

Micro-aerobic, Anaerobic and Two-stage Condition for Ethanol Production by *Enterobacter aerogenes* from Biodiesel-derived Crude Glycerol

Kanokrat Saisaard¹, Irini Angelidaki³ and Poonsuk Prasertsan^{1,2*}

Abstract—The microbial production of ethanol from biodiesel-derived crude glycerol by *Enterobacter aerogenes* TISTR1468, under micro-aerobic and anaerobic conditions, was investigated. The experimental results showed that micro-aerobic conditions were more favorable for cellular growth (4.0 g/L DCW), ethanol production (20.7 g/L) as well as the ethanol yield (0.47 g/g glycerol) than anaerobic conditions (1.2 g/L DCW, 6.3 g/L ethanol and 0.72 g/g glycerol, respectively). Crude glycerol (100 g/L) was consumed completely with the rate of 1.80 g/L/h. Two-stage fermentation (combination of micro-aerobic and anaerobic condition) exhibited higher ethanol production (24.5 g/L) than using one-stage fermentation (either micro-aerobic or anaerobic condition). The two-stage configuration, exhibited slightly higher crude glycerol consumption rate (1.81 g/L/h), as well as ethanol yield (0.56 g/g) than the one-stage configuration. Therefore, two-stage process was selected for ethanol production from *E. aerogenes* TISTR1468 in scale-up studies.

Keywords—crude glycerol, ethanol, micro-aerobic, two-stage, *Enterobacter aerogenes*

I. INTRODUCTION

DUE to the increase in the petroleum price and the environmental concerns about car gases pollution, biodiesel is becoming a developing area of high concern. Biodiesel is defined as fatty acid methyl ester or ethyl ester from vegetable oils or animal fats and used as fuel in diesel engines and heating systems [1]. The principal by-product of biodiesel production is the crude glycerol, which comprises about 10 % (w/v) of the vegetable oils [2]. Glycerol could be applied in food, drug, cosmetic and tobacco industries. However, crude glycerol has very low value, because of its impurities, such as

methanol [3] and the high cost of purification. Hence, the surplus amount is still left unused in the biodiesel plants in Thailand including the biodiesel pilot plant in the University campus.

Glycerol is non-fermentable by most microorganisms, with the exception of group of bacteria including *Bacillus*, *Clostridium*, *Enterobacter*, *Klebsiella* and *Lactobacillus* species [4]. Conversion of crude glycerol to valuable products such as 1,3-propanediol and 2,3-butanediol [5], ethanol, 1,2-propanediol, dihydroxyacetones, hydrogen, polyglycerols, succinic acid was possible [6]. Ethanol and hydrogen from crude glycerol could be produced by *Enterobacter aerogenes* HU-101 under anaerobic condition [7]. Besides ethanol, formic acid was also produced during anaerobic glycerol fermentation by mixed cultures [8] and 1,2-propanediol by *Paenibacillus macerans* [9]. A mutant strain of *Klebsiella pneumoniae* GEM167 could produce higher ethanol concentration (21.5 g/L) and showed higher productivity (0.93 g/L/h) than the wild type strain [10]. The newly isolated bacteria *Kluyvera cryocrescens* S26 could convert biodiesel-derived crude glycerol to ethanol (27 g/L) with high molar yield (0.80 g/g) and productivity (0.61 g/L/h) in batch fermentation under micro-aerobic condition [11]. Glycerol could be converted to ethanol under both micro-aerobic and anaerobic condition. Therefore, this study aims to compare the capability of *Enterobacter aerogenes* TISTR1468 in ethanol production from crude glycerol under micro-aerobic, anaerobic conditions and the combination of both conditions (two-stage process).

II. MATERIALS AND METHODS

Bacteria: *Enterobacter aerogenes* TISTR1468 was purchased from Thailand Institute of Scientific and Technological Research (TISTR).

Crude glycerol: Crude glycerol was obtained from biodiesel production pilot plant at Faculty of Engineering, Prince of Songkla University, Thailand. Biodiesel was produced from waste cooking oil by conventional transesterification batch process using methanol as a reactant and sodium hydroxide as a catalyst [12]. The crude glycerol (containing 45% w/w glycerol) was used as carbon source in the culture medium without purification.

Medium: Preculture medium contained (per liter) 20 g pure glycerol, 5 g yeast extract, 7.0 g K₂HPO₄, 5.5 g KH₂PO₄, 2.1 g

Kanokrat Saisaard, Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Thailand (e-mail: kpoe_20@hotmail.com).

Irini Angelidaki, Department of Environmental Engineering, Technical University of Denmark, Building 113, DK-2800 Kgs. Lyngby, Denmark. (email: iria@env.dtu.dk)

Poonsuk Prasertsan, Department of Industrial Biotechnology, Palm Oil Product and Technology Research Center, Faculty of Agro-Industry, Prince of Songkla University, Thailand (e-mail: poonsuk918@yahoo.com)

(NH₄)₂HPO₄, 0.25 g MgSO₄·7H₂O, 0.021 g CaCl₂·2H₂O, 0.12 g Na₂MoO₄·2H₂O, 2.0 mg nicotinic acid, 0.172 mg Na₂SeO₃, 0.02 mg NiCl₂, and 10 mL trace element solution (0.5 g MnCl₂·4H₂O, 0.1 g H₃BO₄, 0.01g AlK(SO₄)₂·H₂O, 0.001 g CuCl₂·2H₂O and 0.5 g Na₂EDTA (per liter)). The fermentation medium had the same composition as the preculture medium except that 100 g crude glycerol (45% w/w) was used instead of pure glycerol (modified from [7]).

Cultivation conditions: One loopful of culture from the agar slant was inoculated into the preculture medium (200 ml) in a 500 ml flask and cultivated on a rotary shaker (120 rpm) at 37 °C for 18 h. The culture was diluted with the preculture medium to obtain OD₆₀₀ of 0.5. The starter culture (10% v/v) was added into a 3 L fermentor with a 2 L working volume of the fermentation medium adjusted the initial pH to 8.0 and cultivated at 37 °C with agitation speed of 60 rpm. A micro-aerobic condition in the bioreactor was maintained using the aeration rate of 0.5 vvm whereas no aeration for an anaerobic condition. In two-stage condition, first stage was performed for 24 h under micro-aerobic condition (0.5 vvm) and second stage was conducted for another 24 h under anaerobic condition.

Analytical methods: Cell growth was determined as biomass or dry cell weight (DCW) concentration by centrifugation (8000 xg and 4 °C) the sample for 8 min, then washed the cells and dried at 105 °C overnight before weighed [13]. Ethanol concentration was determined by gas chromatography with a flame ionization detector (Hewlette Packard 6890) using a capillary column (model HP 19091N-113) operated at a temperature of 250°C with Helium as carrier gas [7]. Glycerol was spectrophotometrically determined by chromotropic acid method (modified from [14]).

III. RESULTS AND DISCUSSIONS

Micro-aerobic and anaerobic conditions

Time profiles of biomass, ethanol and glycerol concentration are depicted in Fig.1. The fermentation medium contained 100 g/L of crude glycerol (45% w/w), which is equivalent to 45 g/L pure glycerol. Under micro-aerobic condition, the cell concentration increased rapidly during 4-20 h giving a growth rate of 0.22 g/L/h, which was almost stable until the end of cultivation (48 h). The highest biomass production under micro-aerobic condition (4.0 g/L) was 3.3 folds higher than that under anaerobic condition (1.2 g/L) (Fig.1A). This gave the specific growth rate of 0.19 and 0.04 h⁻¹, respectively.

Ethanol was growth-associated product (Fig.1B), as it increased with the increase of biomass. The production of ethanol under micro-aerobic condition (20.7 g/L) was also 3.3 times higher than that under anaerobic condition (6.3 g/L). This gave the ethanol productivity of 0.87 and 0.12 g/L/h, respectively. The glycerol consumption (Fig.1C) was rapid under micro-aerobic condition and was depleted completely in the stationary phase (24 h cultivation). The glycerol consumption rate was 1.80 g/L/h and gave high cell (0.09 g/g) and ethanol concentration (0.47 g/g). Under anaerobic condition, only 19% of glycerol was consumed and converted to small amount of biomass and ethanol with the yield of 0.09

g biomass/g glycerol consumed and 0.72 g ethanol/ g glycerol consumed.

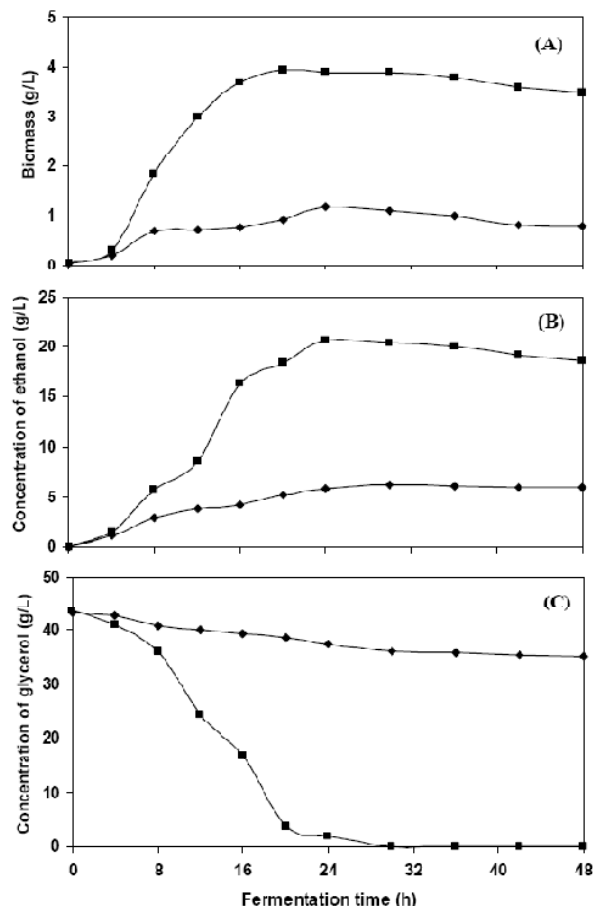


Fig.1 Time course of concentrations of biomass (A), ethanol (B), and residual glycerol (C) during batch cultures of *Enterobacter aerogenes* TISTR1468 under micro-aerobic (■) and anaerobic (◆) conditions

The above results indicated that oxygen could activate the growth of *E. aerogenes* TISTR1468, substrate consumption, ethanol production (20.7 g/L) and ethanol productivity (0.87 g/L/h). The values were similar to the mutant strain of *Klebsiella pneumoniae* (21.5 g/L and 0.93 g/L/h, respectively) [10] and *Kluyvera cryocrescens* S26 (18.33 g/L and 0.92 g/L/h, respectively) [11]. Besides ethanol, the production of 1,3-propanediol from *Klebsiella pneumoniae* was also higher under micro-aerobic condition than anaerobic condition, because of the dissolved oxygen activates faster cell growth and of the gene expression system for enzymes of the citric acid cycle, which provides energy for cell growth [15]. In addition, oxygen supply at high level can enhance the generation of ATP by reducing NADH, which is then used for biomass synthesis. Under micro-aerobic condition, limited amount of oxygen managed to convert NADH generated during cell growth into NAD while maintaining carbon flux into ethanol synthesis.

Enhancement of ethanol production by using two-stage process

The results revealed that micro-aerobic condition was more favorable for cellular growth, ethanol production and glycerol consumption than anaerobic condition. However, generally the ethanol was produced under anaerobic condition [16]. Therefore, combination of micro-aerobic condition for cell production (first stage) and anaerobic condition for ethanol production (second stage) was conducted to enhance the ethanol production. Besides, to our knowledge, up to now there is no report for ethanol production from crude glycerol using two-stage process.

The concentration of biomass, ethanol, and glycerol (Fig.2) under two-stage process, were similar to those obtained under micro-aerobic condition. In the first stage (micro-aerobic condition), the cell concentration increased rapidly within 24 h with the productivity of 0.19 g/L/h, which was 19 times higher than that under anaerobic condition (0.01 g/L/h). The highest biomass production was 4.4 g/L at 32 h. Ethanol production increased rapidly in micro-aerobic condition (0.95 g/L/h) and increased gradually in anaerobic stage (0.15 g/L/h). The highest ethanol concentration was 24.5 g/L. The glycerol consumption was almost complete within the first stage condition and depleted in anaerobic condition.

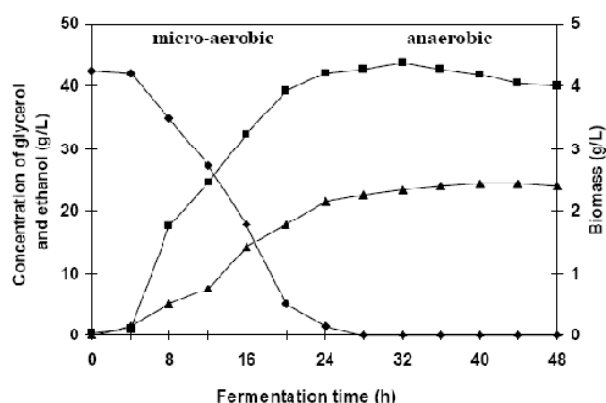


Fig.2 Time course of concentrations of biomass (■), ethanol (▲), and residual glycerol (◆) during batch cultures of *E. aerogenes* TISTR1468 under two-stage condition

TABLE I
COMPARISON OF KINETIC VALUES IN MICRO-AEROBIC AND TWO-STAGE IN BATCH FERMENTATION BY *E. AEROGENES* TISTR1468

	Conditions	
	Micro-aerobic	Two-stage
Specific growth rate (1/h)	0.19 ± 0.02	0.19 ± 0.003
Ethanol productivity (g/L/h)	0.87 ± 0.08	0.68 ± 0.01
Biomass productivity (g/L/h)	0.17 ± 0.02	0.16 ± 0.01
Glycerol consumption rate (g/L/h)	1.80 ± 0.13	1.81 ± 0.11
Ethanol yield (g/g)	0.47 ± 0.01	0.56 ± 0.01
Biomass yield (g/g)	0.09 ± 0.01	0.10 ± 0.003

The experimental results, of the time profile of biomass, ethanol and glycerol concentration under micro-aerobic and two-stage condition were similar. The kinetic parameters are

presented in Table.1. The specific growth rate and ethanol productivity under micro-aerobic condition were higher than those using two-stage process. However, the ethanol yield, glycerol consumption rate and ethanol productivity under the two-stage condition (0.56 g/g, 1.81 g glycerol/L/h and 0.68 g/L/h, respectively) were higher than those obtained from micro-aerobic condition (1.80 g/L/h and 0.47 g/g, respectively). When compared to the ethanol production from glycerol by different microorganisms, it was found that *E. aerogenes* TISTR1468 using the two-stage process exhibited the highest ethanol concentration (Table 2).

IV. CONCLUSION

In this present investigation, the ethanol production by *Enterobacter aerogenes* TISTR1468 using crude glycerol

TABLE II
COMPARISON ETHANOL PRODUCTION FROM GLYCEROL BY DIFFERENCE MICROORGANISMS

Organism	Condition	Ethanol production		Reference
		Concentration (g/L)	Productivity (g/L/h)	
<i>Enterobacter aerogenes</i> HU-101	anaerobic	10	0.83	[7]
<i>Klebsiella oxytoca</i> M5a1	micro-aerobic	19.5	0.56	[17]
<i>Klebsiella pneumoniae</i> GEM167	micro-aerobic	21.5	0.93	[10]
<i>Enterobacter aerogenes</i> TISTR1468	micro-aerobic	20.7	0.87	Present study
<i>Enterobacter aerogenes</i> TISTR1468	two-stage	24.5	0.68	Present study

under the two-stage process was higher than those using micro-aerobic or anaerobic conditions. The highest ethanol concentration of 24.5 g/L and productivity of 0.68 g/L/h were achieved. This is the first report that ethanol was produced under two-stage process using crude glycerol as substrate.

ACKNOWLEDGMENT

This research work was financially supported by the Prince of Songkla University Graduate Studies Grant, Faculty of Agro-Industry and the Graduate School, Prince of Songkla University, Songkhla, Thailand.

REFERENCES

- [1] J. M. Marchetti, V. U. Miguel, and, A. F. Errazu, "Possible methods for biodiesel production," *Renew. Sust. Energy. Rev.*, vol. 11, pp. 1300-1311, 2007.
- [2] M. A. Dasari, P. P. Kiatsimkul, W. R. Sutterlin, and G. J. Suppes, "Low-pressure hydrogenolysis of glycerol to propylene glycol," *Appl. Catalysis.*, vol. 281, no. 1-2, pp. 225-231, 2005.
- [3] J. C. Thompson, and B. B. He, "Characterization of the crude glycerol from biodiesel production from multiple feedstocks," *Appl. Eng. Agri.*, vol. 22, pp. 261-265, 2006.
- [4] Z. M. Zheng, Q. L. Hu, J. Hao, F. Xu, N. N. Guo, Y. Sun, and D. H. Liu, "Statistical optimization of culture condition for 1,3-propanediol by *Klebsiella pneumoniae* AC15 via central composite design," *Bioresour. Technol.* vol. 99, pp. 1052-1056, 2008

- [5] S. Sattayasamitsathit, P. Prasertsan, and P. Methacanon, "Statistical optimization for simultaneous production of 1,3-propanediol and 2,3-butanediol using crude glycerol by newly bacterial isolate," *Process Biochem* vol. 46, pp. 608-614, 2011.
- [6] N. Pachauri, and B. He, "Value-added utilization of crude glycerol from biodiesel production," *A Survey of Current Research Activities. An ASABE Meeting Presentation*, Oregon, 2006.
- [7] T. Ito, Y. Nakashimada, K. Senba, T. Matsui, and N. Nishio, "Hydrogen and ethanol production from glycerol-containing wastes discharged after biodiesel manufacturing process," *J. Biosci. Bioeng.*, vol. 100, no. 3, pp. 260-265, 2005.
- [8] M. F. Temudo, R. Poldermans, R. Kleerebezem, and M. C. M. Loosdrecht, "Glycerol fermentation by (open) mixed cultures: a chemostat study," *Biotechnol. Bioeng.*, vol. 100, pp. 1088-1098, 2008.
- [9] A. Gupta, A. Murarka, P. Campbell, and R. Gonzalez, "Anaerobic fermentation of glycerol in *Paenibacillus macerans*: metabolic pathways and environmental determinants," *Appl. Environ. Microbiol.*, vol. 75, pp. 5871-5883, 2009.
- [10] R. B. Oh, J. W. Seo, S. Y. Heo, W. K. Hong, L. H. Luo, M. H. Joe, D. H. Park, and C. H. Kim, "Efficient production of ethanol from crude glycerol by a *Klebsiella pneumoniae* mutant strain," *Bioresour. Technol.*, vol. 102, pp. 3918-3922, 2011.
- [11] W. J. Choi, M. R. Hartono, W. H. Chan, and S. S. Yeo, "Ethanol production from biodiesel-derived crude glycerol by newly isolated *Kluyvera cryocrescens*," *Appl. Microbiol. Biotechnol.*, vol. 89, pp. 1255-1264, 2011.
- [12] C. Tongurai, S. Klinpikul, C. Bunyakan, and P. Kiatsimkul, "Biodiesel production from palm oil," *Songkla. J. Sci. Technol.*, vol. 23, pp. 831-841, 2001.
- [13] F. Barbirato, S. Astruc, P. Soucaille, C. Camarasa, J. M. Salmon, and A. Bories, "Anaerobic pathways of glycerol dissimilation by *Enterobacter agglomerans* CNCM1210: limitations and regulations," *Microbiol.*, vol. 43, pp. 2423-2432, 1997.
- [14] E. V. Handel, "Suggested modification of micro determination of triglycerides," *Clinical Chem.*, vol 7, no. 3, pp. 249-251, 1961.
- [15] X. Chen, D. J. Zhang, V. T. Qi, S. J. Gao, Z. L. Xiu, and P. Xu, "Microbial fed-batch production of 1,3-propanediol by *Klebsiella pneumoniae* under micro-aerobic conditions," *Appl. Microbiol. Biotechnol.*, vol 63, pp. 143-146, 2003.
- [16] S. S. Yazdani, and R. Gonzalez, "Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry," *Curr. Opin. Biotechnol.*, vol. 18, pp. 213-219, 2007.
- [17] G. Yang, J. Tian, and J. Li, "Fermentation of 1,3-propanediol by a lactate deficient mutant of *Klebsiella oxytoca* under microaerobic conditions," *Appl. Microbiol.*, vol. 73, pp. 1017-1024, 2007.