out at volume of inoculums was 10% from total of glycerol that was fermented and lasted for 3 days. Figure 3 shows the purity of 1,3-Propanediol was 51.2461% when fermentation lasted at 25℃. But when fermentation lasted at 37℃, the purity of 1,3-Propanediol decreased into 27.7948%.

Temperature affects the rate of microbial growth, rate of enzyme synthesis, and rate of enzyme inactivation [17]. Each of bacteria has the optimum, maximum, and minimum temperature. If the environment temperature is less than the minimum temperature or greater than maximum temperature, the activity of enzim would be stopped even at the high temperature will be occur denaturation of enzyme [18]. Enterobacter aerogenes can be found in soil, water, plants, and animals. Temperature growth for these bacteria is 25-37 ℃ [19]. In this study,

the high purity of 1,3-Propanediol was obtained at 25 °C.

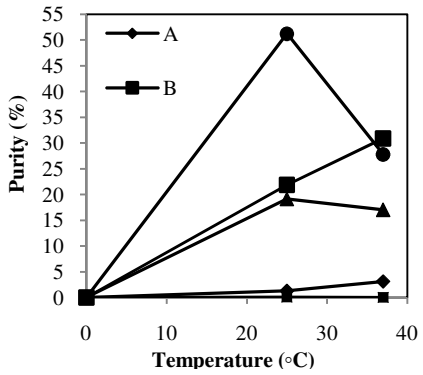

Fig. 3. Effect of Fermentation Tempertaure on The Purity of 1,3-PD (Inoculum Volume: 10% and Fermentation Time: 3 days)

4. CONCLUSION

Enterobacter aerogenes is able to convert glycerol from biodiesel waste into 1,3-Propanediol. Fermentation time, inoculum volume, and fermentation temperature had a profound effects on the activity of *Enterobacter aerogenes* to produced 1,3-Propanediol. The best temperature for fermentation was obtained at 25 ^{o}C with the purity of 1,3-Propanediol was 51.2461%. The best time for fermentation was obtained at 1 day with the purity of 1,3-Propanediol was 46.0685%. And the best inoculum volume for fermentation was obtained at 10% with the purity of 1,3-Propanediol was 31.5919%.

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