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Bioconversion of Crude Glycerol into 1,3-Propanediol(1,3-PDO) with Bioelectrochemical System and Zero-Valent Iron Using *Klebsiella pneumoniae* L17

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Abstract: Crude glycerol is a major byproduct in the production of biodiesel and contains a large number of impurities. The transformation of crude glycerol into valuable compounds such as 1,3-propanediol (1,3-PDO) using clean and renewable processes, like bioconversion, is an important task for the future of the chemical industry. In this study, 1,3-PDO bioproductions from crude and pure glycerol were estimated as 15.4 ± 0.8 and 11.4 ± 0.1 mmol/L, respectively. Because 1,3-PDO is a reductive metabolite that requires additional reducing energy, external supplements of electron for further improvement of 1,3-PDO biosynthesis were attempted using a bioelectrochemical system (BES) or zero-valent iron (ZVI). The conversions of crude and pure glycerol under electrode and iron-based cultivation were investigated for 1,3-PDO production accompanied by metabolic shift and cell growth. The BES-based conversion produced 32.6 ± 0.6 mmol/L of 1,3-PDO with ZVI implementation.

Keywords: crude glycerol; *Klebsiella pneumoniae* L17; 1,3-propanediol (1,3-PDO); bioelectrochemical system (BES); zero-valent iron (ZVI)

1. Introduction

Crude glycerol, a major byproduct of the biodiesel process in the transesterification of animal fats and vegetable oils, has been widely examined for waste conversion to produce value-added chemicals [1,2]. During every 9 kg of biodiesel synthesis, approximately 1 kg of crude glycerol is produced as a byproduct. The composition of crude glycerol is mostly glycerol (over 92%), with some impure ingredients such as water, methanol, and organic matters [3]. Although several successful glycerol conversion studies have been reported, further improvement is still required because of the relatively high purification cost [4]. Pure glycerol costs 7.5 times more than its crude counterpart at the current market price (Table 1) [2,5]; this implies that the additional glycerol purification process is complicated and expensive. Therefore, several studies have reported direct bioconversion of non-purified crude glycerol to value-added products, such as hydrogen, methane, fatty acids, and 1,3-propanediol [6–11].

Table 1. Glycerin technology of economic value. The market price of crude glycerol, pure glycerol, and 1,3-propanediol [2].

	Crude Glycerol	Pure Glycerol	1,3-Propanediol
Price (USD/ton)	170	1275	2000

1,3-Propanediol (1,3-PDO; $C_3H_8O_2$) is an important three-carbon platform chemical for additional chemical synthesis [10], mainly used in the synthesis of polytrimethylene terephthalate (PTT) [12]. The 1,3-PDO market is rapidly growing and expected to reach \$6.2 million by 2021 (146.0 kilotons and 225.9 kilotons by 2014 and 2021, respectively) [13]. Interestingly, biological 1,3-PDO production processes are already competitive against chemical catalytic processes; several industrially applicable bioconversion processes were reported [12,14–16]. Microbial metabolic pathways for the synthesis of 1,3-PDO from glycerol consist of only two-step reactions: glycerol dehydration to 3-HPA (3-hydroxypropionaldehyde with coenzyme B_{12} as a co-factor; $C_3H_6O_2$) and then 3-HPA reduction to 1,3-PDO with NADH as a redox co-factor [13,17]. In other words, NADH regeneration and/or supply of additional reduction energy are essential for the bioconversion of glycerol to 1,3-PDO, based on the stoichiometric net calculation.

Klebsiella pneumoniae is a natural 1,3-PDO producer, having several advantages in 1,3-PDO production [17]. *K. pneumoniae* has highly active glycerol assimilation metabolism in anoxic conditions and well-developed natural 1,3-PDO production pathways [13,17–21]. Several studies reported that electron transfer from electrode to bacteria induced the metabolic shifts of *K. pneumoniae* L17 in a bioelectrochemical system (BES) and increased 1,3-PDO production [13,21,22]. Similarly, zero-valent iron (ZVI) was also demonstrated as the feasible electron source for a *K. pneumoniae* strain for enhancement of 1,3-PDO production [23,24].

In this study, we tried to produce 1,3-PDO using non-purified crude glycerol with a *K. pneumoniae* L17 strain. Moreover, the supplement of an additional electron transfer via ZVI oxidation and the cathode of a bioelectrochemical system was proposed to improve 1,3-PDO productivity. Not only enhanced 1,3-PDO production titer but also metabolic fluxes were identified to understand the effect of both glycerol impurity and additional electron sources by stoichiometric analysis. This study could suggest the possibility of further improvements of the biological 1,3-PDO production process by using non-purified crude glycerol and additional energy sources.

2. Materials and Methods

2.1. Strain and Cultivation

K. pneumoniae L17 was purchased from the China Center for Type Culture Collection (CCTCC). The experimental fermentation medium contains the following composition (per liter): 18.04 g K_2HPO_4 , 1.804 g KH_2PO_4 , 1 g NaCl, 1 g yeast extract, 0.25 g $MgSO_4 \cdot 7H_2O$, 9.2 g pure glycerol or 10 g crude glycerol. To adjust media pH to 7.5, 10% HCl and 5 N NaOH were used. For exogenic electron supply (70 mesh powder, <212 micrometers, ACROS), 0.5 g of zero-valent iron (ZVI) was added. *K. pneumoniae* L17 (hereinafter L17) was cultivated in a shaking incubator at 170 rpm, 30 °C. Aerobic cultivation was carried out in 250 mL flasks with sponge caps, whereas a serum bottle (200 mL) was used for anaerobic cultivation after 10 min of nitrogen purging. Initial cell density was adjusted to 0.05 OD₆₀₀.

2.2. BES Configuration and Operation

H-type 2-chamber BES reactors were used according to procedures described elsewhere [22]. Anode and cathode chambers (310 mL each) were connected with a glass tube bridge and a proton exchange membrane (PEM, Nafion 117, Dupont, Wilmington, DE, USA). Carbon felt (2.5 × 5 cm, Nara Cell-Tech Co., Seoul, Korea) was used for both the anode and cathode. A Ag/AgCl reference electrode was attached to the cathode chamber. The BES reactor was pre-washed with a 10% HCl and 5 N NaOH solution and sterilized with

an autoclave (121 °C, 15 min). After sterilization, the cathode chamber was filled with a growth medium, and the anode chamber was occupied with 50 mM ferrous sulfate hydrate in a 100 mM potassium phosphate solution (pH 7.5). *K. pneumoniae* L17 was pre-cultured in an LB medium overnight and then reactivated in a fermentation medium for 12 h. The cathode chamber was inoculated with pre-cultured L17 (initial OD₆₀₀ = 0.05). Ampicillin was added to the anode and cathode chambers (100 µM) to prevent contamination.

BES reactors were placed in an incubator (30 °C) on a stir plate (100 rpm). Both anode and cathode chambers were purged continuously with nitrogen gas (99.9%) to maintain anaerobic conditions. The chronoamperometry (−0.7 V vs. Ag/AgCl) method was used from a potentiostat (WBCS3000Lee32, WonA Tech, Seoul, Korea) to continuously apply potential externally to the cathode as a working electrode.

2.3. Analytical Methods

Metabolite production and optical density (OD₆₀₀) were determined using a fermentation broth sample from the flask at 0, 5, 10, and 24 h. OD₆₀₀ was measured using UV-Vis spectrophotometer (Optizen POP, Keen Innovative Solutions, Daejeon, Korea) at 600 nm. For HPLC analysis, the liquid samples were centrifuged at 12,000 rpm for 10 min. Then, supernatants were filtered using a syringe filter. The filtered samples were analyzed to identify the metabolite concentrations by HPLC (HP 1160 series, Agilent Technologies, Santa Clara, CA, USA) equipped with a 300 × 7.8 mm Aminex HPX-87H (Bio-Rad, Hercules, CA, USA) column at 65 °C and a refractive index (RI) and photodiode array (PDA) detector, using 2.5 mM H₂SO₄ as the mobile phase (flow rate = 0.5 mL/min).

2.4. Metabolic Flux Analysis (MFA)

The glycerol metabolic flux analysis (MFA) was investigated as previously described [22]. A total of 63 equations have been implemented to determine the metabolic flux, the EMP pathway, PP pathway, tricarboxylic acid cycle (TCA cycle), pyruvate metabolism, energy metabolism, transport reaction, cell growth flux, and electron excretion pathway (Table S1). All the equations for the undetermined metabolic pathways were solved by linear optimization using the MetaFluxNet program [25].

3. Results and Discussion

3.1. 1,3-PDO Production in Flask Experiments

Aerobic and anaerobic flask experiments for 1,3-PDO bioconversion by *K. pneumoniae* L17 were conducted using pure and crude glycerol (100 mM) (Figure 1A). Most glycerol was consumed within 24 h in all conditions, even though the aerobic glycerol consumption rate seemed relatively higher than anaerobic fermentations. Under aerobic conditions, 1,3-PDO productions were much lower as 3.1 ± 0.1 and 2.8 ± 0.1 mM from crude and pure glycerol, respectively (Figure 1B). Previous reports determined that oxygen can strongly interrupt not only glycerol dehydratase (dhaB) but also coenzyme B₁₂ synthesis, which is utilized as an essential co-factor for DhaB in the *K. pneumoniae* strain. On the other hand, 1,3-PDO synthesis was significantly higher in anaerobic conditions as 15.4 ± 0.1 and 11.4 ± 0.9 mM from crude and pure glycerol, respectively (Figure 1B). Byproduct formations were illustrated in Figure 1C,D. Ethanol productions were relatively higher in anaerobic conditions; acetate synthesis was significantly higher in aerobic conditions. Interestingly, relatively higher 1,3-PDO productions were obtained from crude glycerol fermentation in both aerobic and anaerobic conditions. It is supposed that the potential organic and inorganic ingredients contained in crude glycerol (e.g., Na, Ca, K, Mg, P, S, and unidentified organic matters) positively influenced

Different concentrations of glycerol (100, 200, 500, and 1000 mM) were added to anaerobic flasks to identify the effect of initial substrate concentrations with pure glycerol in 1,3-PDO production (Figure 2). After 24 h, 100, 93.8, 60.7, and 23.2% of glycerol were consumed in 100, 200, 500, and 1000 mM pure glycerol bottles, respectively (Figure 2A). 1,3-PDO productions were determined as 11.4 ± 0.1 , 23.3 ± 1.1 , 49.0 ± 1.6 ,

and 74.1 ± 2.9 mM in 100, 200, 500, and 1000 mM glycerol flasks, respectively (Figure 2B). The final cell densities identified as 1.9 ± 0.02 , 2.0 ± 0.01 , 1.5 ± 0.0 , and 1.5 ± 0.0 mM from 100, 200, 500, and 1000 mM, respectively (Figure 2C). The other byproduct production was presented in Figure S1.

Figure 1. Productivity of 1,3-PDO, ethanol, and acetate according to oxygen condition using crude and pure glycerol. (A) Glycerol consumption and productivity of (B) 1,3-PDO, (C) ethanol, and (D) acetate. Pure glycerol conversion in anaerobic conditions, crude glycerol conversion in anaerobic conditions, pure glycerol conversion in aerobic conditions, and crude glycerol conversion in aerobic conditions expressed as Ana-P, Ana-C, Aer-P, and Aer-C, respectively.

Glycerol is usually considered a reduced substrate in comparison with glucose. Thus, anaerobic glycerol oxidation can elevate the cellular NADH/NAD⁺ ratio and then shift metabolic fluxes. Under anaerobic conditions, a high concentration of initial glycerol induced overflow metabolism toward reductive pathways in *Klebsiella* species, resulting in increased 1,3-PDO production yield [27,28]. Figure 2D presented 1,3-PDO production yield. The fermentation result indicates glycerol-derived overflow metabolism with higher glycerol concentration (in 1000 mM glycerol fermentation) in the *K. pneumoniae* L17 strain. According to overflow metabolism, glycerol consumption, 1,3-PDO production, and cell growth seemed to be reduced after 24 h (Figure 2C).

1,3-PDO production using crude glycerol was attempted with different concentrations (100, 200, 500, and 1000 mM) (Figure 3). Glycerol consumption rates, within 24 h, presented as 100.0, 63.9, 33.6, and 18.9% with 100, 200, 500, and 1000 mM, respectively (Figure 3A). 1,3-PDO productions were determined as 16.1 ± 0.3 , 28.8 ± 0.2 , 12.6 ± 0.4 , and 13.1 ± 0.1 mM with 100, 200, 500, and 1000 mM of crude glycerol (Figure 3B). Interestingly, significantly lower glycerol consumption and 1,3-PDO production rates were identified in high-crude glycerol conditions, 500 and 1000 mM. In addition, cell density was presented as significantly inhibited (lower than 0.1 OD₆₀₀). This might be the toxic effect of micronutrients or methanol added as the catalyst in biodiesel production [29,30]. 1,3-PDO

production yields are illustrated in Figure 3D. Although high concentrations interrupted glycerol metabolism by overflow metabolism and/or toxicity, relatively higher 1,3-PDO production yields were identified in 100 and 200 mM crude glycerol fermentation compared with pure glycerol fermentation (Table 2). In crude glycerol, micronutrients exist, such as fatty acids, magnesium, zinc, and iron, which can help anoxic metabolism by utilizing cell components or co-factors [26]. Because large-scale 1,3-PDO biosynthesis generally uses the constant glycerol concentration (lower than 200 mM), unpurified glycerol can be directly applicable to 1,3-PDO production [11–13,23].

Figure 2. Production difference due to pure glycerol concentration 100, 200, 500, and 1000 mM. (A) Glycerol consumption, (B) 1,3-PDO production, (C) optical density (600 nm), and (D) 1,3-PDO production yield (mol/mol).

Table 2. Production difference due to pure and crude glycerol concentration 100, 200, 500, and 1000 mM.

Type of Glycerol	Initial Glycerol	Glycerol	1,3-PDO	Produced PDO/ Consumed Glycerol Ratio
	Concentration	Consumption	Production	
	(mM)	(mM)	(mM)	
Pure glycerol	100	110.2 ± 0.1	11.4 ± 0.1	0.10
	200	197.6 ± 1.0	23.3 ± 1.1	0.14
	500	310.0 ± 3.6	49.0 ± 1.7	0.16
	1000	235.3 ± 3.8	74.1 ± 3.0	0.31
Crude glycerol	100	119.6 ± 0.1	16.1 ± 0.3	0.15
	200	132.9 ± 2.9	28.8 ± 0.2	0.29
	500	168.2 ± 7.4	12.6 ± 0.4	0.06
	1000	191.7 ± 2.4	13.1 ± 0.1	0.07

Figure 3. Production difference due to crude glycerol concentration 100, 200, 500, and 1000 mM. (A) Glycerol consumption, (B) 1,3-PDO production, (C) optical density (600 nm), and (D) 1,3-PDO production yield (mol/mol).

3.2. Flux Balance Analysis (FBA) of Crude and Pure Glycerol

To understand the metabolic dynamics of *K. pneumoniae* L17 between pure and crude glycerol (100 mM), stoichiometric and metabolic flux balance analyses were conducted (Figure 4). Glycerol metabolism can be categorized as oxidation and reduction, referred to as glycerol oxidative and reductive pathways, respectively. In the fermentation flask with pure glycerol as a substrate, the ratio between glycerol oxidative and reductive pathways was presented as 81:19, whereas the metabolic flux seemed to be shifted toward more reduction pathways (62:38) when crude glycerol was used as a sole carbon source instead of pure glycerol. 1,3-PDO and ethanol are major metabolites of anaerobic glycerol metabolism in *K. pneumoniae* strains, representing oxidation and reduction pathways. Although both metabolites are synthesized by NADH-consuming aldehyde reductases (propanediol aldehyde reductase or acetaldehyde reductase for 1,3-PDO or ethanol, respectively), net NADH balances (from glycerol to products) implied that 1,3-PDO synthesis (net -1 NADH) would be more effective to reduce cellular redox balance than ethanol synthesis (net 0 NADH) (Equations (1) and (2)) [13].

This metabolic shift indicates that intracellular metabolism of *K. pneumoniae* L17 was significantly affected by minor components contained in crude glycerol for several reasons. Even after the extraction process of biofuel, organic materials still existed, such as free fatty acids (FFA), soaps, and fatty acid methyl esters (FAMES). These could be utilized

as cell components; thus, bacteria can reduce glycerol oxidative pathways to synthesize additionally. Another hypothesis is that providing multiple substrates (e.g., sugar) can increase carbon flux starting from DHAP. This can over stack glycerol oxidative pathway intermediates, or the anaerobic assimilation of other substrates can increase intracellular redox balance (NAD(P)H/NAD(P)⁺). The increase in cellular redox state can induce glycerol reductive metabolism [14,31].

Figure 4. The metabolic flux analysis of crude and pure glycerol was calculated using the meta flux net program.

3.3. Electron Uptake for Improvement of 1,3-PDO Production

Previous works have demonstrated that 1,3-PDO production can be improved with additional reducing energy supplies by cathodic electron transfer in BES or oxidation of ZVI [13,32]. 1,3-PDO productions using both 100 mM of crude and pure glycerol were examined in a cathode chamber in BES reactors with -0.7 V (vs. Ag/AgCl) potential and non-BES (electrically disconnected reactor). Most glycerol was consumed within 10 h, while different 1,3-PDO productions were identified as 2.1 (32.5 ± 0.6 mM) and 2.5 (28.3 ± 0.5 mM) times higher than flask fermentation with crude and pure glycerol, respectively (Figure 5). Similarly, 1,3-PDO production increases were determined with 200 mM glycerol substrate as 2.2 (76.5 ± 1.1 mM, crude glycerol) and 2.3 (67.3 ± 3.2 mM, pure glycerol) in the same BES. Kim et al. suggest that current uptake triggers the metabolic shift toward glycerol reductive metabolism in the *K. pneumoniae* L17 strain [13]. Even though crude glycerol contained several minor components, this might not affect the electron transferring and/or electrode-driven metabolic shifting in 1,3-PDO production (Figure 6).

Installation of the electrode into the bioreactor enables us to regulate the cellular redox state without chemical additives but brings technical issues, such as bioreactor design and process development. Instead, addition of undissolved iron molecules, such as ZVI, into bioreactors can make similar effects in conventionally used bioreactors or bottles. As the fermentation results with ZVI as the electron donor, 1.9 (28.9 ± 0.2 mM) and 2.2 (24.8 ± 1.6 mM) times higher 1,3-PDO productions were determined with both crude and pure glycerol substrate, respectively (Figure 5). With the doubled initial glycerol concentration (200 mM), the increase in 1,3-PDO was also presented as 2.5 (67.3 ± 3.2 mM) and 2.6 (76.5 ± 1.1 mM) times higher with crude and pure glycerol, respectively (Figure 6). The

results indicate that ZVI can support glycerol reduction via transferring additional reducing energy, both with non-purified and purified glycerol, similar to BES-applied fermentations.

Figure 5. Crude and pure glycerol (100 mM) applied to BES and ZVI. Productivity of 1,3-PDO and glycerol consumption using (A) BES, (B) ZVI, and (C) 1,3-PDO production with and without ZVI and BES for crude and pure glycerol (P: pure glycerol; C: crude glycerol).

Figure 6. Crude and pure glycerol (200 mM) applied to BES and ZVI. Productivity of 1,3-PDO and glycerol consumption using (A) BES, (B) ZVI, and (C) 1,3-PDO production with and without ZVI and BES for crude and pure glycerol (P: pure glycerol; C: crude glycerol).

Current 1,3-PDO bioproduction processes have already been well developed; however, it is required to improve the productivity with not only traditional upstream (e.g., strain engineering) and downstream (e.g., process engineering) development but also other

approaches. Non-purified substrate utilization might be the one option to increase bioconversion efficiency with regard to the economic aspects. Table 1 presented that the price of crude glycerol is just 13% of the price of pure glycerol; the crude glycerol fermentation process can reduce the fermentation cost by using a much cheaper substrate. Providing additional reducing energy via both cathodic electrodes and metallic granules would be the next step for 1,3-PDO bioproduction because of redox imbalance between the substrate and product. Although there are several demonstrations of the improvement of 1,3-PDO productivity using BES or ZVI, this research presented the applications in non-purified glycerol conversion. The results implied that both electron supply modes can be applied not only in 1,3-PDO production but also in several reductive fermentations using glycerol as a substrate.

4. Conclusions

In this study, we examined the 1,3-PDO bioproduction process using non-purified crude glycerol as a substrate. Fermentation results indicated that significantly higher 1,3-PDO production was identified with crude glycerol (15.4 ± 0.8 mM) compared to pure glycerol (11.4 ± 0.1 mM). Metabolic flux balance analysis revealed that micronutrients, included in crude glycerol, induced the metabolic shift toward glycerol reductive pathways. Furthermore, additional reducing energy supplemented by cathodic electrodes improved 1,3-PDO production (32.5 ± 0.6 and 28.3 ± 0.5 mM with crude and pure glycerol, respectively) or ZVI (28.9 ± 0.2 and 24.8 ± 1.6 mM with crude and pure glycerol, respectively). The results demonstrated the possibility of crude glycerol utilization in 1,3-PDO production and further improvement of productivity with BES and ZVI-based bioconversion.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/en14206806/s1>, Figure S1: Comparison OD and pH of 100 mM pure and crude glycerol in flask. (A) cell growth (OD) and pH and (B) lactate production; Table S1: The reactions and results of in silico metabolic flux analysis.

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