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Efficient Production of Bioethanol from Waste Glycerol Using Microwave Irradiation Induced Mutant *Escherichia coli.*

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ABSTRACT

Crude glycerol, an inevitable byproduct during biodiesel production, is emerging as a potential feedstock for fermentation, due to its availability and a reasonable price. The anaerobic digestion of glycerol derived from biodiesel manufacturing, in which COD was found to be 1010 g/kg, was studied in batch laboratory-scale reactors. Dissimilation of glycerol by *Escherichia coli* is strictly linked to their capacity to synthesize the highly reduced product 1,3-propanediol (1,3-PDO). The present study is focus on the development of adaptive mutant strains of *Escherichia coli* EC-MW (ATCC 11105), through microwave irradiation, at frequency 2.45 GHz and irradiation time 5 min pulse irradiation. The mutants were used for high bioethanol production from glycerol feedstock. Consequently, glycerol oxidative pathway (bioethanol) enhanced upon the parallel reduction in the 1,3-propanediol (1,3-PDO) pathway. The modified *E. coli* strains were able to increase bioethanol production upon fermentation reaching the level 280 g/L.

Keywords: Waste Glycerol, Biodiesel, Bioethanol, Anaerobic Fermentation, *Escherichia coli*, Microwave Induced Mutation

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INTRODUCTION

The increasing problem of the CO_2 emissions besides some energy security concerns has strengthened the interest in alternative, non-petroleum-based sources of energy. Bioethanol and biodiesel are the only suitable and renewable primary energy resource than can provide alternative transportation fuels (Hamelinck, et al., 2005; Sun and Cheng, 2002). Biodiesel appears to be one of the most promising and feasible alternatives among the nonconventional sources of energy; however, the biodiesel production ends up with a huge amount of glycerol (Canakci and Sanli, 2008; EPA, 2013). Other advantages of biodiesel compared to petro diesel include their higher flash point, non-toxic, and essentially free of sulfur and aromatics (Ramírez-Verduzco, et al., 2011). So far, biodiesel is still far more expensive than conventional petroleum-derived diesel due to the higher feedstock and processing costs. By creating a continuous process it may be possible to reduce the production cost and hence lower the overall cost of biodiesel process, making the price of biodiesel more competitive (Ma and Hanna, 1999; Srivastava and Prasad, 2000; Knothe, et al., 2005; Pahl, 2005; Van Gerpen, 2005). Therefore, it is essential to develop viable methods to dispense or utilize this anticipated huge quantity of glycerol and to add value to the biodiesel production chain (Pagliaro, et al., 2007; Zhou, et al., 2008; Serafim, et al., 2011). These motives have triggered rigorous research in recent years to find out the novel applications of this cheap and off grade chemical (Serafim, et al., 2011; Lu, et al., 2013; Shen, et al., 2012; Ayoub and Abdullah, 2013). Although crude glycerol can be used as an ingredient in various fields, for example, in the food industry, pharmaceutical and cosmetic industries, but the demand for crude glycerol in these processes is limited. Raw glycerol obtained from biodiesel manufacture contains impurities, and its purification process will, therefore be costly due to the requirement of separation units, which require energy input. However, crude glycerol is widely available and cheap and offers new opportunities for energy use (Duane and Katherine, 2007; Yuksel, et al., 2010). In recent years there has been a lot of interest in pursuing ways to the improve bioethanol yield during fermentation as well as increasing throughput and decreasing costs. From an economic point of view, glycerol, a non-edible feedstock, has been identified to be a more sustainable biomass source for bioethanol production (Chaudhary, et al., 2012). Glycerol can substitute traditional carbohydrates, such as sucrose, glucose, and hence, one of many promising applications to take advantage of the glycerol surplus is its bioconversion to ethanol through microbial fermentation (Posada and Cardona, 2010).

Glycerol is non-fermentable by most microorganisms, with the exception of a group of bacteria including *Bacillus, Clostridium, Enterobacter, Klebsiella and Lactobacillus species* (Zheng, et al., 2008). However, the potential for using these organisms at industrial level is limited due to issues such as pathogenicity, requirement of strict anaerobic conditions, and the need for supplementation with rich nutrients (Yazdani and Gonzalez, 2007). Glycerol fermentation by *Klebsiella, Citrobacter, Enterobacter, Clostridia and Lactobacilli* results in the accumulation of two main products, 1,3- propanediol (1,3-PDO) and acetate, while lactate, formate, succinate and ethanol are produced as secondary products (Da Silva, et al., 2009; Saxena, et al., 2009). 1, 3-PDO concentrations in the range of around 40 - 100 g/L have been obtained with these producers (Celinska, 2010). Recently, Gonzalez et al. (2008), demonstrated anaerobic fermentation of glycerol by *Escherichia coli*, a species that had long been considered to be incapable of glycerol utilization. The major constraint limiting bioethanol production in wild type *E.coli* is the growth inhibition by 1,3-PDO metabolite and other toxics compounds, Therefore, it's a necessity to disruption the synthesis toward 1,3-PDO, to improve flux of bioethanol metabolite pathway in *E. coli* bacteria. In recent years, anaerobic and micro-aerobic fermentation of crude glycerol has been well established for the higher yield of bioethanol by the construction of different recombinants *E. coli* (Stephanopoulos, 2007; Gonzalez, et al., 2008; Murarka, et al., 2008; Dharmadi, et al., 2006; Durnin, et al., 2009; Suhaimi, et al., 2012; Chaudhary, et al., 2011; Nikel, et al., 2010). A high production of bioethanol metabolically requires a significant re-engineering of metabolic pathway to derive bioethanol (Shah, et al., 2013). Oh, et al., (2011), reported that bioethanol production from glycerol was greatly enhanced upon fermentation by the mutant strain which was obtained by Gamma irradiation, to a maximum production level of 21.5 g/L, with a productivity of 0.93 g/L/h. while the 1,3-propanediol and acetate decreased to 0.2 and 1.0 (g/l) respectively.

Recently, microwaves have received increased attention due to their ability to complete chemical reactions in very short times; rapid heating; cost savings due to lower energy consumption, time and work space savings; precise and controlled processing; selective heating; volumetric and uniform heating; improved quality and properties; and effects not achievable by conventional means of heating (Clark and Sutton,1996; Caddick and Fitzmaurice, 2009; Ku, et al., 2002; De la Hoz, et al., 2005; Roberts and Strauss, 2005; Varma,

1999; Giguere, et al.,1986). Microwaves transfer energy into materials by dipolar polarization, ionic conduction and interfacial polarization mechanisms to cause localized and rapid superheating of the reaction materials. Microwave irradiation has been used to sterilize (Uchiyama, et al., 2005; Prakash, et al., 1997) and to stimulate seedling growth in plants (Bhaskara, et al., 1995). Recently, either low power or high power (for very short duration, and with a provision of cooling to avoid heating) MW irradiation has been used in new applications such as for mutagenesis in plants (Jangit, et al., 2010) and microorganisms (Lin, et al., 2012; Miao, et al., 2010), protein unfolding (George, et al., 2008), and enzyme immobilization (Wang, et al., 2011). Several bacterial strains have also been mutated successfully by microwave irradiation (Banik, et al., 2006). Doran, et al., (2009) reported that microwave irradiation could enhance gene and *oligonucleotide* delivery and induce effective exon skipping in *myoblasts*. Garajvrhovac, et al., (1991) reported that pulsed microwaves can be the cause of genetic and cell alterations. Li, et al., (2010) mutated the *Trichoderma viride* by microwave to enhance cellulose production. Accordingly, there is a possible non-thermal effect involved with the microwave irradiation. The non-thermal effect by the microwave irradiation could arise from the interference of cell metabolic activities, and energy absorption and DNA/RNA molecule rotation in response to the microwave (Wu and Yao, 2010). Investigation on the effect of low power microwave (2450 MHz, 90 W) on growth and enzyme activity of different bacteria positively suggested existence of microwave specific non-thermal effects. Pectinase activity in *P. carotovora* and *B. subtilis* was greatly affected by microwave treatment (Mishra, et al., 2013).

The potential of microwave irradiation as a substantial tool for strain improvement through mutagenesis has not been exploited as widely as the use of ultra-violet (UV) radiation. Identifying the frequencies, power range and exposure duration in microwave region, which are most suitable for mutagenesis among microbes will certainly be of interest to fermentation industries. In light of the paucity of knowledge surrounding the existence of specific MW effects on bacteria, the aim of the present study was to investigate the electromagnetic effects of MW irradiation under carefully defined and controlled parameters. In this present study, development of glycerol-utilizing mutant *Escherichia coli* (ATCC 11105) strain for production of bioethanol using low power microwave (2450 MHz, 90 W) will be carried out. The *E. coli*, is used as a host strain for the production of bioethanol from glycerol, because it is very amenable to industrial applications, easy to handle and grows well. Wild type *E. coli* strains are rarely used for glycerol-derived bioethanol production as they are be incapable of glycerol utilization as carbon source. The mutant *E. coli* bacterial strain will be used to compare with the wild-type counterpart to evaluate its capability to grow in a high glycerol concentration and of resisting product (ethanol) inhibition.

MATERIALS AND METHODS

Bacterial Strain and Chemicals

Waste crude glycerol was supplied by Sime Derby biodiesel plant at Carey Island, Klang Malaysia. Lyophilized *Escherichia coli* (ATCC 11105) strain was obtained from American Type Culture Collection (ATCC). The Mueller Hinton Broth and physiological saline were purchased from Sigma-Aldrich, USA. Glycerol Stock Solution supplied from Cayman Chemical Company, USA. Sodium hydroxide provided by Sigma-Aldrich, USA. All deionized water used was supplied from ELGA Lab Water (UK), ultrapure water purification system in our laboratory. All the chemicals used were of analytical grade and used as received.

Culture Media and Cultivation Of Bacteria

Lyophilized Escherichia coli (ATCC 11105) was grown at 37 °C for 72 h. at 150 rpm on a rotary shaker (Lab Companion Shaker-300, Korea) in 900-ml capped bottle containing 250 ml of sterilized medium of Mueller Hinton Broth (Beef infusion solids 2.0% (g/l), Starch 1.5% (g/l), and Casein Hydro lysate, 17.5% (g/l)). The media was sterilized by autoclaving it for 20 min at 121 °C using bench top centrifuge (Model No.25X-2, All American Company). The culture propagation was stopped when sufficient growth of bacterial cells was achieved based on optical density at 600 nm (GENESYS 10 UV-Vis spectrophotometer-Thermo-Fisher). Sample from cultivations were collected and centrifuged at (6000 rpm, 20 min) using table top centrifuge ((Hettich Zentrifugen Rotofix 32, Germany). The resulting cell pellet was washed one time with 0.9 % (w/v) of physiological saline followed by two more washings with ultrapure water and re-centrifuged, until no broth was detected inside the cells pellet. The resulting pellets (12-ml) were divided into two portions. A 2-ml aliquot of the cells were re-suspended in 1-ml of NaCl, and was store at 4°C for the immediate use during further

experiments. The second portion was transferred immediately into 5-6 tube containing 50 % (v/v) pure glycerol as stock solution and physiological saline in a ratio (1:3), than kept in the -20 °C freezer for longer storage.

Microwave Irradiation Assisted Mutation of Escherichia Coli

The microwave irradiation was conducted in a microwave oven (Samsung CE2877-N, Korea) with operating frequency of 2.45 GHz and variable power levels of 100 W, 180 W, 300 W, 450 W, 600 W and 850 W. The freshly obtained *Escherichia coli* bacterial cells were put in a 3-ml screw capped polypropylene tube and exposed to short duration (1 min) of microwave irradiation at a frequency of 2.45 GHz and output power was at 180 W in cyclic manner. In each cycle; the cells pellets was exposed for 1 min and the sample was allowed to cool on ice for a period of 1 min. This was repeated for 15 cycles. After microwave exposure, the bacterial cell suspensions concentrations were determined by optical density measurement at 600 nm. The cell suspension was kept at 4°C before being used for the fermentation of the glycerol.

Preparation of Crude Glycerol

The crude glycerol used as substrate was the glycerol- containing waste discharged after the biodiesel manufacturing process at the Sime Derby Biodiesel plant (Klang, Malaysia). The waste glycerol was stored in a sealed plastic container and left at room temperature. Prior the trials, the crude glycerol was heated up by using hot plate heater to melt the raw material. The crude glycerol was left for one hour to settle down at room temperature. In general, this crude glycerol contained glycerol, water, methanol, salts and fatty acids.

Crude Glycerol Partial Pre-Treatment

To use the crude glycerol as raw material for bioconversion process, the substrate was previously treated partially in two different ways: The procedure were explored by (Saifuddin, et al., 2013) in a two sequential phase by a combination of chemical and bio adsorption methods. Basically, these stages starts with using microwave assisted acidification process and the next process was utilized by a bio adsorbent synthesized from dead yeast cells immobilized on chitosan. A typically, the partial recovered of crude glycerol stream obtained was about 93.1-94.2 % (w/w) with clear color.

Bioreactor Process For Bacteria Cultivation

Batch fermentation mode was performed in this study with basic fermenter reactor unit of 2-liter working volume vessel and 3-liter total volume (Bio Gene bioreactor fermenter model - biogene T type, India). The fermentation flask was removed from the reactor and was first sterilized by autoclaving at 121°C for 20 min. The partially purified glycerol (carbon source) was also sterilized separately using the autoclave. The flask was assembled into the reactor and filled with the partially purified glycerol. The headspace was purged with nitrogen gas for 2 min to create anaerobic condition. The inoculum at 10% was added to the fermenter. The fermentation mixture was stirred at speed 200 rpm for 4-5 days, the temperature was set to 37°C, and a constant pH was maintained at 6.5 by the automatic addition of (2M) NaOH. Fermentation was stopped when cell growth was in the late phase where no further glycerol consumption was detected. All the results were recorded in duplicate.

Analytical Methods

Identification of Bacteria Growth And Products

Cell concentration was measured via optical density at a wavelength of 600 nm (OD_{600nm}) using a spectrophotometer and 1 cm cuvette (GENESYS 10 UV-Vis spectrophotometer). The spectrophotometer was zeroed with sterile broth. Fermentation samples were taken at twenty-four-hour intervals. The fermentation samples were spun down to separate cells from the supernatant by centrifuged at 10000 rpm for 10 min. The supernatant was filtered. Glycerol concentration was measured by using the glycerol assay kit (Megazyme, K-GCROL), and ethanol concentration was measured by using the ethanol assay kit (Megazyme, K-ETOH).

Fourier Transformed Infrared (FTIR) Spectra Measurement

The FTIR spectra measurements were performed using the Shimadzu FTIR spectrophotometer -model IRPrestige-21 (Shimadzu Corparation Japan) equipped with temperature controlled DLATGS (deuterated, L-alanine doped triglycine sulfate) detector. The scan settings were set as follows; resolution: 4 cm^{-1}, accumulation: 20 scans, measurement mode: transmittance (%T), wave number 2000 to 700 cm^{-1}. In a typical analysis, a drop of sample (using a Pasteur pipette) from various samples was deposited on the surface of the horizontal attenuated total reflectance (ATR) crystal disc (Diamond Type II crystal) at controlled ambient temperature (23°C). A background measurement of air spectrum was performed. Spectra were processed using IR solution-window based software version 1.4 (Shimadzu). After every scan, a new reference air background spectrum was taken. After sample, the liquid was removed with dry tissue and the surface of crystal disc was washed with acetone, and finally it was dried and cleaned with tissue.

RESULT AND DISCUSSION

Effect Of Microwave Irradiation Power And Exposure Time On E. coli Strains

Fermentative utilization of glycerol by *Escherichia coli* for the production of biofuels has been getting a lot of attention in recent years. *E. coli* is generally considered an easy and safe species to work with for various industrial processes. Wild-type *E. coli* cannot grow anaerobically on glycerol, because the redox potential is imbalanced with accumulation of high levels of NADH (Murarka, et al., 2008). The *E. coli* is also not efficient in converting glycerol to ethanol due to the existence of many inefficient pathways. In this study, normal wild-type strain of *E. coli*, which is known as a poor microbe for bioethanol production from glycerol (as sole carbon source) was subjected to short microwave irradiation treatment (1 min) at a frequency of 2.45 GHz and output power was at 180 W in cyclic manner (total 15 cycles). The MW irradiation was performed to investigate the role of low power and the exposure time of MW on the *E. coli* mutation. Duration of MW exposure seems to be a major determinant of MW effect on living cell. The time of exposure and power density are correlated in a way that decrease in power density could be compensated by increase in duration of exposure. From previous observations by Shamis, et al., (2011), it was postulate that the application of MW radiation might cause disruption of the cellular membrane so that the cytosolic fluids within the *E. coli* cells are able to pass through the membrane. This effect, however, appeared to be temporary, as the shape of the cells appears to have recovered within 10 min after the application of MW radiation. This was most likely due to the reabsorption of the fluids into cells. From this study and also previous report (Shamis, et al., 2011), it was shown that the specific MW effect was not bactericidal under these experimental conditions. Accordingly, there is a possible non-thermal effect involved with the microwave irradiation. The non-thermal effect by the microwave irradiation could arise from the interference of cell metabolic activities, and energy absorption and DNA/RNA molecule rotation in response to the microwave. In this study MW irradiation on bacterial growth was performed to investigate the role of low power and the exposure time of MW on the *E. coli* mutation. The approach for detecting MW-induced conformational changes of *E.coli* was by means of ATR-FTIR (attenuated total reflection Fourier-transform infrared). Figure 1 (a & b) shows the FT-IR absorption spectra of *E. coli* and its modified mutant strain with bands at 2000–800 cm^{-1}. The MW irradiation at (2.45 GHz; 180W) for 5 minutes pulse, had induced some changes in the *E. coli* strains. The typical signals of the polysaccharides were exhibited at 1650 cm^{-1}. The MW radiated *E. coli* showed distinct strong signal stretching at 1650, 1400, 1250 and 1100 cm^{-1}. The FT-IR spectra of the MW induced E. coli showed that the stretching vibration band at 1650 cm^{-1} was narrowed compared to the wild type *E. coli*. The main functional groups of mutant *E. coli*, including the C = O stretching at 1650 cm^{-1}, δ O-H bending at 1400 cm^{-1}, δ O-H bending at 1250 cm^{-1}. The band at 940 cm^{-1} corresponds to the pyranose units of the polysaccharide and proves that the cyclic pyranosyl rings were not destroyed by microwave radiation. As low intensity MW are believed not to possess sufficient energy for breaking chemical bonds directly. in this way, the effect vibrational energy generate from microwave irradiation penetrate of electromagnetic waves into intercellular compounds that caused the mutation, alternative mechanisms of interaction between MW and cells entities are likely to prevail.

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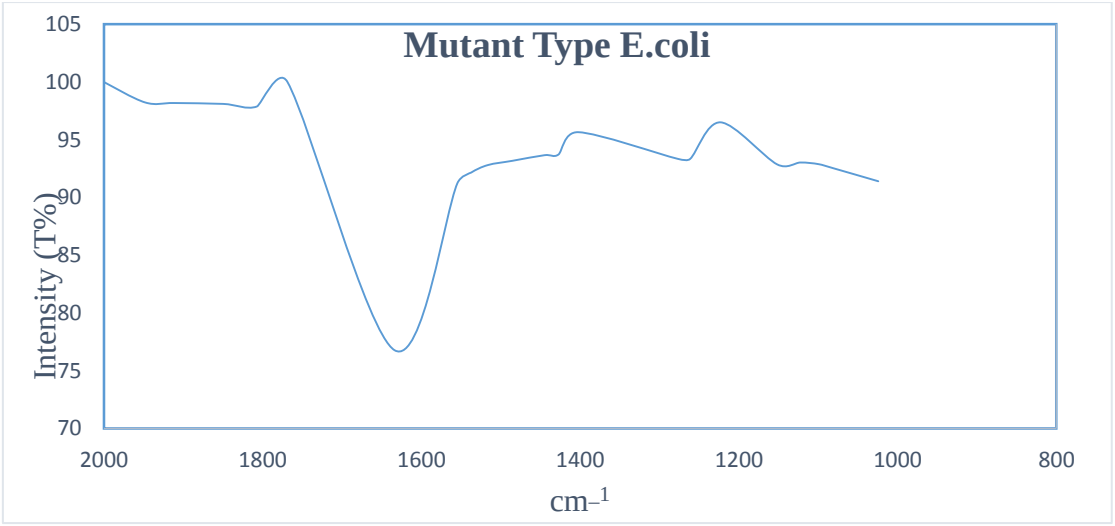

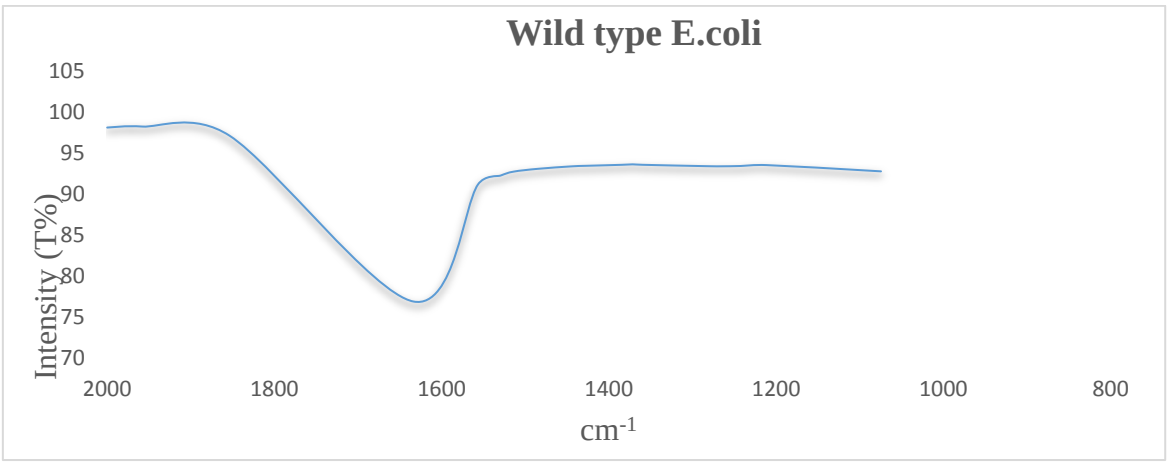

Figure 1: Representative FTIR spectra of the wild type EC-non-MW (a) and a mutant type EC-MW (b)

Optimization Bioethanol Production From Glycerol By Escherichia Coli (EC-MW)

Although glycerol may be converted into many useful products by micro-organisms, fuel, such as bioethanol may be one of the best target products due to its sufficiently large market value; i.e., the demand for fuel is nearly inexhaustible (Sabourin-Provost; Hallenbeck, 2009). The chemicals used in the transesterification process can also be present in the glycerol. Since a wide variety of feed stocks being utilized in biodiesel production, the composition of crude glycerol can vary due to residues and impurities being carried over to the by-product. These residues can inhibit the growth of micro-organisms and thus affect product formation. The process for converting glycerol should be robust to the impurities and variability of crude glycerol offering a cost-effective solution for converting this waste. Therefore, partial purification of the crude waste glycerol had been performed in this study, which was reported as previously by (Saifuddin, et al., 2013). *Escherichia coli* is able to convert glycerol to bioethanol, however, yields are relatively low because bioethanol is only a secondary product of the fermentation. In order to improve glycerol conversion to bioethanol and increase production quantity in the fermentation processes, two batch fermentations were performed in this study. Two different strains, i.e.; the wild type *E. coli* (ATCC 11105) (labeled as EC non-MW) and the other was mutant strain of *E. coli* (exposed to MW radiation) (labeled as EC-MW). The anaerobic condition was chosen as it was reported previously that anaerobic condition was more appropriate for bioethanol production from glycerol (Yazdani and Gonzalez, 2008; Gonzalez, et al., 2008; Durnin, et al.,2009; Suhaimi, et al., 2012; Nikel, et al., 2010). The results presented in this work (Figure 2) showed that the *E. coli* EC-MW was able to degrade partially purified glycerol with inherent production of 1,3- propanediol (1,3-PDO) that was much lower than that produced by the wild type *E. coli*. (non- microwave strain). A typical profile for glycerol, bioethanol and weight cells production during fermentation of glycerol by strain *E. coli* (ATCC 11105) is shown in Figure 2(a). It can be seen that the *E. coli* utilized glycerol within 120 h and yielded about 90 g/ L

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bioethanol from 280 g/L glycerol feedstock. In contrast (Figure 2 b), the mutant type strain EC-MW, produced 280 g/L bioethanol in 100 h from 270 g/L partially purified glycerol. Table 1 gives a comparison of bioethanol production from glycerol by different microorganisms. This study has shown that the new strains are better adapted to consumed glycerol and effectively convert the concomitant bioethanol yield more than the wild type *E coli* strain. Thus the glycerol metabolic pathway in EC-MW was likely modified, and probably certain enzymes systems that are essential for the conversion of glycerol to 1,3 POD, have been altered or deactivated. The inactivation of the 1,3 PDO pathway had led to the increase in activity of the ethanol production pathway. In other word, non-thermal process caused alteration of the rate of enzyme catalyzed reactions inside the bacterial cells for 1,3 PDO, and this allowed the increase in catalytic activity of alcohol dehydrogenase which in turn was responsible for the increase in bioethanol production. Ability of MW irradiation to increase the enzymatic activity of bacterial suspensions has also been demonstrated in members of the family *Enterobacteriaceae* (Spencer, et al., 1985). Lin, et al., (2012) explored the effect of MW at (400 W; 3 mins) on *L. rhamnosus;* and reported that MW irradiation caused the enzymatic activities of the various enzymes (malate/lactate dehydrogenase, pyruvate kinase, and NAD- dependent aldehyde dehydrogenases), responsible for the production of L-lactic acid production to be enhanced which subsequently gave a 50 % increase in L-lactic acid production yield. They also reported that the mutant generation was found to be stable up to 9 generations. (Lin, et al., 2012).

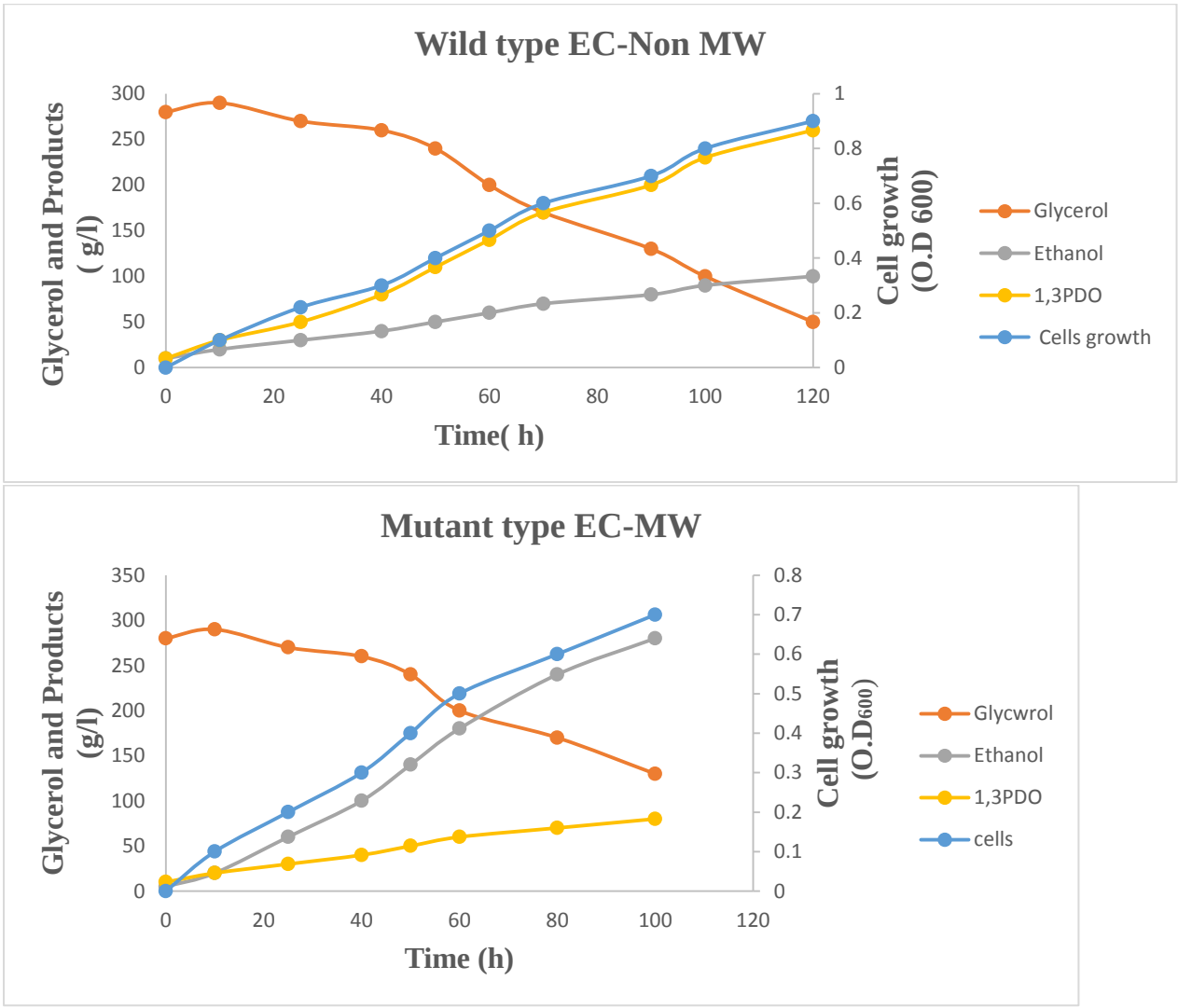

Figure 2: Batch fermentation by *Escherichia coli* EC-Non MW (a) and EC-MW (b) strains, showing bioethanol production levels, Residual glycerol levels, cell growth level, 1,3-PDO levels.

Table 1: Comparison of bioethanol production from glycerol by different microorganisms.

Organism	Fermentation method	Bioethanol Concentration g/l	Bioethanol Productivity g/l/h	Reference
Enterobacter aerogenes Hu-101	Batch	10	0.83	Ito, et al., 2005
Escherichia coli EH05	Batch	20.7	0.22	Durnin, et al., 2009
Klebsiella oxytoca M5al	Fed-batch	19.5	0.56	Yang, et al., 2007
Klebsiella pneumoniae M5a1	Fed-batch	18	0.28	Cheng, et al., 2007
K. pneumoniae GEM167	Fed-batch	21.5	0.93	Oh, et al., 2011
K. pneumoniae GEM167/pBRpdc-adh	Fed-batch	25.0	0.78	Oh, et al., 2011
Escherichia coli (EC-MW)	Batch	280	2.8	Present study

CONCLUSION

Lately, the normal, wild type *E. coli* (ATCC 11105) was examined for its ability to convert waste glycerol from biodiesel production to bioethanol. However, the ethanol yield has been very poor. The present study shows the utilization of non-conventional microwave irradiation method as a clean and green approach toward the development of modified *E. coli* strains to maximize bioethanol production from glycerol. The microwave irradiated *E. coli* (EC-MW) has been shown to be robust and efficient biocatalyst for conversion of glycerol to bioethanol. It generated 280 g/L bioethanol within 100 h, at optimum temperature and agitation speed of 37°C and 200 rpm, respectively, with minimal production 1,3 PDO. Utilization of crude glycerol in fermentation processes account in reduction of manufacturing costs and higher yield product of bioethanol than those obtained from sugar substrates. Further work is necessary to optimize the condition toward improving the bacteria tolerance in the partially purified waste glycerol to obtain high productivity, since the technology is effective and easy to apply. The results from this study provide further references when applying microwave irradiation to alter the enzymes activities in the bacteria strain and hence the phenomenon of non-thermal microwave effect could be appreciated clearly.

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REFERENCES

Ayoub, M.; Abdullah, A.Z., (2013), Diglycerol synthesis via solvent-free selective glycerol etherification process over lithium-modified clay catalyst, Chem.Eng. J., Vol. 225, pp.784–789.

Banik, S.; Bandyopadhyay, S.; Ganguly, S.; Dan, D., (2006), Effect of microwave irradiated Methanosarcina barkeri DSM-804 on biomethanation, Bioresour. Technol., Vol. 97, pp.819-823.

Bhaskara, R.M.; Kushalappa, A.; Raghavan, G.; Stephenson, M., (1995), Use of microwave energy for the eradication of seedborne diaporthe phaseolorum in soybean and its effect on seed quality, J. Microw. Power Electromagn. Energy, Vol. 30, pp.199-204.

Caddick, S.; Fitzmaurice, R, (2009), Microwave enhanced synthesis, Tetrahedron, Vol.65, pp.3325–3355.

Canakci, M.; Sanli, H., (2008), Biodiesel production from various feedstocks and their effects on the fuel properties, J. Ind. Microbiol. Biotechnol., Vol. 35, pp.431–441.

Celiñska, E., (2010), Debottlenecking the 1, 3-propanediol pathway by metabolic engineering, Biotechnol Adv, Vol. 28, pp.519–530, doi:10.1016/j.biotechadv.2010.03.003.

Chaudhary, N.; Ngadi, M. O.; Simpson, B. K.; Kassama, L. S., (2011), Biosynthesis of ethanol and hydrogen by crude glycerol fermentation using Escherichia coli, Adv. Chem. Eng. Sci., Vol. 1, pp. 83-89.

Chaudhary, N.; Ngadi, M.O.; Simpson, B., (2012), Comparison of Glucose, Glycerol and Crude Glycerol Fermentation by Escherichia Coli K12, Journal Bioprocessing & Biotechniques, S1:001 doi:10.4172/2155-9821.S1-001.

Cheng, K.K.; Zhang, J.A.; Liu, D.H.; Sun, Y.; Liu, H.J.; Yang, M.D.; Xu, J.M.,(2007), Pilot-scale production of 1,3-propanediol using Klebsiella pneumoniae, Process Biochem, Vol. 42, pp.740–744.

Cheng, K.K.; Zhang, J.A.; Liu, D.H.; Sun, Y.; Liu, H.J.; Yang, M.D.; Xu, J.M.,(2007), Present state and perspective of downstream processing of biologically produced 1, 3-propanediol and 2, 3-butanediol, Process Biochem, Vol. 42, pp.740–744.

Clark, D.E.; Sutton, W.H., (1996), Microwave processing of materials, Annual Review of Materials Science, Vol.26, pp.299–331.

Da Silva, G.P.; Mack, M.; Contiero, J., (2009), Glycerol: A promising and abundant carbon source for industrial microbiology, Biotechnology Advances, Vol. 27, pp.30–39.

De la Hoz, A.; Diaz-Ortiz, A.; Moreno, A., (2005), Microwaves in organic synthesis, Thermal and non-thermal microwave effects, Chem Soc Rev, Vol.34, pp.164–178.

Dharmadi, Y.; Murarka, A.; Gonzalez, R., (2006), Anaerobic fermentation of crude glycerol by Escherichia coli: a new platform for metabolic engineering, Biotechnol.Bioeng., Vol. 94, pp. 821-829.

Dharmadi, Y.; Murarka, A.; Gonzalez,R., (2006),Anaerobic fermentation of glycerol by Escherichia coli: a new platform for metabolic engineering, Biotechnol.Bioeng, Vol. 94, pp.821-829.

Doran, T.J.; Lu, P.J.; Vanier, G.S.; Collins, M.J.; Wu, B.; Lu, Q.L., (2009),Microwave irradiation enhances gene and oligonucleotide delivery and induces effective exon skipping in myoblasts, Gene ther.,Vol. 16(1), pp.119-126.

Duane, T.J.; Katherine, A.T., (2007), Environ Prog, Vol.26, pp.338-48.

Durnin, G.; Clomburg, J.; Yeates, Z.; Alvarez, P. J. J.; Zygourakis, K.;Campbell, P.; Gonzalez, R., (2009), Understanding and harnessing the microaerobic metabolism of glycerol in Escherichia coli, Biotechnol. Bioeng., Vol. 103, pp.148-161.

EPA, (2013), The U.S. Environmental Protection Agency (EPA) has set a target of 1.28 billion gallon biodiesel to be included in diesel fuel markets by (2013) which ensures the production of a huge amount of glycerol. 09/14/2012.

Garaj-Vrhovac, V.; Horvat, D.; Koren, Z., (1991), The relationship between colony-forming ability, chromosome aberrations and incidence of micronuclei in V79 Chinese hamster cells exposed to microwave radiation, Mutat. Res. Lett.,Vol. 263(3), pp.143-149.

George, D. F.; Bilek, M. M.; McKenzie, D. R., (2008), Non-Thermal effects in the microwave induced unfolding of proteins observed by chaperone binding, Bioelectromagnetics, Vol. 29(4), pp.324-330.

Giguere, R.J.; Bray, T.L.; Duncan, S.M.; Majetich, G., (1986), Application of commercial microwave ovens to organic synthesis, Tetrahedron Lett, Vol. 27, pp.4945–4948.

Gonzalez, R.; Murarka, A.; Dharmadi, Y.; Yazdani, S. S., (2008), A new model for the anaerobic fermentation of crude glycerol in enteric bacteria: trunk and auxiliary pathways in Escherichia coli, Metab. Eng., Vol. 10, pp. 234-245.

Gonzalez, R.; Murarka, A.; Dharmadi, Y.; Yazdani, S.S., (2008), a new model for the anaerobic fermentation of glycerol in enteric bacteria: trunk and auxiliary pathways in Escherichia coli, Metab. Eng., Vol. 10, pp. 234-245.
Hamelinck, C.N.; van Hooijdonk, G.; Faaij, A.P.C., (2005), Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term, Biomass Bioenergy, Vol. 28, pp. 384–410.

Ito, T.; Nakashimada, Y.; Senba, K.; Matsui, T.; Nishio, N., (2005), Hydrogen and ethanol production from glycerol-containing wastes discharged after biodiesel manufacturing process, J. Biosci. Bioeng., Vol. 100,pp. 260–265.

Jangid, R. K.; Sharma, R.; Sudarsan, Y.; Eapen, S.; Singh, G.; Purohit, A.K., (2010), Microwave treatment induced mutations and altered gene expression in Vigna aconitifolia. Biologia Plantarum, Vol. 54(4), pp.703-706.

Knothe, G.; Van Gerpen, J.H.; Krahl, J., (2005), the Biodiesel Handbook, AOCS Press, Champaign, IL.
Ku, H.S.; Siores, E.; Taube, A.; Ball, J.A.R.,(2002), Productivity improvements through the use of industrial microwave technologies, Computers & Industrial Engineering ,Vol. 42(2–4), pp.281–290.

Li, J.; Zhang, S.; Shi, S.; Huo, P., (2011), mutational approach for N2-fixing and P-solubilizing mutant strains of Klebsiella pneumoniae RSN19 by microwave mutagenesis, World Journal of Microbiology and biotechnology, Vol.27(6), pp. 1481-1489.

Li, X.H.; Yang, H.J.; Roy, B.; Park, E.Y.; Jiang, L.; Wang, D.; Miao, Y.G., (2010), Enhanced cellulase production of the Trichoderma viride mutated by microwave and ultraviolet, Microbiol. Res., Vol. 165(3), pp.190-198.

Lin, H.; Chen, X.; Yu, L.; Xu, W.; Wang, P.; Zhang, X.; Li, W.; Li, C.; Ren, N., (2012),Screening of Lactobacillus rhamnosus strains mutated by microwave irradiation for increased lactic acid production, African Journal of Microbiology Research, Vol. 6(31), pp. 6055-6065.

Lin, H.; Chen, X.; Yu, L.; Xu, W.; Wang, P.; Zhang, X.; Ren, N., (2012), Screening of Lactobacillus rhamnosus strains mutated by microwave irradiation for increased lactic acid production, African Journal of Microbiology Research, Vol.6(31), pp. 6055-6065.

Lu, P.; Wang, H.; Hu, K., (2013), Synthesis of glycerol carbonate from glycerol and dimethyl carbonate over the extruded CaO-based catalyst, Chem. Eng. J.,Vol. 228, pp.147–154.

Ma, F.R.; Hanna, M.A., (1999), Biodiesel production: a review, Bioresour. Technol., Vol. 70, pp. 1-15.

Miao, Y. G.; Li, X. H.; Yang, H. J.; Roy, B.; Park, E. Y.; Jiang, L. J.; Wang, D., (2010), Enhanced cellulase production of the Trichoderma viride mutated by microwave and ultraviolet, Microbiological Research, Vol.165(3), 190-198.

Mishra, T.; Kushwah, P.; Dholiya, K.; Kothari, V., (2013), Effect of low power microwave Radiation on microorganisms and other life forms, Advances in Microwave and Wireless Technologies, Vol. 1 (1), pp.4-11, DOI: 10.12966/amwt.05.02.2013.

Murarka, A., Y.; Dharmadi, S. S.; Yazdani; Gonzalez, R. , (2008), Fermentative utilization of glycerol by Escherichia coli and its implications for the production of fuels and chemicals,Appl. Environ. Microbiol., Vol.74, pp.1124–1135.

Murarka, A.; Dharmadi, Y.; Yazdani, S. S.; Gonzalez, R., (2008), Fermentative utilization of crude glycerol in Escherichia coli and its implication for the production of fuels and chemicals, Appl. Environ. Microbiol., Vol.74, pp. 1124-1135.

Murarka, A.; Dharmadi, Y.; Yazdani, S.S.; Gonzalez, R., (2008), Fermentative utilization of Glycerol by Escherichia coli and its Implications for the Production of Fuels and Chemicals, Appl. Environ. Microbiol., Vol. 74, pp. 1124-1135.

Murarka, A.; Dharmadi, Y.; Yazdani, S.S.; Gonzalez, R., (2008), Fermentative utilization of Glycerol by Escherichia coli and its Implications for the Production of Fuels and Chemicals, Appl. Environ. Microbiol., Vol. 74(4), pp. 1124-1135.

Nikel, P. I.; Ramirez, M. C.; Pettinari, M. J.; Mendez, B. S.; Galvagno, M. A., (2010), Methanol synthesis from crude glycerol by Escherichia coli redox mutants expressing adhE from Leuconostoc mesenteroides, J. Appl. Microbiol., Vol. 109, pp. 492-504.

Oh, B-R.; Seo, J-W.; Heo, S-Y.; Hong, W-K.; Luo, L-H.; Joe, M-H.; Park, D-H.; Kim, C-H., (2011), Efficient production of ethanol from crude glycerol by a Klebsiella pneumonia mutant strain, Bioresource Technology, Vol. 102, pp.3918–3922.

Oh, B-R.; Seo, J-W.; Heo, S-Y.; Hong, W-K.; Luo, L-H.; Joe, M-H.; Park, D-H.; Kim, C-H., (2011), Efficient production of ethanol from crude glycerol by a Klebsiella pneumonia mutant strain, Bioresource Technology, Vol. 102, pp.3918–3922.

Pagliaro, M.; Ciriminna, R.; Kimura, H.; Rossi, M.; Pina, C.D., (2007), from glycerol to valueadded products, Angew. Chem. Int. Ed., Vol. 46, pp. 4434–4440.

Pahl, G., (2005), Biodiesel: Growing a New Energy Economy, Chelsea Green Publishers, White River Junction, VT.

Posada, J.A.; Cardona, C.A., (2010), Design and analysis of fuel bioethanol production from raw glycerol, Energy, Vol.35, pp.5286-5293.

Prakash, A.; Kim, H.J.; Taub, I.A., (1997), Assessment of microwave sterilization of foods using intrinsic chemical markers, J. Microw. Power Electromagn. Energy, Vol. 32, pp.50-57.

Ramírez-Verduzco, L.F.; García-Flores, B.E.; Rodríguez-Rodríguez, J.E.; Jaramillo-Jacob, A.D.R., (2011), Prediction of the Density and Viscosity in Biodiesel Blends at Various Temperatures, Fuel, Vol. 90, pp.1751–1761.

Roberts, B.; Strauss, C.R., (2005), toward rapid, “green”, predictable microwave assisted synthesis, Acc Chem Res, Vol.38, pp.653–661.

Sabourin-Provost, G.; Hallenbeck, P.C.; (2009), high yield conversion of a crude glycerol fraction from biodiesel production to hydrogen by photofermentation, Bioresource Technology, Vol.100, pp.3513-7.

Saifuddin, N.; Refal, H.; Kumaran, P., (2014), Rapid Purification of Glycerol by-product from Biodiesel Production through Combined Process of Microwave Assisted Acidification and Adsorption via Chitosan Immobilized with Yeast, Research Journal of Applied Sciences, Engineering and Technology, Vol. 6(5), pp.1032-1041.

Saxena, R.K.; Anand, P.; Saran, S.; Isar, J., (2009), Microbial production of 1, 3-propanediol: recent developments and emerging opportunities, Biotechnol Adv, Vol. 27, pp.895–913. doi:10.1016/j.biotechadv.2009.07.003.

Serafim, H.; Fonseca, I.M.; Ramos, A.M.; Vital, J. ; Castanheiro, J.E., (2011), Valorization of glycerol into fuel additives over zeolites as catalysts, Chem. Eng. J., Vol. 178, pp. 291–296.

Shah, P.; Chiu, F-S.; Lan, J.C-W., (2013), Aerobic utilization of crude glycerol by recombinant Escherichia coli for simultaneous production of poly 3-hydroxybutyrate and bioethanol, Journal of Bioscience and Bioengineering, Vol. xx, No. xx, pp. 1-8.

Shamis, Y.; Taube, A.; Mitik-Dineva, N.; Croft, R.; Crawford, R.J.; Ivanova, P., (2011), Specific Electromagnetic Effects of Microwave Radiation on Escherichia coli Appl. Environ. Microbiol., Vol. 77 (9), pp.3017-3023.
Shen, L.; Yin, H.; Wang, A.; Feng, Y.; Shen, Y.; Wu, Z.; Jiang, T., (2012), Liquid phase dehydration of glycerol to acrolein catalyzed by silicotungstic, phosphotungstic, and phosphomolybdic acids, Chem. Eng. J.,Vol. 180, pp.277–283.

Spencer, R.C.; Hafiz, S.; Cook, C., (1985), Effect of microwave energy on the metabolism of enterobacteriaceae, J Med Microbiol., Vol.19 (2), pp.269-72.

Srivastava, A.; Prasad, R., (2000), Triglycerides-based Diesel Fuels, Renew. Sustain. Energy Rev., Vol. 4, pp.111-133.

Stephanopoulos, G., (2007), Challenges in engineering microbes for biofuels production, Science, Vol. 315, pp. 801-804.

Suhaimi, S. N.; Phang, L. Y.; Maeda, T.; Abd-Aziz, S.; Wakisaka, M.; Shirai, Y.; Hassan, M. A., (2012), Bioconversion of glycerol for bioethanol production using isolated Escherichia coli SS1, Braz. J. Microbiol., Vol. 43, pp. 506-516.

Sun, Y.; Cheng, J., (2002), Hydrolysis of lignocellulosic materials for ethanol production: a review, Bioresour. Technol., Vol. 83, pp. 1–11.

Uchiyama, K.; Ujihira, M.; Mabuchi, K.; Takahira, N.; Komiya, K.; Itoman, M., (2005), Development of heating method by microwave for sterilization of bone allografts, J. Orthop. Sci., Vol.10, pp.77-83.

Van Gerpen, J.H., (2005), Biodiesel Processing and Production, Fuel Processing Tech., Vol. 86, pp.1097-1107.
Varma, R.S., (1999), Solvent- free organic syntheses using supported reagents and microwave irradiation, Green Chem, Vol.1, pp.43–55.

Wang, A.; Wang, M.; Wang, Q.; Chen, F.; Zhang, F.; Li, H.; Zeng, Z.; Xie, T., (2011), Stable and efficient immobilization technique of aldolase under consecutive microwave irradiation at low temperature, Bioresource Technology, Vol. 102(2), pp.469-474.

Wu, Y.; Yao, M., (2010), Inactivation of bacteria and fungus aerosols using microwave irradiation, Journal of Aerosol Science, Vol. 41, pp. 682–693.

Yang, G.; Tian, J.; Li, J., (2007), Fermentation of 1, 3-propanediol by a lactate deficient mutant of Klebsiella oxytoca under microaerobic conditions, Appl. Microbiol. Biotechnol., Vol. 73,pp. 1017–1024.

Yazdani, S. S.; Gonzalez, R., (2008), Engineering Escherichia coli for the efficient conversion of crude glycerol to ethanol and co-products, Metab. Eng., Vol. 10, pp.340-351.

Yazdani, S.S. and Gonzalez, R., (2007), Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry, Curr.Opin. Biotechnol., Vol. 18, pp. 213–219.

Yazdani, S.S.; Gonzalez, R., (2007), Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry, Curr Opin Biotechnol, Vol.18, pp.213-219.

Yuksel, A.; Koga, H.; Sasaki, M.; Goto, M., (2010), Hydrothermal electrolysis of glycerol using a continuous flow reactor, Ind Eng Chem Res, Vol.49, pp.1520-5.

Zheng, Z.M.; Hu, Q.L.; Hao, J.; Xu, F.; Guo, N.N.; Sun, Y.; Liu, D.H., (2008), Statistical optimization of culture conditions for 1, 3-propanediol by Klebsiella pneumoniae AC15 via central composite design, Bioresour. Technol., Vol. 99, pp. 1052–1056.

Zhou, C.H.; Beltramini, J.N.; Fana, Y.X.; Lu, G.Q., (2008),Chemoselective catalytic conversion of glycerol as a biorenewable source to valuable commodity chemicals, Chem. Soc. Rev., Vol. 37, pp. 527–549.