

# Biosynthesis of Ethanol and Hydrogen by Glycerol Fermentation Using *Escherichia coli*

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## Abstract

Production of high value products from glycerol via anaerobic fermentation is of utmost importance for the biodiesel industry. The microorganism *Escherichia coli* (*E. coli*) K12 was used for fermentation of glycerol. The effects of glycerol concentration and headspace conditions on the cell growth, ethanol and hydrogen production were investigated. A full factorial experimental design with 3 replicates was conducted in order to test these factors. Under the three headspace conditions tested, the increase of glycerol concentration accelerated glycerol fermentation. The yields of hydrogen and ethanol were the lowest when glycerol concentration of 10 g/L was used. The maximum production of hydrogen was observed with an initial glycerol concentration of 25 g/L at a final concentration of hydrogen was 32.15 mmol/L. This study demonstrated that hydrogen production negatively affects cell growth. Maximum ethanol yield was obtained with a glycerol concentration of 10 g/L and was up to 0.40 g/g glycerol under membrane condition headspace. Statistical optimization showed that optimal conditions for hydrogen production are 20 g/L initial glycerol with initial sparging of the reactor headspace. The optimal conditions for ethanol production are 10 g/L initial glycerol with membrane.

**Keywords:** Fermentation, Ethanol, Hydrogen, Glycerol, Cell Growth, Reactor Headspace

## 1. Introduction

The continuous use of fossil fuels is globally accepted as not sustainable largely because of the underlying environmental factors. The depletion of natural resources and the accumulation of greenhouse gasses poses' significant threat to global warming. Schenk *et al.* [1] reported that the greenhouse gasses in the environment have already exceeded "dangerously higher" threshold of 450 ppm CO<sub>2</sub>-e. These startling findings are leading governments to establish regulations to reduce CO<sub>2</sub> emissions. Furthermore, the increase dependency on foreign oil is of grave concern to national securities and economic stability.

Converting biomass-derived oil to biofuels is a viable substitute for petroleum-based liquid fuel. Glycerol is a by-product that is produced in abundance in biodiesel fuel production. Yazdani [2] reported that 10 lb of crude glycerol is generated for every 100 lb of biodiesel fuel produced by transesterification of vegetable oils or ani-

mal facts. The current growth in the biodiesel industry results to surplus glycerol being produced, thus the price of crude glycerol has dropped substantially [2]. The collapsed glycerol price has led to the shutdown of many glycerol producing plants, hence negatively affected the economic viability of the biodiesel industry [3]. As a result, glycerol becomes a waste stream instead of being a desirable co-product of significant economic value [2] and in compliance to environmental regulation the burden of cost for disposal of the effluent became a liability for plants [4]. Thus, improving the bioconversion methods of the low-cost glycerol into higher valued product will provide an incentive for the commercialization of glycerol into biofuel.

Glycerol (C<sub>3</sub>H<sub>8</sub>O<sub>3</sub>; κ = 4.7) is a better source of carbon compared to the common fermentable sugars (glucose C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>; κ = 4.0; xylose C<sub>5</sub>H<sub>10</sub>O<sub>5</sub>; κ = 4.0) for fermentation [5,6]. The common sugars (glucose, xylose etc) additionally produce succinate, and propanediols during fermentation when compared to glycerol [2,7]. The con-

version of glycerol to a higher value product requires channelling the processes through chemical and biological pathways. Glycerol fermentation by organisms are mediated by two-branch-pathway, which results in the synthesis of the glycolytic intermediate dihydroxyacetone (DHA), dihydroxyacetone phosphate (DHAP) and the fermented product 1,3-propanediol (1,3-PDO) [2,8,9]. In effect the biological conversion process improves the function of chemical catalysis to synthesise crude glycerol with high levels of contaminants [2]. The microbial fermentation of glycerol can be either aerobic or anaerobic process; the anaerobic fermentation is preferred because of the low capital and operational costs of anaerobic fermenters.

The microbial conversion of glycerol to various compounds has been investigated by many authors [10-14]. Several microorganisms have been used to ferment glycerol into 1,3-propanediol (1,3-PDO) which is a basic ingredient for polyester production [15]. *Klebsiella pneumonia* has been cited as an excellent producer of 1,3-PDO [16], however, the presence of oxygen inhibits the metabolic pathway of converting glycerol into ethanol [17]. The *Citrobacter* species is another microbe that received many interest for fermenting glycerol into 1,3-PDO [18], hence *Clostridium* species are also good candidates for similar reasons [19]. However, the pathogenicity, the need for strict anaerobic conditions, lack of genetic tools and requirement of rich nutrients can be limiting factors for the use of these microorganisms [20]. *Esherishia coli* (*E. coli*) is one of the most commonly used host organisms for metabolic engineering and industrial applications, because it is easy to manipulate genetically and can produce wide variety of anaerobic fermentation products [10].

Dharmadi [21], Hu and Wood [10] reported that *E. coli* ferments glycerol in a low pH medium, and the fermentation is dependent on CO<sub>2</sub> availability. These authors reported that oxidation of formate hydrogen lyase (FHL) produces CO<sub>2</sub> which severely impedes the fermentation process and block the FHL activity. Eighty percent (80%) of glycerol initially presented in the media is consumed within 84 h of the active growth period, producing 86% ethanol and 7% succinic acid with minor fraction of acetate and no detectable formate or lactate [22]. Yazdani *et al.* [22] created two strains of *E. coli* for the co-production of ethanol-hydrogen and ethanol-formate. They also reported high yield of ethanol with both strains and minimal synthesis of the by-products such as succinate and acetate. The yield observed in their study was higher than those obtained with other organisms. The accumulation of hydrogen was observed, and is problematic to the metabolic recycling and generates an unfavourable internal redox state [3]. Thus, controlling

and preventing hydrogen accumulation during glycerol fermentation is significant in glycerol production [20].

*Esherishia coli* can be used as a microorganism for a high-yield production of ethanol from low cost glycerol. However, there is lack of adequate information in literature on the ideal culture and effective fermentation conditions. Therefore, the aim of this research is to study effect different growth conditions and how it affects ethanol and hydrogen production during glycerol fermentation with *E. coli* in lab-scale bioreactors.

## 2. Materials and Methods

### 2.1. Microorganism and Maintenance

*Esherishia coli* MG1655 (ATCC 700926) was obtained from Cedar Lane Labs. The M9 minimal medium contained 990 ml distilled water, 2 ml of MgSO<sub>4</sub>, 10 ml of 20% glucose, 6.0 g Na<sub>2</sub>HPO<sub>4</sub>, 3.0 g KH<sub>2</sub>PO<sub>4</sub>, 0.5 g NaCl and 1.0 g NH<sub>4</sub>Cl were mixed. Agar (3.6 g) was added to 240 ml of this medium and autoclaved and subsequently poured into Petri dishes. Sixty millilitres of the medium was used to rehydrate the bacteria pellet. The bacteria was then transferred to the plates and grown overnight at 37°C. Single colonies were then replaced onto Luria Bertani agar plates and incubated overnight at 37°C. Single colonies selected from these agar plates were used to generate 80% glycerol stock solutions which were then stored at -80°C.

At the beginning of each reactor run, a sterile pipette tip was used to transfer cells from glycerol stock into 5 ml of Luria Bertani media broth and incubated overnight at 37°C. During the exponential growth phase, a sample was drawn and plated onto Luria Bertani agar plates and incubated at 37°C in anaerobic state inside an anaerobic jar (Oxoid Anaerojar, AG0025A) for 24 hours.

### 2.2. Inoculums and Culture Medium

A modified rich buffer (10X MOPS) minimal media kit (Teknova, M2106) was used to make MOPS minimal media and supplemented with 10 ml of 0.132 N<sub>2</sub>HPO<sub>4</sub> and 1 ml 1 mol sodium selenite adjusted to pH 6.3 to serve as experimental media. The media was also supplemented with 5 g/L yeast extract and 10 g/L tryptone. Hungate tubes (Bellco Glass, 2047-16125) filled with media with 10 g/L glycerol as carbon source and inoculated with a single colony taken from the anaerobically grown agar plate. The hungate tubes were incubated at 37°C until an initial optical density of 0.1 O.D was reached. Each run required 2.25 L of media supplemented with 10, 25 or 50 g/L of glycerol in a bioreactor and inoculated to maintain an initial O.D of 0.05.

## 2.3. Analytical Methods

Biomass was measured by measuring dry weight which was in turn correlated with optical density. A 15 ml sample was collected and the optical density measured at 550 nm in spectrophotometer zeroed with sterile broth. The sample was then centrifuged for 10 minutes at 10 000 rpm. The pellet was collected and washed twice with 35 ml distilled water. It was then dried at 105°C for 24 hours and weighed. The supernatant was collected and stored for analysis at -20°C.

Glycerol concentration was determined using the glycerol assay kit (Megazyme, K-GCROL). Ethanol concentration was measured using the ethanol assay kit (Megazyme, K-ETOH). Hydrogen (H<sub>2</sub>) concentration of the headspace was measured by taking samples through a septa with a syringe and direct injection into an HP Packard Series II 5890 GC with TCD detector and Porapak Q Molecular Sieve 5A column with argon carrier at 75 psig. A one point calibration was carried out to determine the relationship between peak size and H<sub>2</sub> concentration.

## 2.4. Experimental Design

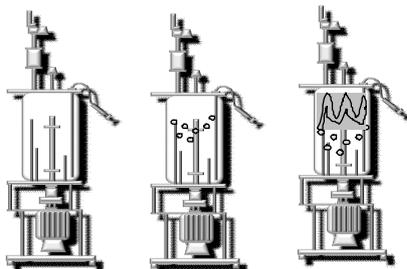
A full (2 × 3) factorial design with 3 replicates was used. The factors were glycerol concentration and headspace and three levels were 10, 25, 50 g/L and initial sparging, continuous sparging membrane, respectively, were used as shown in **Table 1**. Statistical analysis was conducted with SAS<sup>®</sup> version 9.1 (SAS, 2003) statistical software package was used for data analysis.

## 3. Results and Discussion

### 3.1. Bioprocessing of Ethanol

**Table 1. Experimental design and set-up of the bioreactors.**

Factor	Levels		
	Low	Medium	High
Glycerol Concentration	10 g/L	25 g/L	50 g/L
Headspace	Initial Sparge	Continuous Sparge	Membrane



A typical profile for hydrogen, ethanol and dry weight production during fermentation of glycerol by *E. coli* strain MG1655 (ATCC 700926) is shown of **Figure 1**. Exponential production of hydrogen was observed during the first 12 h of fermentation and maximum concentration was achieved after 84 h of fermentation. Glycerol was almost completely consumed within the 84 hrs of fermentation when only 0.55 g/L of the initial 9.6 g/L of glycerol remained. The remaining time after the 84 h apparently falls within the stationary period. Dharmadi *et al.* [21] reported that within 84 h, 80% of the glycerol initially present in the medium was consumed, a trend different from this study large due to different microbial strains used as well as varied experimental conditions. Cell concentration in the first 36 h was observed to be 0.5 g/L and sluggishly reached 0.73 g/L at the end of 120 h fermentation. This could be attributed to the accumulation of hydrogen, thus inhibit development of biomass [20].

Glycerol concentration has an important impact from the economic point of view. It is desirable to avoid including more water than necessary in the system in order to minimize the energy consumption for heating and pumping in the reactor [23]. **Figure 2** shows the comparison between cell growth and H<sub>2</sub> production in three different glycerol concentrations. The highest final dry weight concentration was observed at glycerol concentration of 25 g/L followed by 50 g/L and 10 g/L. This result is different from the results obtained by Zhu *et al.* [24] who reported that high levels of glycerol significantly inhibit cell growth. This difference may have occurred due to the different strains of *E. coli* used. The effect of headspace may have also contributed to some of the differences. The lowest final cell dry weight and highest hydrogen concentration were observed under initial sparging headspace condition. Continuous sparging and the membrane conditions promoted the stripping of fermentative gases from the process, hence the hydrogen production was limited [20]. Statistical analysis revealed that glycerol concentration and headspace condition significantly (at the 5% level) affected cell dry weight, ethanol and hydrogen production. Although hydrogen is a growth-associated product (as shown from **Figure 1**), it inhibits the growth of *Escherichia coli* during glycerol fermentation. In general, as higher concentrations of hydrogen are produced, there was a decrease in the final cell dry weight concentrations.

**Figure 3** shows the comparison between cell growth and ethanol production in three different glycerol concentrations. As expected, final ethanol concentrations are somewhat related to cell growth since ethanol production is growth-dependent. The highest amount of ethanol produced and the highest cell dry weight were in the mem-

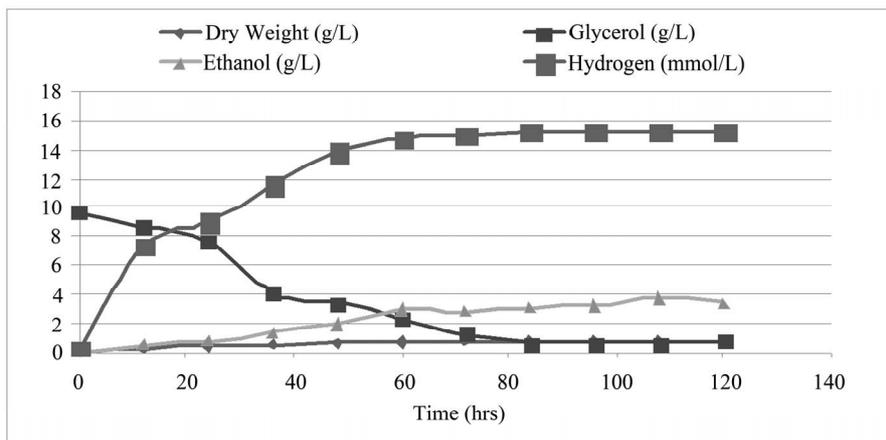


Figure 1. Profile for consumption of glycerol and production of H<sub>2</sub>, biomass and ethanol for initial glycerol concentration of 10 g/L and initial sparging headspace condition.

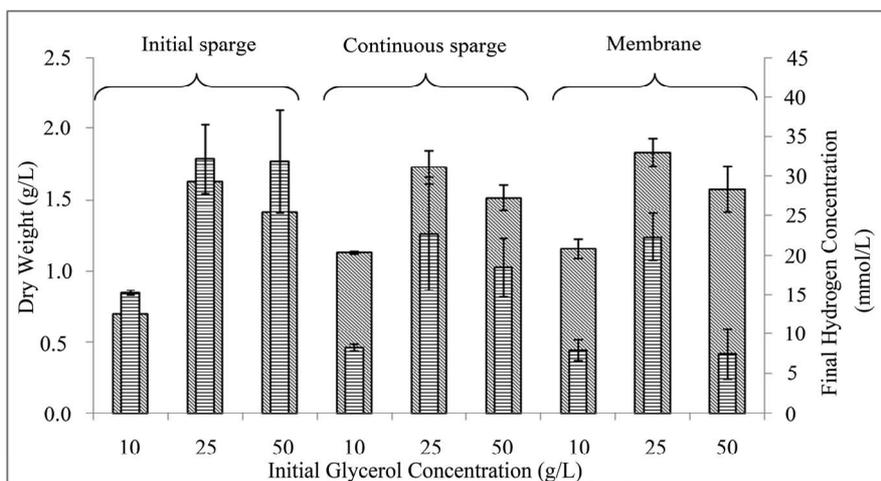


Figure 2. Comparison between hydrogen (▨) and cell growth (▩) from glycerol fermentation under the three headspace conditions.

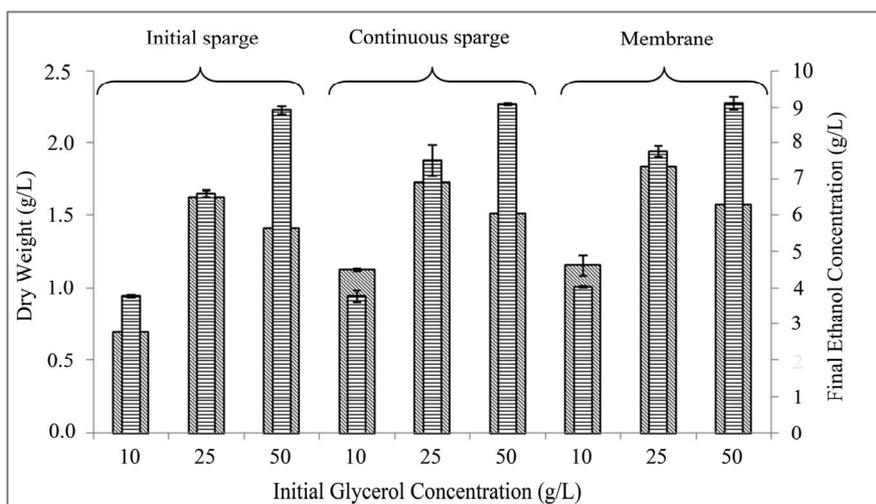


Figure 3. Comparison between ethanol (▨) and cell growth (▩) from glycerol fermentation under the three headspace conditions.

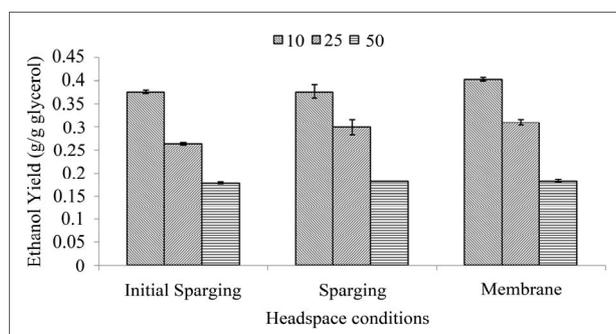
brane headspace condition. The highest ethanol yield of 9 g/L was at the 50 g/L glycerol concentration.

Starting with glycerol concentration of 25 g/L, Ito *et al.* [25] reported lower final ethanol concentration of 0.80 g/L. **Figure 4** shows the relationship between ethanol yield and headspace condition for the three different initial concentrations of glycerol tested. Ethanol yield was calculated by dividing the final ethanol concentration by the amount of glycerol used up. Since ethanol is considered as the most desirable product over the other fermented products, the ethanol yield can provide useful information concerning the treatments studied namely the headspace conditions and glycerol concentration. The highest ethanol yield was obtained from the use of a membrane with sparging as a headspace condition of initial glycerol concentration (10 g/L). A portion of the glycerol was used in the synthesis of cell mass while the remainder is used to synthesis the fermentation products principally ethanol. Thus, the best ethanol and H<sub>2</sub> production can be obtained using a membrane in the headspace with continuous sparging and starting with 10 or 25 g/L of glycerol concentration, respectively. A better understanding of the optimal conditions for glycerol fermentation for the production of hydrogen and ethanol can be done by statistical analysis of the results of the full factorial experimental design.

### 3.2. Process Optimization

Initial glycerol concentration and headspace conditions have different effects on ethanol and H<sub>2</sub> yields. A wide range of yields were obtained in the study. Hydrogen yield ranged from 0.02 to 1.58 mmol/g whereas ethanol yield ranged from 0.17 to 0.4 g/g. The optimum set of process variables were determined by applying the optimization tool in SAS software on the experimental data.

**Table 2** shows that to maximize ethanol yield, the conditions were 10 g/l as initial glycerol concentration and membrane as headspace condition. The corresponding yield obtained under the condition was 0.40. To maximize



**Figure 4.** Ethanol yield with different headspace and initial glycerol concentrations.

**Table 2.** Optimized hydrogen and ethanol yield from regression models.

Initial glycerol concentration	Headspace condition	H <sub>2</sub> Yield (mmol/L)	Ethanol Yield
20	initial	1.40	0.32
10	initial	1.38	0.37
30	initial	1.29	0.26
40	initial	1.06	0.21
20	cont	0.95	0.33
10	cont	0.93	0.38
20	memb	0.86	0.34
30	cont	0.84	0.28
10	memb	0.84	0.39
30	memb	0.75	0.29
50	initial	0.71	0.16
40	cont	0.62	0.23
40	memb	0.52	0.24
50	cont	0.27	0.18
50	memb	0.18	0.19

H<sub>2</sub> yield, the conditions were 20 g/l as initial glycerol concentration and initial sparging as headspace condition. The yield obtained in this condition was 1.40 mmol/g.

### 4. Conclusions

This work evaluated the effect of glycerol concentration, argon sparging and the use of silicon rubber membrane in lab-scale bioreactor using *E. coli* for glycerol fermentation and ethanol and H<sub>2</sub> production. Biomass production up to 1.82 g/L was obtained when using the membrane with 25 g/L of glycerol concentration and the cell growth was seen to be affected negatively by H<sub>2</sub> production. Ethanol yield was calculated by dividing the final ethanol concentration and the amount of glycerol consumed. The highest ethanol yield was obtained with continuous sparging in the headspace and 10 g/L initial glycerol concentration and was up to 0.38 g/g. H<sub>2</sub> production was up to 32.15 mmol/l; this was obtained under initial sparging conditions with 25 g/L initial glycerol concentration. The study showed that that the highest ethanol yield was obtained under membrane condition and 10 g/L initial glycerol concentration; therefore, for ethanol production, these conditions should be further investigated. For hydrogen production, another viable route of research, the highest H<sub>2</sub> yield was obtained with membrane condition and 25 g/L glycerol concentration. Statistical analysis and process optimization showed that the data indicates that the optimal conditions for hydrogen production are potentially 20 g/L initial glycerol condition with initial sparging for a yield of 1.40 mmol/g of glycerol. Similar analysis for ethanol production showed a potential optimal condition of 10 g/L initial glycerol with membrane headspace condition for a yield up to 0.40 g/g.

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## 6. References

- [1] P. M. Schenk, S. R. Thomas-Hall, E. Stephens, U. C. Marx, J. H. Mussgnug, C. Posten, O. Kruse and B. Hankamer, "Second Generation Biofuels: High-Efficiency Microalgae for Biodiesel Production," *Bioengineering Research*, Vol. 1, No. 1, 2008, pp. 20-43.
- [2] S. S. Yazdani and R. Gonzalez, "Anaerobic Fermentation of Glycerol: A Path to Economic Viability for the Biofuels Industry," *Current Opinion in Biotechnology*, Vol. 18, No. 3, 2007, pp. 213-219. [doi:10.1016/j.copbio.2007.05.002](https://doi.org/10.1016/j.copbio.2007.05.002)
- [3] M. McCoy, "Glycerin Surplus," *Chemical & Engineering News*, Vol. 84, No. 6, 2006, p. 7. [doi:10.1021/cen-v084n006.p007a](https://doi.org/10.1021/cen-v084n006.p007a)
- [4] T. Willke and K. D. Vorlop, "Industrial Bioconversion of Renewable Resources as an Alternative to Conventional Chemistry," *Applied Microbiology and Biotechnology*, Vol. 66, No. 2, 2004, pp. 131-142. [doi:10.1007/s00253-004-1733-0](https://doi.org/10.1007/s00253-004-1733-0)
- [5] G. Durmin, J. Clomburg, Z. Yeates, P. J. J. Alvarez, K. Zygourakis, P. Campbell and R. Gonzalez, "Understanding and Harnessing the Microaerobic Metabolism of Glycerol in *Escherichia coli*," *Biotechnology and Bioengineering*, Vol. 103, No. 1, 2008, pp. 148-161. [doi:10.1002/bit.22246](https://doi.org/10.1002/bit.22246)
- [6] J. Nielsen, J. Villadsen and G. Liden, "Bioreaction Engineering Principles," Kluwer Academic/Plenum Publishers, New York, 2003, pp. 60-73.
- [7] K. S. Tyson, J. Bozell, R. Wallace, E. Peterson and L. Moens, "Biomass Oil Analysis: Research Needs and Recommendations," Technical Report, National Renewable Energy Laboratory, US Department of Energy. NREL/TP-510-34796, 2004.
- [8] I. Booth, "Glycerol and Methylglyoxal Metabolism." In: F. C. Neidhardt, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger, Eds., *Escherichia coli and Salmonella: Cellular and Molecular Biology*, ASM Press, Washington D.C., 2005. [www.ecosal.org](http://www.ecosal.org)
- [9] O. M. Bouvet, P. Lenormand, E. Ageron, and P. A. Grimont, "Taxonomic Diversity of Anaerobic Glycerol Dissimilation in the Enterobacteriaceae," *Research Microbiology*, Vol. 146, No. 4, 1995, pp. 279-290. [doi:10.1016/0923-2508\(96\)81051-5](https://doi.org/10.1016/0923-2508(96)81051-5)
- [10] H. Hu and T. K. Wood, "An Evolved *Escherichia coli* Strain for Producing Hydrogen and Ethanol from Glycerol," *Biochemical and Biophysical Research Communications*, Vol. 391, No. 1, 2010, pp. 1033-1038. [doi:10.1016/j.bbrc.2009.12.013](https://doi.org/10.1016/j.bbrc.2009.12.013)
- [11] G. Sabourin-Provost and P. C. Hallenbeck, "High Yield Conversion of a Crude Glycerol Fraction from Biodiesel Production to Hydrogen by Photofermentation," *Bioresour. Technol.*, Vol. 100, No. 14, 2009, pp. 3513-3517. [doi:10.1016/j.biortech.2009.03.027](https://doi.org/10.1016/j.biortech.2009.03.027)
- [12] W. Parawira, "Biotechnological Production of Biodiesel Fuel Using Biocatalysed Transesterification: A Review," *Critical Review Biotechnology*, Vol. 29, No. 2, 2009, pp. 82-93. [doi:10.1080/07388550902823674](https://doi.org/10.1080/07388550902823674)
- [13] B. Katryniok, S. Paul, M. Capron and F. Dumeignil, "Towards the Sustainable Production of Acrolein by Glycerol Dehydration," *ChemSusChem*, Vol. 2, No. 8, 2009, pp. 719-730. [doi:10.1002/cssc.200900134](https://doi.org/10.1002/cssc.200900134)
- [14] T. Maeda, V. Sanchez-Torres and T. K. Wood, "Enhanced Hydrogen Production from Glucose by Metabolically Engineered *Escherichia coli*," *Applied Microbiology and Biotechnology*, Vol. 77, No. 4, 2007, pp. 879-890. [doi:10.1007/s00253-007-1217-0](https://doi.org/10.1007/s00253-007-1217-0)
- [15] Z.-X. Wang, J. Zhuge, H. Fang and B. A. Prior, "Glycerol Production by Microbial fermentation: A Review," *Biotechnology Advances*, Vol. 19, No. 3, 2001, pp. 201-223. [doi:10.1016/S0734-9750\(01\)00060-X](https://doi.org/10.1016/S0734-9750(01)00060-X)
- [16] G. L. Zhang, B. B. Ma, X. L. Xu, C. Li and L. W. Wang, "Fast Conversion of Glycerol to 1,3-Propanediol by a New Strain of *Klebsiella pneumoniae*," *Biochemical Engineering Journal*, Vol. 37, No. 3, 2007, pp. 256-260. [doi:10.1016/j.bej.2007.05.003](https://doi.org/10.1016/j.bej.2007.05.003)
- [17] B. B. Ma, X. L. Xu, G. L. Zhang, L. W. Wang, M. Wu and C. Li, "Microbial Production of 1,3-Propanediol by *Klebsiella pneumoniae* XJPD-Li Under Different Aeration Strategies," *Applied Biochemistry and Biotechnology*, Vol. 152, No. 1, 2009, pp. 127-134. [doi:10.1007/s12010-008-8220-5](https://doi.org/10.1007/s12010-008-8220-5)
- [18] T. Homann, C. Tag, H. Biebl, W. D. Deckwer and B. Schink, "Fermentation of Glycerol to 1,3-Propanediol by *Klebsiella* and *Citrobacter* Strains," *Applied Microbiology and Biotechnology*, Vol. 33, No. 2, 1990, pp. 121-126. [doi:10.1007/BF00176511](https://doi.org/10.1007/BF00176511)
- [19] C. W. Forsberg, "Production of 1,3-Propanediol from Glycerol by *Clostridium acetobutylicum* and Other *Clostridium* Species," *Applied and Environmental Microbiology*, Vol. 53, No. 4, 1987, pp. 639-643.
- [20] A. Murarka, Y. Dharmadi, S. S. Yazdani and R. Gonzalez, "Fermentative Utilization of Glycerol by *Escherichia coli* and Its Implications for the Production of Fuels and Chemicals," *Applied and Environmental Microbiology*, Vol. 74, No. 4, 2008, pp. 1124-1135. [doi:10.1128/AEM.02192-07](https://doi.org/10.1128/AEM.02192-07)
- [21] Y. Dharmadi, A. Murarka and R. Gonzalez, "Anaerobic Fermentation of Glycerol by *Escherichia coli*: A New Platform for Metabolic Engineering," *Biotechnology and Bioengineering*, Vol. 94, No. 5, 2006, pp. 821-829. [doi:10.1002/bit.21025](https://doi.org/10.1002/bit.21025)
- [22] S. S. Yazdani and R. Gonzalez, "Engineering *Escherichia coli* for the Efficient Conversion of Glycerol to Ethanol and Co-products," *Metabolic Engineering*, Vol. 10, No. 6, 2008, pp. 340-351. [doi:10.1016/j.ymben.2008.08.005](https://doi.org/10.1016/j.ymben.2008.08.005)
- [23] A. J. Byrd, K. K. Pant and R. B. Gupta, "Hydrogen Production from Glycerol by Reforming in Supercritical Water Over Ru/Al<sub>2</sub>O<sub>3</sub> Catalyst," *Fuel*, Vol. 87, No. 13-14, 2008, pp. 2956-2960. [doi:10.1016/j.fuel.2008.04.024](https://doi.org/10.1016/j.fuel.2008.04.024)
- [24] M. M. Zhu, P. D. Lawman and D. C. Cameron, "Improving 1,3-Propanediol Production from Glycerol in a Metabolically Engineered *Escherichia coli* by Reducing Accumula-

tion of sn-Glycerol-3-Phosphate,” *Biotechnology Progress*, Vol. 18, No. 4, 2002, pp. 694-699.  
[doi:10.1021/bp020281+](https://doi.org/10.1021/bp020281+)

- [25] T. Ito, Y. Nakashimada, K. Senba, T. Matsui and N. Nishio, “Hydrogen and Ethanol Production from Glyc-

erol-Containing Wastes Discharged after Biodiesel Manufacturing Process,” *Journal of Bioscience and Bioengineering*, Vol. 100, No. 3, 2005, pp. 260-265.  
[doi:10.1263/jbb.100.260](https://doi.org/10.1263/jbb.100.260)