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# Development of ethanol production from cooking oil glycerol waste by mutant *Enterobacter aerogenes*

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**Bacterial strains, capable of producing ethanol by using cooking oil glycerol waste (derived from biodiesel production) as sole C-source were screened from soil samples. Based on 16S rRNA sequence, the selected strain was identified as *Enterobacter aerogenes* G2WG. The sequence was submitted to GenBank and the accession number KU315428 was obtained. The genetic nature of the wild type strain was altered for enhancing ethanol production using UV irradiation and chemical method. Three steps of mutation treatment were developed. Finally, the best strain of *Enterobacter aerogenes* V90M11 was obtained. As *E. aerogenes* is a facultative anaerobic microbe, it can growth well and produce high yield of ethanol in low aeration condition. At 15 g/l glycerol, *E. aerogenase* V90M11 converted glycerol waste and pure glycerol into 6.7 and 3.62 g/l ethanol respectively. The highest ethanol (14.77 g/l) was gained from 30 g/l of glycerol waste in batch process at initial pH 8, and 35°C temperature with micro-aeration. It was equivalent to 0.984 mol ethanol/mol glycerol. Fed-batch process was investigated by adding 200 ml (30 g/l glycerol) and 400 ml (55 g/l glycerol) of fresh T-glycerol medium to the culture after 24h fermentation process. It was found that higher ethanol yield of around 33% and 59% was obtained from fed batch than batch process, with initial glycerol of 15 g/l, respectively.**

**Key words:** Ethanol, cooking oil glycerol waste, *Enterobacter aerogenes*, mutation, UV, chemical treatment.

## INTRODUCTION

Nowadays, surplus glycerol is derived from the high demand of biodiesel production around the world. 16 billion liters by 2020 will be generated (OECD/FAO 2015). Glycerol can be used in various industries, such as cosmetics, food and drinks, pharmaceutical, paint, toiletries, plastics, leather, and textile. Glycerol, a major by-product in biodiesel manufacturing process, usually has 55-90% impurity that consists of unconverted methanol and triglycerides, catalyst residue, biodiesel, soaps and other contaminants (Meher et al., 2006). However, it is very expensive to purify, especially crude glycerol from cooking oil. It is now left as a waste stream that costs much to store and dispose. Chemical and biological process of converting biodiesel glycerol waste

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to high value-added products is currently being examined. However, biological approaches both in anaerobic or aerobic process draw more attention than chemical process. Glycerol ($C_3H_8O_3$; K=4.67) is a better c-source than common sugars (glucose ($C_6H_{12}O_6$) : K=4.0; xylose ($C_5H_{10}O_5$): K=4.0) used for fermentation (Chaudhary et al., 2011; Durnin et al., 2009; Nielsen et al., 2003) because it has higher average degree of reduction per carbon (K). Many authors have studied the microbial conversion of crude glycerol to various compounds, such as hydrogen, furmaric acid ($C_4H_4O_4$), succinic acid ($C_4H_6O_4$), acetic acid ($C_2H_4O_2$), lactic acid ($C_3H_6O_3$), citric acid ($C_6H_8O_7$), glyceric acid ($C_3H_6O_4$), amino acid, 1,3-propanediol ($C_3H_8O_2$), 1,3-dihydroxyacetone ($C_3H_6O_3$), biosurfactant, and ethanol ($C_2H_6O$) (Li et al., 2013; Volker et al., 2011; Cha et al., 2015; Chanthoom et al., 2016).

Bioethanol is an alternative fuel derived from fermentation process of sugar, starch, cellulose and also biodiesel glycerol waste (Ito et al., 2005). Mostly species in the family of Enterobacteriaceae, such as *Klebsiella pneumonia* (Wu et al., 2011; Oh et al., 2011), *Klebsiella variicola* (Suzuki et al., 2014), *Kluyvera cryocrescens* (Choi et al., 2011) including *Escherichia coli* (Ito et al., 2005) and *Enterobacter aerogenes* (Lee et al., 2012) are able to convert glycerol to ethanol. Ito et al. (2005) reported that hydrogen and ethanol were mainly products of *E. aerogenes*. Besides this, *E. coli* can use glycerol at optimum conditions to produce ethanol with minimum hydrogen accumulation (Dharmadi et al., 2006). Furthermore, a yeast strain of *Pachysolen tannophilus* was able to convert glycerol to ethanol of 17.5 g/l using 5% (v/v) crude glycerol (Liu et al., 2012). However, most wild type strains produce low yield of ethanol. In order to increase ethanol production yield, two approaches of a genetically modification and a non-genetically modified were used.

Recently, Nwachukuw et al. (2012) reported the successful development of a mutant *E. aerogenes* using six-tube-subculture-generations method. The mutant strain can grow in a high content of 20% glycerol medium. It utilizes 39 g/l glycerol from 50 g/l pure glycerol in the medium, yielding 20 g/l ethanol at 120h. Liu et al. (2012) improved ethanol production in a 2 staged-batch, adding crude glycerol. Ethanol of up to 28.1 g/l was gained. High-ethanol-tolerant mutant and ribosome engineering technique were also used to improve ethanol production using *Klebsiella variicola* TB-83D. It was found that high ethanol (34 g/l) was achieved by adding yeast extract and combining with corn steep liquor (Suzuki et al., 2015). A mutant strain of *Klebsiella pneumonia* GEM167 was derived from γ-irradiation. Maximum ethanol production of 21.5 g/l from pure glycerol was obtained by those mutant strains (Oh et al., 2011). Saisaard et al. (2011) studied the micro-aerobic, anaerobic and two-stage conditions for ethanol production from biodiesel-derived crude glycerol using *E. aerogenes* TISTR1468. The results revealed that more cell growth and ethanol production (20.7 g/l) were obtained in micro-aerobic condition than in anaerobic condition (6.3 g/l ethanol).

In this study, UV and hydroxylamine treatments were used to improve the wild type strain of bacteria that can use cooking oil glycerol waste derived from biodiesel as c-source and convert it to higher ethanol yield. Batch and fed batch processes were also studied in the fermenter with micro-aeration condition.

## MATERIALS AND METHODS

### Cooking oil glycerol waste pre-treatment

Cooking oil glycerol waste, a by-product of biodiesel production from cooking oil was used in this study. It was derived from Faculty of Engineering, Khon Kaen University. The impurity was removed by pre-treatment with 8 N HCl until pH 7 (Tianfeng et al., 2013) was obtained. The precipitate was easily removed from the mixture by heating to a temperature of about 70°C for 30 min. It was left to cool down at room temperature in separating funnel. Two distinct phase layers of the solution were separated. Organic salt and free fatty acid (top phase) were removed and T-glycerol at the bottom phase was kept. Glycerol content was determined by the method of Bondioli and Bella (2005).

### Microorganism and culture medium

Bacterial wild type strain, G2WG capable of converting cooking oil glycerol to ethanol was isolated from soil sample. The strain was maintained in YMG (yeast extract, 3 g/l; malt extract, 3 g/l; peptone, 5 g/l; glucose, 1 g/l; pure glycerol, 9 ml/l; agar, 15 g/l) in screw capped glass tube at 4°C. The pre-cultures were also cultivated in 5 ml YMG broth medium at 35°C for 24 h in a screw capped tube. Anaerobic fermentation was performed in 60 ml serum bottles containing 27 ml GB medium. It was done by transferring 3 ml of seed inoculum with sterilized syringe and incubated at 35°C. The GB (glycerol base) medium contains (per liter) yeast extract of 2 g; $K_2HPO_4$, 1.6 g; $NaH_2PO_4$, 0.85 g; $(NH_4)_2SO_4$, 2 g; $MgSO_4.7H_2O$, 0.4 g; $ZnSO_4.7H_2O$, 0.04 g, and pure glycerol, 20 ml. The medium was adjusted to pH 8 before sterilization at 121°C for 15 min.

### Batch fermentation mode

Batch fermentation process was carried out in fermenter (Biostat B, B. Braun, Biotech International, Germany) containing GB medium with working volume of 1.1 L. Seed inoculum was prepared in YMG medium with 10% of working volume. Temperature (35°C) and agitation speed of 250 rpm, with minimum air supplied were controlled continuously. Glycerol and ethanol concentrations were analyzed from the supernatant.

### Fed batch fermentation mode

Two types of fed batch processes were studied in Biostat fermenter. Firstly, 200 ml of GB medium (crude glycerol equivalent to 30 g/l pure glycerol) was added in the fermenter at 24 h after adding 100 ml of seed inoculum (YMG medium). At the beginning, the bioreactor contained 800 ml GB medium (15 g/l crude glycerol). Secondly, GB medium (700 ml) containing 20 g/l of glycerol waste was filled in the fermenter. GB fresh medium (400 ml) containing 55

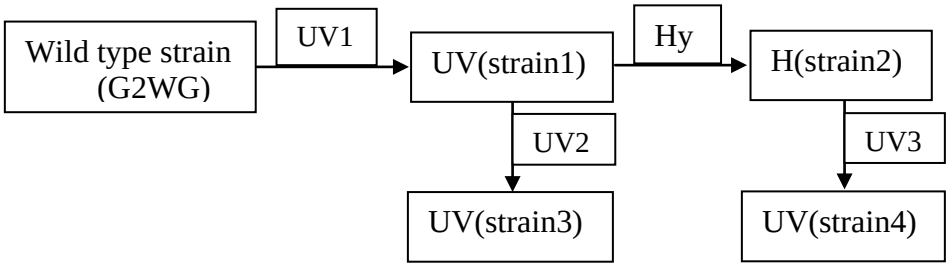


**Figure 1.** Schematic diagram of UV light (90 lux) and chemical treatment (160 mM hydroxylamine, Hy) for improving higher ethanol yield from *E. aerogenase.*

g/l glycerol waste was added after 24 h of adding seed inoculum (YMG 100 ml). The fermentation process was controlled as described in the batch process.

#### Mutation with UV and chemical treatment

UV light and hydroxylamine were used as mutagen. The selected wild type strain was grown on YMG liquid medium for 24 h before it was exposed beneath 90 lux of UV light at various times (UV1). The cell dilution of 0.1 ml ($10^{-4}$ to $10^{-6}$) was spread on GB agar plate and incubated at 35°C. The single colony was subsequently transferred to the medium and ethanol was tested after inoculation in serum bottle for 24 h. The best isolated UVstrain1 was treated with 160 mM hydroxylamine (Hy) at various times and spread on GM agar plate for single colony selection. Ethanol production was also determined from the selected colony using the same procedure as previously described in serum bottle. UV pretreatment (UV2 and UV3) was done with UV(strain1) and H (strain 2), and UV (strain 3) and UV (strain 4) were obtained respectively. Ethanol production from the selected colonies at each step was determined, and the most efficient strain for further study was selected. Mutation diagram of this study is shown in Figure 1.

#### Analytical methods

The fermentation broth was collected and centrifuged (Minispin plus, Germany) at 10,000 rpm for 5 min. The supernatant was kept in deep freezer (Sanyo, Japan) at -20°C until ethanol and glycerol were determined. Ethanol was analyzed by gas chromatography (14A Model Shimadzu, Japan) equipped with flame ionization detector. The column was packed with 80-100 mesh of Porapack Q. The temperature of the injector and detector was 200°C and the column temperature was 180°C. Isopropanal, used as internal standard, was added in equal volume of the sample and 1ul of the mixture was injected. Glycerol concentration was analyzed by the method of Bondioli and Bella (2005) using spectrophotometer (DU® 700series UV/VIS, Beckman Coulter, USA). All chemicals used in microbial medium analytical methods were of laboratory and analytical grades, respectively.

#### Bacterial strain identification

The total genomic DNA of the selected wild type strain was extracted. The 16S rRNA was amplified by PCR technique (thermal cycler Model Px2, Thermo Scientific Hybaid, USA), with 27F (5´-AGAGTTTGATCCTGGCTGAG-3´) and 1492R (5´-TACGGCTACCTTGTTACGACTT-3´) as primers. The PCR conditions are as follows: initial denaturation at 95°C for 5 min; 30 cycles of denaturation for 45 s at 94°C; annealing for 60 s at 55°C; extension for 60 s at 72°C; and the final extension step at 72°C for 5 min. The PCR product was sequenced with 2 pairs primer of 27F with 1492R and 518F (5´-CCAGCAGCCGCGGTAATACG-3´) and 800R (5´-TACCAGGGTATCTAATCC-3´). Big dye terminator cycle sequencing kit was used with Applied Biosystem model 37230XL, USA. The DNA sequence obtained was aligned with GenBank database. Phylogenetic tree was reconstructed by PUAP* version 4.0b10 (Swofford, 1993).

#### Statistical analysis

The data of the experiments were done with three replicates using the same sample, and were reported as means with standard deviations.

## RESULTS AND DISCUSSION

### Bacterial screening and identification

Ethanol producing bacteria were obtained from various soil samples. The best strain that was able to produce ethanol from cooking oil glycerol waste was tested and selected. In order to identify the species of the selected strain molecular technique of 16S rRNA base pairs was used. GenBank database was retrieved by blastn dialog boxes. There was 99 to 100% similarity among the various Enterobacteraceae strains of GenBank database. Some strains of *E. aerogenease* including the related species were selected for phylogenetic tree construction. Neighbor joining tree (Figure 2) showing bacterial strain G2WG was placed in *E. aerogenes* clade. Then it was named *Enterobacter aerogenes* G2WG. The gene sequencing was submitted to National Center for Biotechnology Information (NCBI) and accession number KU315428 was obtained.

### UV and chemical treatment of mutants

From previous study (data not shown), it was found that the wild type strain produced high ethanol at 35°C, pH 8, 2 g/l $(NH_4)_2SO_4$ and 20 g/l glycerol. In order to increase the ethanol yield, firstly the wild type bacterium, *E.*

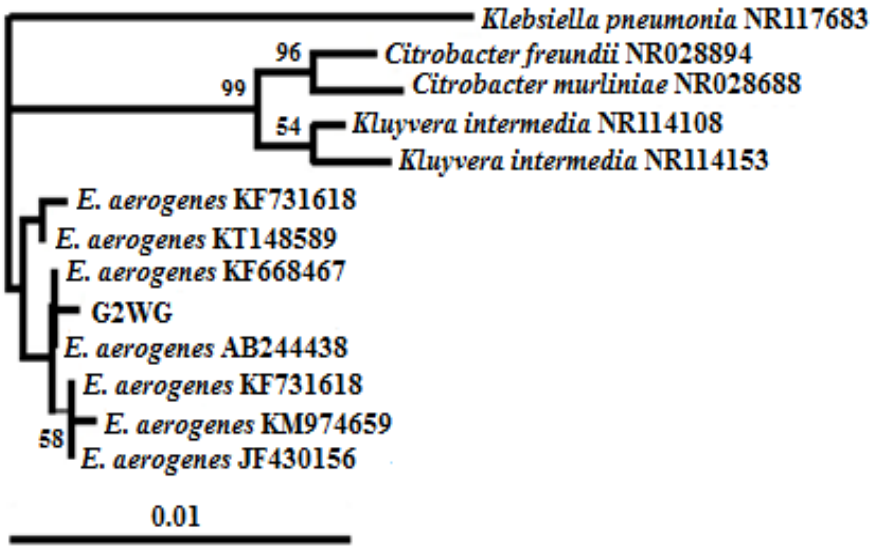


**Figure 2** Neighbor joining tree for 16S rRNA of the strain G2WG and the sequences from GenBank database reconstructed by HKY85 genetic distance method with 1416 bp, using PUAP* (Swofford, 1993). The numbers at nodes indicated bootstrap values of 1000 replicates. The tree was rooted with Klebsiella *pneumonia* NR117683. Scale represented 0.01 changes per site.

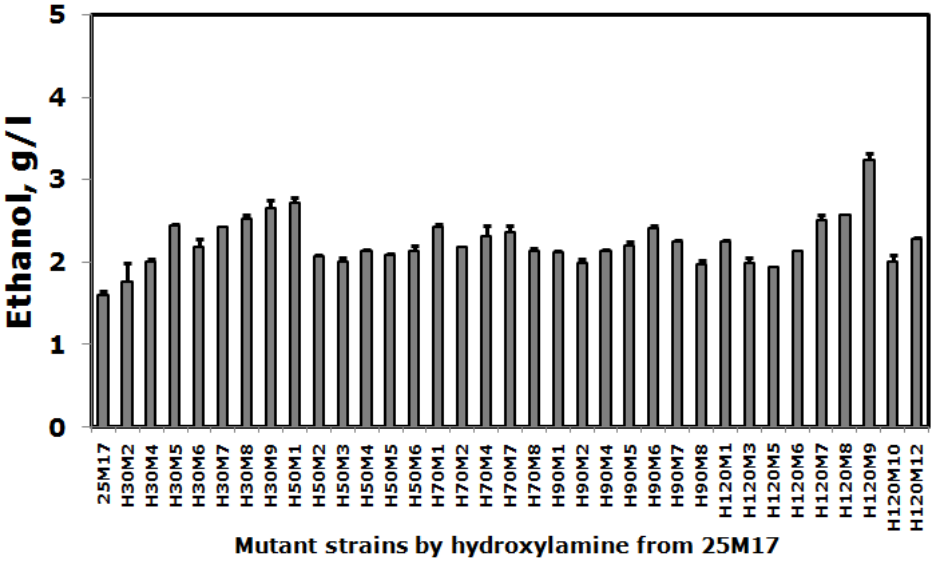


**Figure 3.** Ethanol production at 24 h from T-glycerol medium by various mutant strains that were derived from the mutant strain of 25M17 that was treated with hydroxylamine and compared to the parental strain of 25M17 that was derived from the mother strain of *E. aerogenes* G2WG.

*aerogenes* G2WG was exposed under UV light (90 lux) for 13, 25, 37 and 50 minutes. Ethanol produced from T-glycerol medium by 48 isolates was selected. The highest ethanol production was gained from the strain of 25M17 (UVstrain1, data not shown). Then it was selected and treated with hydroxylamine at different times. The results found that the highest ethanol was produced from the mutant strain of H120M9 (Hstrain2) as shown in Figure 3. This finding indicated that ethanol produced from the mutant strain at the second step (by chemical treatment) was 1.8 fold higher than the first treatment step. However, mutation was done again under UV light with 2

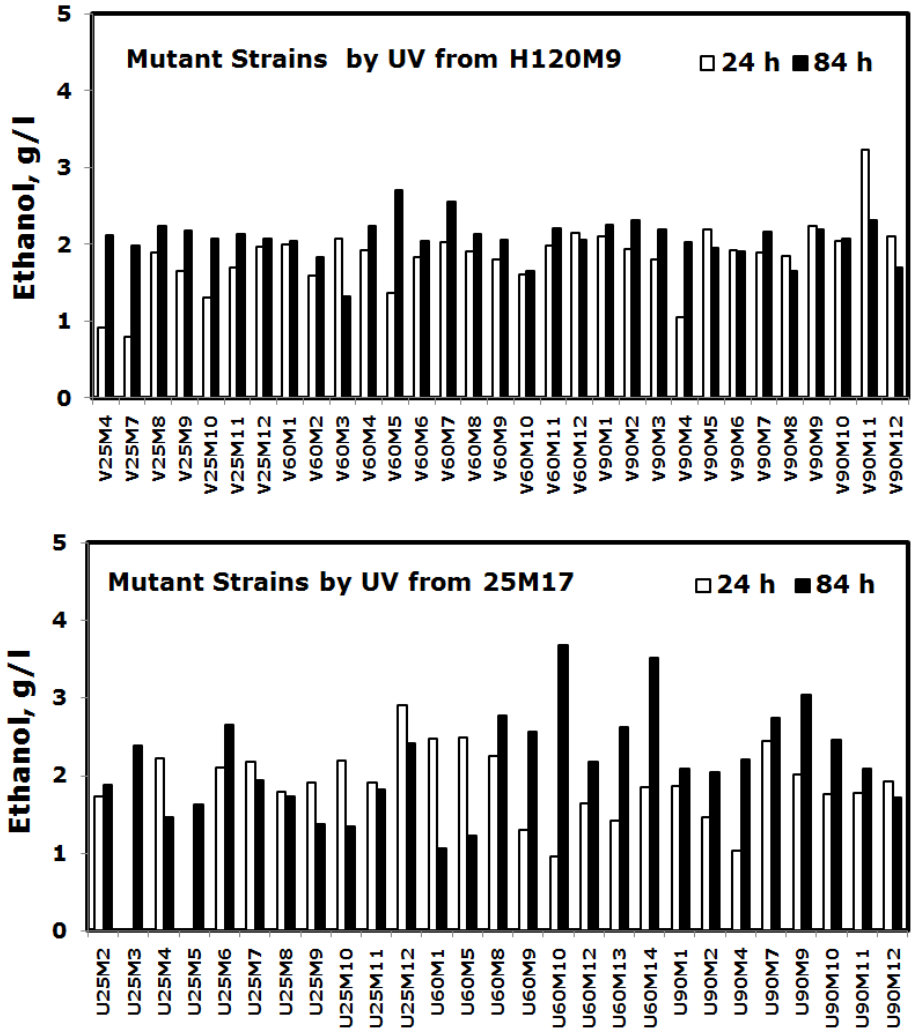


**Figure 4.** Ethanol production at 24 and 84 h from T-glycerol medium by the 2nd and the 3rd step of mutant strains under UV explosion to the strain of 25 M17 and H120M9, respectively.

strains of 25M17 and H120M9. It was found that ethanol concentrations from the selected mutant strains from those 2 strains were not higher than the second step of mutation with hydroxylamine (Figure 4). However, only one strain of V90M11 (UVstrain 4) derived from H120M9 produced maximum ethanol (3.2 g/l) at 24 h. While some of the mutant strains produced ethanol at 84 h higher than 24 h (U60M10 and U60M14) and slightly higher than that obtained from the strain of V90M11. Because the highest ethanol was produced by the mutant strain of V90M11 at 24 h, then it was chosen for further studied.

## Ethanol production in batch process

The wild type and the mutant strain were compared based on their ability to produce ethanol from crude glycerol in batch fermentation mode. The results are shown in Figure 5. It was found that crude glycerol was used rapidly by both strains within 24 h. Glycerol at the initial concentration of 15 g/l was consumed completely by the mutant strain within 12 h (Figure 5B), and the remaining glycerol was left at 4.16% from the initial concentration of 30 g/l (Figure 5C); while, 60.6% of the initial glycerol was used by the wild type strain (Figure 5A). The mutant strain showed more efficient utilization of glycerol than the wild type strain. Consequently, ethanol produced from the mutant strain was higher than the wild type. Ethanol produced by the mutant strain from glycerol at the initial concentration of 15 and 30 g/l was 8.2 and 14.8 g/l (at 48 h), respectively. While only 6.74 g/l of ethanol (at 40 h) was produced by the wild type strain. These results indicated that glycerol utilization and ethanol production by the mutant strain (*E. aerogenes* V90M11) were more efficient than those of the wild type strain of *E.aerogenes* G2WG. Chanthoom et al., (2016) studied ethanol

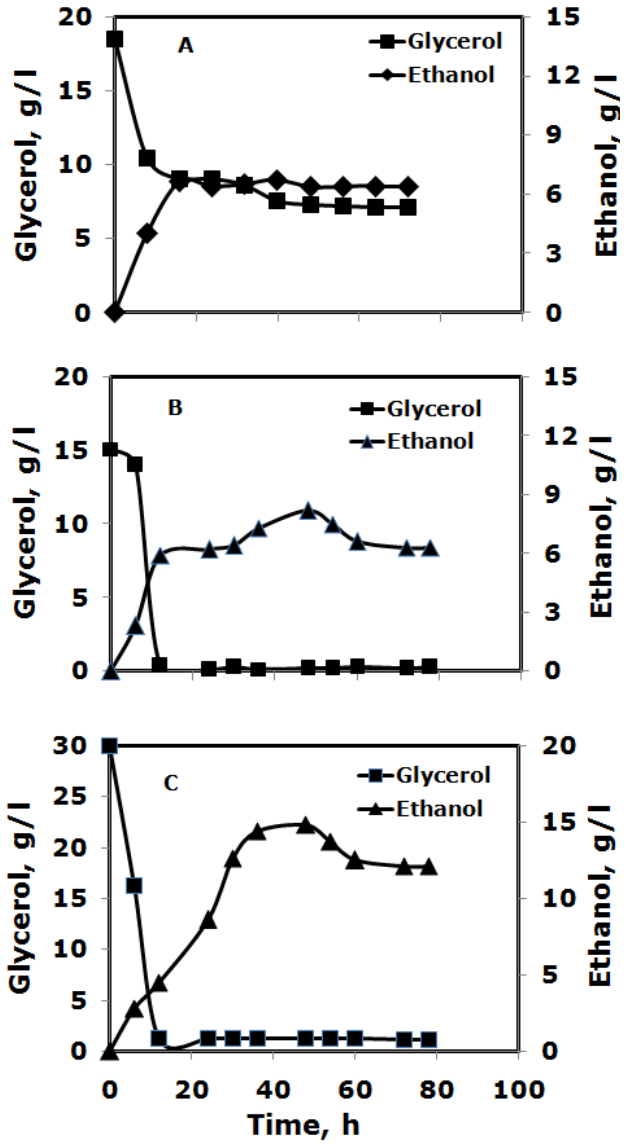


**Figure 5.** Batch fermentation for ethanol production from cooking oil glycerol waste (T-glycerol) medium by *E. aerogenes* G2WG and mutant strain of *E. aerogenes* V90M11. The wild type strain of *E. aerogenes* G2WG was cultivated in 18 g/l of glycerol waste (A). The mutant strain (*E. aerogenes* V90M11) was grown in the medium containing 15 g/l glycerol waste (B); and 30 g/l (C).

production from biodiesel-derived crude glycerol using *E. aerogenes*. They found that 40 g/l glycerol inhibited the substrate. The highest ethanol produced was 9.38 from 25 g/l of crude glycerol at 24 h, with productivity of 0.39 g/l/h. This led to the increment of productivity in batch culture of this study from 0.171 to 0.307 g/l/h when glycerol used was increased from 15 to 30 g/l, respectively (Table 1). Furthermore, they observed that *E. aerogenase* produced higher ethanol from crude glycerol than pure glycerol. This result corresponded to that of our study.

**Ethanol production in fed-batch**

In order to increase the ethanol production yield, two runs of fed batch process were investigated. Firstly, 15 g/l glycerol of 800 ml T-glycerol was filled in the fermentation vessel. After 24 h of inoculation with the mutant strain, 200 ml of T-glycerol medium (30 g/l of glycerol) was added to the culture. The fermentation profiles are shown in Figure 6A. It was indicated that the glycerol was used up at 24 h, and it was used up again at 48 h after adding the fresh medium at 24 h. Meanwhile, 6.5 g/l of ethanol was produced at 24 h, and increased to 10.9 g/l at 48 h after adding fresh medium. Ethanol concentration was decreased to 9.8 g/l at 84 h during the end of the fermentation. In Figure 6B, fed batch fermentation was exhibited with 20 g/l glycerol in 750 ml T-glycerol medium. For 24 h after adding the inoculum (*E. aerogenes* V90M11), 400 ml of fresh T-glycerol medium containing 55 g/l of glycerol was admixed. Glycerol was diluted in the fermenter and at that time the concentration was at 22 g/l. Before adding fresh medium, ethanol was produced up to 11.22 g/l. And then it was dropped to 7.8 g/l when the medium was diluted by adding fresh medium. After that ethanol concentration was increased to 13.07 g/l at the fermentation time of 36 h. Then it was gradually decreased to 7.68 g/l and remaining 1.1 g/l glycerol at 78 h (Figure 6B).

Glycerol can be used as C-source for ethanol production by various microorganisms as shown in Table 1. In order to produce high yield of ethanol, mutation techniques were used. In this study a new mutant strain, *E. aerogenes* V90M11 was successfully developed. T-glycerol was easily used and higher ethanol yield was produced than pure glycerol by *E. aerogenes* V90M11. This finding agrees with the report of Nwachukwu et al. (2012), Lee et al. (2012) and Chanthoom et al. (2016). Furthermore, Lee et al. (2012) reported that glycerol concentration higher than 20 g/l might be due to the limited glycerol uptake and utilization by *E. aerogenes* because of osmotic pressure of glycerol content between intracellular and extracellular. Besides this, the inhibition of ethanol production in *E. aerogenes* with 1% salts of NaCl and KCl was discussed. All bacterial strains of Enterobacteraceae are facultative anaerobe. Oh et al. (2011) found that maximal ethanol production was obtained at 0.5 vvm aeration by the mutant strain *Klebsiella pneumonia* GEM167, while high aeration (2 vvm) promoted highest cell growth. Furthermore, Wong et al. (2014) reported that microaerobic with 5% oxygen supplied to the fermentation system enhanced ethanol production from glycerol by the metabolic engineering strain of *Escherichia coli*. There are several methods to enhance high yield production of ethanol. Thapa et al. (2013), using co-fermentation of c-sources, discovered that the medium containing 16 g/l manitol and 20 g/l glycerol increased six times ethanol (32.10 g/l) than that containing glycerol alone (5.23 g/l) in anaerobic fermentation conditions.

**Table 1.** Comparative studies on ethanol production from crude glycerol waste by microorganisms.

| Microorganism | Fermentation mode/ (initial glycerol, g/l) | Ethanol production (g/l) | Productivity (g/l/h) | Reference |
|---|---|---|---|---|
| *Enterobacter aerogenes* (Mutant) | Batch (26 g/l) | 16 | 0.33 | Nwachukwu, et al., 2012 |
| *Pachysolen tannophilus* (Wild type) | 3-stage-batch/ (70 g/l) | Phase 1: 18.7 | 0.16 | Liu et al., 2012 |
| | (70 g/l) | Phase 2: 27.5 | 0.18 | |
| | (70 g/l) | Phase 3: 28.1 | 0.06 | |
| *Klebsiella pneumonia* (Mutant) | 3-repeated-fed-batch (20 g/l) | 24.6 | 0.492 | Oh et al., 2011 |
| *Kluyvera cryocrescens* (Wild type) | Batch (90 g/l) | 27 | 0.61 | Choi et al., 2011 |
| *Enterobacter aerogenes* (Wild type) | Batch (20 g/l) | 6.62 | 0.275 | Lee et al., 2012 |
| *Enterobacter aerogenes* | Batch (25 g/l) | 9.38 | 0.39 | Chanthoom et al., 2016 |
| *Enterobacter aerogenes* (Mutant) | **Batch(15 g/l)** | **8.2** | **0.171** | **This study** |
| | **Batch (30 g/l)** | **14.77** | **0.307** | |
| | Fed-batch (15 g/l) | 10.9 | 0.227 | |
| | Fed-batch (20 g/l) | 13.07 | 0.363 | |

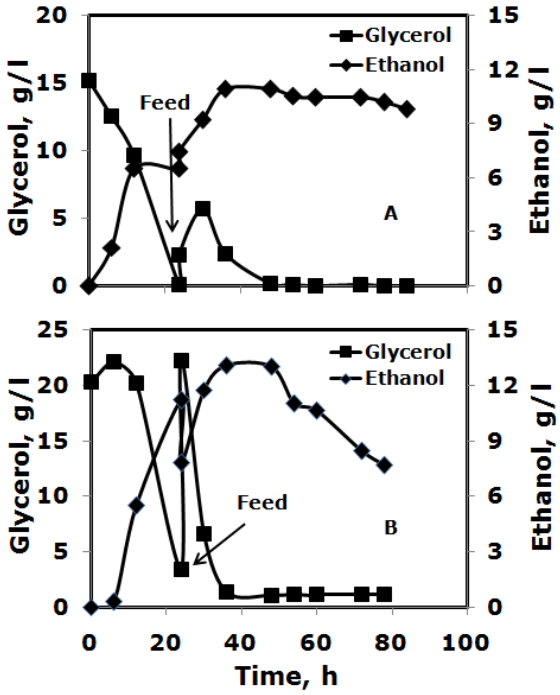


**Figure 6.** Ethanol production by the strain of *E. aerogenes* V90M11 from T-glycerol medium in fed-batch fermentation. The fresh medium containing different concentration of T-glycerol was added at 24 h of the fermentation period. Fresh medium 200 ml containing 30 g/l of T-glycerol was added into the culture that had the initial concentration of 15 g/l T-glycerol medium, A; Fresh medium 400 ml containing 55 g/l T-glycerol medium was added into the culture that had the initial concentration of 20 g/l T-glycerol medium, B.

## Conclusion

Cooking oil glycerol waste from biodiesel is composed more of impurity than crude glycerol from uncooked oil. However, partial purification glycerol waste by acid treatment can be used as c-source for ethanol production and more ethanol production than pure glycerol at the same concentration. This finding also corresponds to the results of Suzuki et al. (2014). Besides this, mutation with UV and chemical are also powerful tools for improving the wild type strain of *E. aerogenes* to produce higher ethanol yield. Fed batch process is a process for improving ethanol yield. However, glycerol concentration should be controlled for it not to be higher than substrate inhibition. Fed batch experiments should be studied more.

## Conflict of Interests

The authors have not declared any conflict of interests.

## ACKNOWLEDGMENTS

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## REFERENCES

Bondioli P, Bella LD (2005). An alternative spectrophotometric method for the determination of free glycerol in biodiesel. Eur. Lipid Sci. Technol. 107(13):153-157.

Cha HG, Kim YO, Choi WY, Kang DH, Lee HY, Jung KH (2015). Ethanol production from glycerol using immobilized Pachysolen tannophilus during microaerated repeated-batch fermentatior culture. J. Microbiol. Biotechnol. 25:366-374.

Chanthoom K, Tanikkul P, Sirisukpoka U, Pisutpaisal N (2016). Ethanol production from biodiesel-derived crude glycerol by *Enterobacter aerogenes*. Chem. Eng. Trans. 50:211-216.

Chaudhary N, Ngadi MO, Simpson BK, Kassama LS (2011). Biosynthesis of ethanol and hydrogen by glycerol fermentation using

*Escherichia coli.* Adv. Chem. Eng. Sci. 1:83-89.
Choi WJ, Hartono MR, Chan WH, Yeo SS (2011). Ethanol production from biodiesel-derived crude glycerol by newly isolated *Kluyvera cryocrescens*. Appl. Microbiol. Biotechnol. 89(4):1255-1264.
Dharmadi Y, Murarka A, Gonzalez R (2006). Anaerobic fermentation of glycerol by *Escherichia coli*: a new platform for metabolic engineering. Biotechnol. Bioeng. 94(5):821-829.
Durnin G, Clomburg J, Yeates Z, Alvarez PJJ, Zygourakis K, Campbell P, Gonzalez R (2009). Understanding and harnessing the microaerobic metabolism of glycerol in *Escherichia coli*. Biotechnol. Bioeng. 103(1):148-161.
Ito T, Nakashimada Y, Senba K, Matsui T, Nishio N (2005). Hydrogen and ethanol production from glycerol-containing wastes discharged after biodiesel manufacturing process. Biosci. Bioeng. 100(3):260-265.
Lee SJ, Kim SB, Kang SW, Han SO, Park C, Kim SW (2012). Effect of crude glycerol-derived inhibitors on ethanol production by *Enterobacter aerogenes*. Biopro. Biosyst. Eng. 35(1):85-92.
Li C, Lesnik KL, Liu H (2013). Microbial conversion of waste glycerol from biodiesel production into value-added products. Energies 6:4739-4768.
Liu X, Jensen PR, Workman M (2012). Bioconversion of crude glycerol feedstocks into ethanol by *Pachysolen tannophilus*. Bioresour. Technol. 104(1):579-586.
Meher LC, Sagar DV, Naik SN (2006). Technical aspects of biodiesel production by tranesterification-a review. Renew. Sustain. Energy Rev. 10(3):248-268.
Nielsen J, Villadsen J, Liden G (2003). Bioreaction engineering principles. New York: Kluwer Academic/Plenum Publishers, pp. 60-73.
Nwachukwu RES, Shahbazi A, Wang L, Ibrahim S, Worku M, Schimme K (2012). Bioconversion of glycerol to ethanol by a mutant *Enterobacter aerogenes*. AMB Express 2:20.
OECD (2015). OECD-FAO Agricultural Outlook 2015-2024. Food and Agriculture Organization of the United Nations. OECD Piblishing. [cited 2016 Oct. 8] Available from: http://www.fao.org/3/a-i4738e.pdf
Oh BR, Seo JW, Heo SY, Hong WK, Luo LH, Joe MH, Park DH, Kim CH (2011). Efficient production of ethanol from crude glycerol by a *Klebsiella pneumonia* mutant strain. Bioresour. Technol. 102(4):3918-3922.
Saisaard K, Angelidaki I, Prasertsan P (2011). Micro-aerobic, anaerobic and two-stage condition for ethanol production by Enterobacter aerogenes from biodiesel-derived crude glycerol. World Acad. Sci. Eng. Technol. 77:795-798.
Suzuki T, Nishikawa C, Seta K, Shigeno T, Nakajima-Kambe T (2014). Ethanol production from glycerol-containing biodiesel waste by *Klebsiella variicola* shows maximum productivity under alkaline condition. New Biotechnol. 31(3):246-253.
Suzuki T, Seta K, Hara E, Shigeno T (2015). Improved ethanol tolerance and ethanol production from glycerol in a streptomycin-resistance *Klebsiella variicola* mutant obtained by ribosome engineering. Bioresour. Technol. 176(1):156-162.
Swofford DL (1993). PUAP: Phylogenetic Analysis Using Parsimony, Version 4.0b10. Illinois Natural History Survey, Champaign, IL.
Thapa LP, Lee SJ, Yang XG, Yoo HY, Kim SB, Park C, Kim SW (2013). Co-fermentation of carbon sources by Enterobacter aerogenes ATCC 29007 to enhance the production of bioethanol. Bioprocess Biosyst. Eng. 37(6):1073-1084.
Tianfeng C, Huipeng L, Hua Z, Kejian L (2013). Purification of crud glycerol from waste cooking oil based biodiesel production by orthogonal test method. China Pet. Process. Petrochem. Technol. 15:48-53.
Volker FW, Steffen NL, Tobias MM (2011). Charpter 18: Use of Glycerol in Biotechnological Applications. In. Montero G, Stoytcheva M. (ed) Biodiesel-Quality, Emissions and By-Products. Publisher InTech. CCBY 3.0 license. Science, Technology and Medicine open access content. Publish, read and share novel research. Pp. 305-340.
Wong MS, Li M, Black RW, Le TQ, Puthli S, Campbell P, Monticello DJ (2014). Microaerobic conversion of glycerol to ethanol in Escherichia coli. Appl. Environ. Microbiol. 80(10):3276-3282
Wu HJ, Lin YH, Lo YC, Chen CY, Chen WM (2011). Converting glycerol into hydrogen, ethanol, and diols with a *Klebsiella* sp. HE1 strain via anaerobic fermentation. J. Taiwan Inst. Chem. Eng. 42(1):20-25.