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Ethanol Production form Biodiesel-derived Crude Glycerol by *Enterobacter Aerogenes*

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Glycerol is an inevitable byproduct of biodiesel production that has become an attractive carbon source for fermentation processes due to its availability, low price and high degree of reduction. This study demonstrated the potential of utilising the glycerol surplus through conversion to ethanol. In this work, glycerol was used as a feedstock for ethanol batch fermentation process by *Enterobacter aerogenes*, under pH 7.0 and 30°C. *E aerogenes* favored oxidative- over reductive pathways and yielded the ethanol as a main fermentative product. The profiles of glycerol utilization rate, ethanol production rate, and specific growth with respect to the glycerol concentration and the ethanol production were similar for both types of the glycerol. Substrate inhibitory effect was found at 40 g L^{-1} initial glycerol concentration. Impurities in the crude glycerol posed no negative impact to *E aerogenes*, and apparently raised the ethanol concentration and yield by 32 and 21% compared those with the pure glycerol fermentation at the optimum glycerol concentration. The highest ethanol production was 204 mM on 25 g L^{-1} crude glycerol. The current findings showed the potential application of *E aerogenes* in a large scale ethanol production from the crude glycerol.

1. Introduction

The rise of energy demand results the rapid growth of biodiesel industry.The biodiesel, widely used as a substitute of diesel, can be produced from a variety of renewable feedstocks such as vegetable oils, animal fats, and used oils. In the biodiesel production process, fatty acids in either vegetable oils or animal fats are converted to of fatty acid methyl or ethyl esters in tranesterification reaction. Large amounts of crude glycerol are formed as the main by-product in biodiesel production and constitutes approximately 10% (w w-1) of the total biodiesel generated (Johnson and Taconi, 2007). The low value crude glycerol, with concentration in the range of 35-50%, is required further purification processes to remove impurities such as water, methanol, soap and fat to raise the glycerol concentration over 80% before marketing. This surplus crude glycerol has not only greatly disturbed the market price but has also caused environmental problem since it is required treatment prior to discharging directly into the environment (da Silva et al., 2009). Considerable efforts have been developed biological methods to convert the crude glycerol to various valuable chemicals and fuels including 1, 3- propanediol, hydrogen, succinic acid, dihydroxyacetone, and ethanol.
Renewable and environmental friendly fuel ethanol has been extensively produced and used as an alternative energy source to replace fossil fuels, and the demand for ethanol has been steadily. The ethanol can be produced from a variety of feedstocks such as sugars (e.g. glucose and sucrose), starch, grains,

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lignocelluloses, and glycerol. Glycerol seems to be a promising feedstock for the ethanol production compared with the corn. Yazdani and Gonzalez (2007) demonstrated that the cost of ethanol production from glycerol is about 40% less than that of production from corn-derived sugars, considering both feedstock demand and operational costs. It has been shown that glycerol, as the sole carbon and energy source, can be fermented by number of wild-type bacteria including Bacillus, Clostridium, Enterobacter, Klebsiella, Citrobacter, Propionibacterium, Anaerobiospirillum and Lactobacillus species (Papanikolaou et al., 2004; Yazdani and Gonzalez, 2007). From all of the species, only E. aerogenes is able to convert glycerol to ethanol as a main product (Ito et al., 2005). Glycerol can be converted to a wide range of high value biochemicals and biofuels such as 1, 3- propanediol (Wong et al., 2011; Rossi et al., 2012), hydrogen (Ito et al., 2005; Wu et al., 2010), succinic acid (Lee et al., 2001), ethanol (Ito et al., 2005; Vikromvarasirit and Pisutpaisal, 2011), and dihydroxyacetone (Hongbo and Thomas, 2010). In this work, the production of ethanol by E. aerogenes was conducted in a simple medium without supplement of yeast extract, tryptone or peptone. The influences of glycerol types, and initial concentrations on the kinetic data including specific growth rate; ethanol production rate, and yield; and glycerol utilization rates were evaluated. Substrate and product inhibitory effects on the ethanol production process were also tested.

2. Materials and methods

2.1 Microorganism and medium

E. aerogenes was obtained from the Institute of Science Research and Technology of Thailand (TISTR). *E. aerogenes* was aerobically cultured to obtain high cell density for fermentation experiments (Chantoom et al., 2014). Laboratory grade glycerol (QRec, Auckland, New Zealand) or crude glycerol (Trang Plam Oil Co., Ltd., Thailand) were used as carbon source and energy sources. The crude glycerol consisted of 63.9% glycerol, 642,127 mg total COD L^{-1}, conductivity of 0.1 µS cm^{-1}, and pH 8.9. Culture medium (Vikromvarasiri and Pisutpaisal, 2011) was prepared in distilled water with the following constituents (per liter); 7.0 g K_2HPO_4, 5.5 g KH_2PO_4, 1.0 g $(NH_4)_2SO_4$, 0.25 g $MgSO_4.7H_2O$, 0.021 g $CaCl_2.2H_2O$, 0.12 g $Na_2MoO_4.2H_2O$, 2.0 mg nicotinic acid, 0.172 mg Na_2SeO_3, 0.02 mg $NiCl_2$ and 10 mL of trace element solution containing 0.5 g $MnCl_2.4H_2O$, 0.1 g H_3BO_4, 0.01 g AlK $(SO_4)_2.H_2O$, 0.001 g $CuCl_2.2H_2O$ and 0.5 g Na_2EDTA. The medium was autoclaved at 121°C, 15 psi for 15 min before use.

2.2 Ethanol fermentation

The concentrated cell (2.5 mL) was dispensed into the 100-mL serum bottles containing 50-mL culture medium. The cell concentration in each bottle was fixed at 1.50 g dry cell weight L^{-1}. Experiments were setup under varying initial glycerol concentrations for both pure and crude glycerol, and under fixed pH 7.0 and incubation temperature of 30 °C. In this study, each experiment was carried out in duplicate and the data shown for each condition are representative results of independent tests that were duplicate. After all components were dispensed in the bottles, the rubber plug and aluminum cap were closed and nitrogen gas was purged into the bottle content, to achieve anaerobic condition, through the 0.45 µm syringe filter prior to incubation at 30 ^{o}C with rotary shaking at 150 rpm. Liquid sample of 1.5 mL was collected periodically from the bottles during 240 h fermentation and centrifuged (8,000 rpm, 4 ^{o}C, 15 min) and filtered through 0.45 µm cellulose acetate membrane to obtain supernatant for analysis of glycerol, ethanol and fermentative products. Gas sample in reactor headspace was also collected for analysis of gas compositions.

2.3 Analytical procedures

Ethanol and other fermentative products were quantified by gas chromatography (Shimadzu GC-7A, Japan) equipped with a flame ionization detector and a Stabilwax DA capillary column (Restek Corporation, USA). The temperatures of the injection port and detector were maintained at 240°C (Nathao et al., 2013). Biogas content (H_2, CH_4, and CO_2) was determined by gas chromatograph (Shimadzu GC-2014, Japan) equipped with a thermal conductivity detector (TCD) with a Unibeads C 60/80 column (GL Sciences, Inc., Tokyo, Japan). Glycerol concentration was determined by a high performance liquid chromatograph (Agilent LC1200 Series, USA) equipped with a UV/RI detector, and an Aminex HPX-87H column of 300 x 7.8 mm. (Bio-Rad, USA). Sample of 20 µL was injected into the HPLC system and eluted with 5 mM H_2SO_4, and 0.6 mL min^{-1} flow rate, and column temperature of 60^{o}C.

3. Results and discussion

3.1 Pure glycerol

E. aerogenes was cultured under initial pH 7.0 and growth conditions with ammonium as a nitrogen source and only glycerol as a sole carbon source without supplement of yeast extract and tryptone/peptone. Time profiles of cell growth; concentrations of glycerol, volatile fatty acids, and ethanol; gas content; and pH were

monitored during 240 hr fermentation. The growth of E. aerogenes increased after inoculation and reached log- and stationary phases after 48-h and 56-h fermentation. Similar profiles of glycerol utilization and ethanol production were closely corresponding to the growth characteristics (data not shown). Regardless of the initial glycerol concentration, ethanol was the main products of glycerol fermentation compared with organic acids (e.g. formic, acetic, propionic and lactic acids), and negligible 1,3-propandiol was detected. This results strongly indicated that oxidative- is more favourable than reductive pathways. These observations are in agreement with other studies (Lee et al., 2012), which used a pure culture as an inoculum, but in contradiction with those, which used a mixed consortium as the inoculum. Temudo et al. (2008) and Vikromvarasiri et al. (2014) reported the shift of fermentative products as the glycerol concentration increased when the mixed consortium was used as the inoculum. Figure 1 shows that glycerol utilized, and ethanol production were highly related and greatly influenced by the initial concentration of the glycerol with a maximum ethanol produced of 130 mM at the 20 g L-1 initial glycerol concentration. The ethanol production increased as the increase of glycerol concentrations from 10 to 20 g L-1, abruptly dropped at 40 g L-1 glycerol, and gradually decreased beyond this glycerol concentration. Similar trends of glycerol utilization (GUR), ethanol production (EPR), and specific growth rates (SGR) as a function of glycerol concentration were found (Figure 2). Results indicated the optimum glycerol concentration was 20 g L-1, and inhibitory effect, directly on glycerol utilization, ethanol production and the growth of E. aerogenes, obviously appeared at 40 g L-1 glycerol. The kinetic data of ethanol production, glycerol utilization, and the growth of E. aerogenese apparently showed non-linear correlation with the ethanol produced (Figure 3) suggesting there is no toxic effect of ethanol up to 137 mM on the E. aerogenes. Molar ethanol yield in the range of 65-71 % for the glycerol concentration range of 10-60 g L-1, but sharply dropped to 25 % when the glycerol concentration was increased to 120 g L-1 (Table 1).

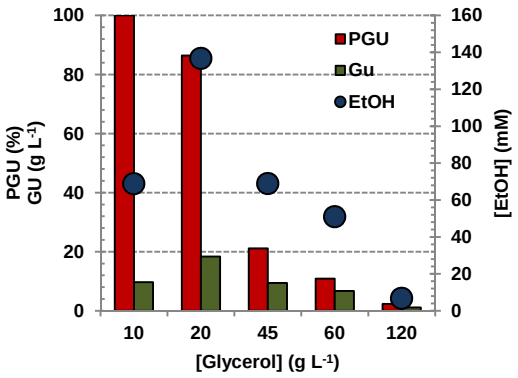

Figure 1: Percent glycerol utilization (PGU), glycerol utilized (GU), and ethanol concentration after 40 h fermentation of varying concentrations of pure glycerol. Symbols represent mean values of duplicate measurements, error bars represent one standard deviation.

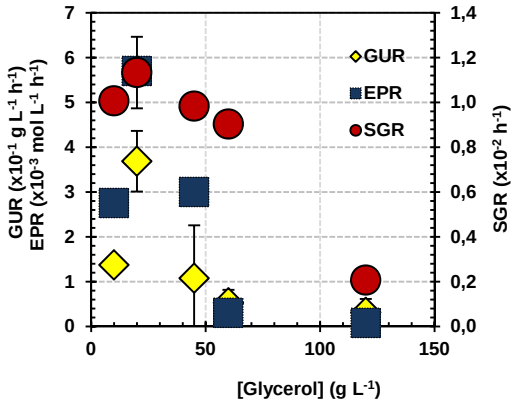

Figure 2: Glycerol utilization rate (GUR), ethanol production rate (EPR), and specific growth rate (SGR) of ethanol fermentation under the varying concentrations of pure glycerol. Symbols represent mean values of duplicate measurements, error bars represent one standard deviation.

Table 1: Kinetic parameters of pure glycerol fermentation

[Glycerol] g L^{-1}	Glycerol			Ethanol			Cell
	Rate g L^{-1} h^{-1}	Utilization[1] g L^{-1}	Utilization[1] %	Concentration[1] mM	Rate mol L^{-1} h^{-1}	Yield[1] mol mol^{-1}	Specific growth rate h^{-1}
10	0.137	10.0 ±0.2	100.0±0.0	68.8±8.0	0.0017	0.65±0.07	0.011
20	0.369	18.4 ±1.6	91.0±9.6	137.7±8.4	0.0034	0.69±0.02	0.013
45	0.108	9.0 ±0.6	21.1±1.0	68.8±4.7	0.0017	0.68±0.09	0.010
60	0.053	6.5 ±0.2	10.9±0.3	51.2±2.2	0.0013	0.71±0.04	0.009
128	0.031	1.1 ±0.1	0.9±0.2	6.7±1.4	0.0002	0.25±0.03	0.003

[1]calculated after 40 h fermentation period

Maximum glycerol utilised of 18.4 g L^{-1} at 20 g L^{-1} initial glycerol concentration. However, less glycerol was utilised and ethanol production was decreased as the glycerol concentrations were increased beyond this point. Szymanowska-Powałowska (2015) found that the pure and crude glycerol concentrations above 90 and 70 g L^{-1} inhibited the growth and the production of 1,3-prodiol by *Clostridium butyricum* DSP1, while Ito et al. (2005) and Lee et al (2012) reported that glycerol utilization and ethanol production by *E. aerogenes* were limited at the crude glycerol concentration exceeding 25 and 20 g L^{-1}, respectively. The differences between results of experiments regarding the glycerol toxic concentrations were probably mainly due to types of bacterial strains, including different selection factors used in the screening, and differences in the composition of carbon providing substrate (Ringel et al., 2012; Szymanowska-Powałowska et al., 2013). Because glycerol is an osmotically active substance with a significant influence on the osmotic potential of a fermentation medium, it may limit the production capacity of microorganisms (Nicolaou et al., 2010). Bacterial cells possibly encountered osmotic stress at high glycerol concentration and its subsequent detrimental effect on cellular transport systems, modified the fluidity of the plasma membrane (Liu et al., 2011; Diaz-Montano et al., 2010) and ultimately caused the cell growth and the ethanol production.

3.2 Crude glycerol

Similar experimental setup was carried out using crude glycerol but a lower initial concentration range of 5-15 g L^{-1} was used in order to avoid the substrate inhibitory phenomenon. The time profiles of cell growth; concentrations of glycerol, and fermentative products showed similar characteristics when compared with those under pure glycerol fermentation (data not shown). All kinetic parameters were increased as the function of the initial glycerol concentration (Figure 4, 5) and ethanol produced (Figure 6). Ethanol yield was found in the range of 0.9-1.0 mol mol^{-1} (Table 2), which is 20-30% higher than that of the pure glycerol fermentation with the corresponding initial glycerol concentrations. Results indicated that the impurities (e.g. ash, methanol, and salts) pose no negative impact of the growth *E. aerogneses* and ethanol production, and the presence of other carbon sources (e.g. free fatty acids), electron sources or supplement nutrients in the crude glycerol seemed to contribute the ethanol production (Thompson and He, 2006). Previous works (Jitrwung and Yargeau, 2011; Lee et al., 2012; Liu et al., 2012) reported higher ethanol production and ethanol yield using the crude glycerol carped to the pure glycerol, but Ito et al. (2005) showed the yields from ethanol from pure glycerol are higher those for the crude glycerol. The current findings strongly supported the crude glycerol derived from the biodiesel process can be fermented in the sequential process of industrial ethanol production without additional cost of glycerol purification.

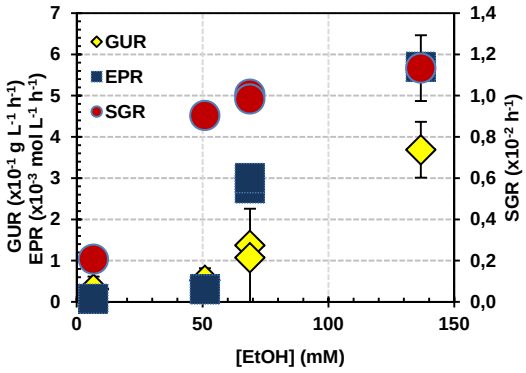

Figure 3: Glycerol utilization rate (GUR), ethanol production rate (EPR), and specific growth rate (SGR) of ethanol fermentation versus the produced ethanol. Symbols represent mean values of duplicate measurements, error bars represent one standard deviation.

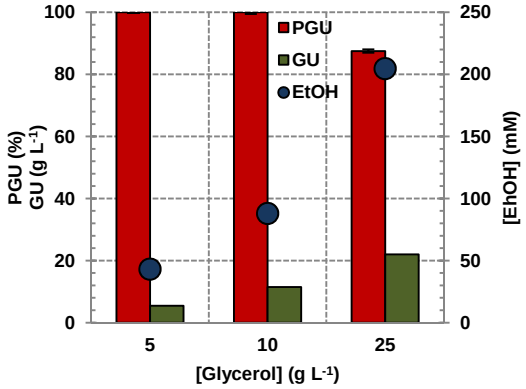

Figure 4: Percent glycerol utilization (PGU), glycerol utilized (GU), and ethanol concentration after 24 h fermentation of varying concentrations of crude glycerol. Symbols represent mean values of duplicate measurements, error bars represent one standard deviation

Table 2: Kinetic parameters of crude glycerol fermentation

[Glycerol] g L^{-1}	Glycerol			Ethanol			Cell
	Rate g L^{-1} h^{-1}	Utilization[1] g L^{-1}	Utilization[1] %	Concentration[1] mM	Rate mol L^{-1} h^{-1}	Yield[1] mol mol^{-1}	Specific growth rate h^{-1}
5	0.020	3.6 ±0.2	71.9±0.0	43.1±2.3	0.0018	1.00±0.08	0.003
10	0.015	7.3 ±0.1	73.2±0.0	87.9±2.2	0.0037	0.90±0.06	0.016
25	0.219	20.8 ±0.1	83.0±0.0	204.6±5.1	0.0085	0.90±0.05	0.024

[1]calculated after 24 h fermentation period

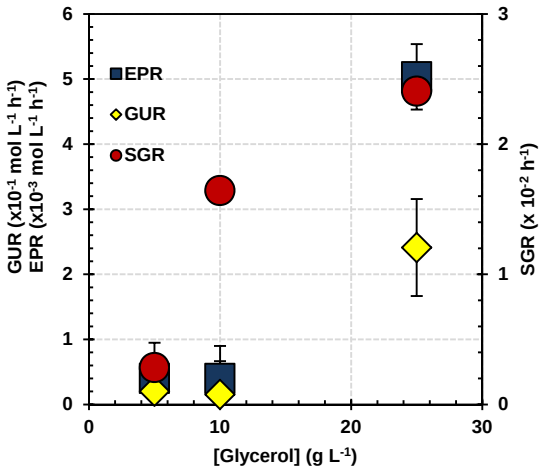

Figure 5: Glycerol utilization rate (GUR), ethanol production rate (EPR), and specific growth rate (SGR) of ethanol fermentation under the varying concentrations of crude glycerol. Symbols represent mean values of duplicate measurements, error bars represent one standard deviation.

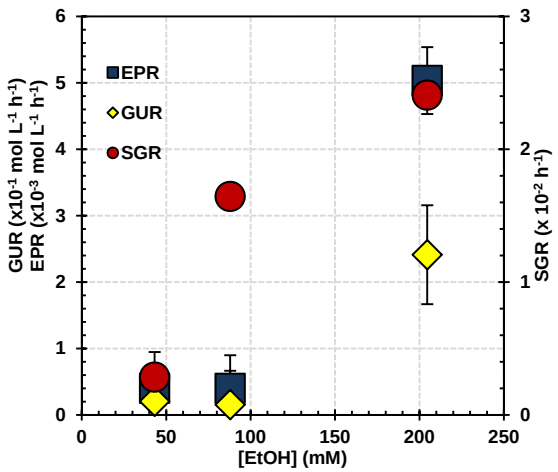

Figure 6: Glycerol utilization rate (GUR), ethanol production rate (EPR), and specific growth rate (SGR) of ethanol fermentation versus the produced ethanol. Symbols represent mean values of duplicate measurements, error bars represent one standard deviation.

4. Conclusions

Feasible conversion of biodiesel-derived crude glycerol into ethanol required low cost substrates and an economically competitive bioprocess. In the current study, a cost-effective, sustainable and green bioprocess was developed for the conversion of increased quantities of crude glycerol into ethanol. The crude glycerol showed a promising feedstock for the ethanol fermentation by *E aerogenes*, which favored the oxidative pathway and yields ethanol as the main fermentative products. Types and initial glycerol concentration showed greatly influence on the ethanol production, bacterial growth, and other kinetic parameters. Substrate inhibitory effect was found at the glycerol concentration above 40 g L^{-1}, while impurities in the crude glycerol showed no negative impact on the ethanol production. The crude glycerol seems to favour the ethanol fermentation regarding the produced ethanol concentration and yield.

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