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Effect of Biodiesel-derived Raw Glycerol on 1,3-Propanediol Production by Different Microorganisms

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Abstract The microbial production of 1,3-propanediol (1,3-PD) from raw glycerol, a byproduct of biodiesel production, is economically and environmentally advantageous. Although direct use of raw glycerol without any pretreatment is desirable, previous studies have reported that this could cause inhibition of microbial growth. In this study, we investigated the effects of raw glycerol type, different microorganisms, and pretreatment of raw glycerol on the production of 1,3-PD. Raw glycerol from waste vegetable-oil-based biodiesel production generally caused more inhibition of 1,3-PD production and microbial growth compared to raw glycerol from soybean-oil-based biodiesel production. In addition, two raw glycerol types produced from two biodiesel manufacturers using waste vegetable oil exhibited different 1,3-PD production behavior, partially due to different amounts of methanol included in the raw glycerol from the two biodiesel manufacturers. *Klebsiella* strains were generally resistant to all types of raw glycerol while the growth of *Clostridium* strains was variably inhibited depending on the type of raw glycerol. The 1,3-PD production of the *Clostridium* strains using acid-pretreated raw glycerol was significantly enhanced compared to that with raw glycerol, demonstrating the feasibility of using raw glycerol for 1,3-PD production by various microorganisms.

Keywords Raw glycerol · 1,3-Propanediol · Fermentation · Pretreatment

Introduction

Glycerol is produced as a byproduct of saponification [1]. Recently, since the demand for biodiesel has increased in the world market, the production of glycerol has also increased (10 kg of biodiesel yields 1 kg of glycerol) [2]. Consequently, a sharp decrease of glycerol

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cost (by tenfold) has also been observed [1]. Therefore, using glycerol for the production of various chemicals and bio-fuel is advantageous both economically and environmentally.

Because of its lower cost, glycerol is now being considered as a feedstock for industrial fermentation [3]. One of its many applications is the bioconversion of glycerol to high-value products by microorganisms [4]. Glycerol can be utilized to produce 1,3-propanediol (1,3-PD) [2], succinic acid [5], propionic acid [6], ethanol [7], butanol [8], and hydrogen [9]. Among these, the demand for 1,3-PD, which is the monomer for polypropylene terephthalate, has been increasing [10]. Various microorganisms, including *Klebsiella pneumoniae*, *Citrobacter freundii*, *Clostridium butyricum*, and *Clostridium pasteurianum*, can produce 1,3-PD [10]. Among them, *C. butyricum* and *K. pneumoniae* are capable of converting glycerol to 1,3-PD [11, 12].

Although many microorganisms can convert glycerol to 1,3-PD, previous studies have shown that the use of raw glycerol could inhibit the growth of microorganisms. For example, *C. butyricum* strains obtained from bacterial culture collections did not grow on raw glycerol in batch culture [13]. Furthermore, Chi et al. reported that algal growth was inhibited when crude glycerol was used without pretreatment [14]. In our preliminary experiments, we observed that raw glycerol was not consumed by *C. pasteurianum* DSM 525 without pretreatment (data not shown). However, a newly isolated *C. butyricum* strain can convert raw glycerol to 1,3-PD with high yields [15].

The objective of this study was to investigate the effects of the type of raw glycerol, pretreatment of raw glycerol, and the use of different microorganisms on the production of 1,3-PD. The production of 1,3-PD was determined with pure glycerol, raw glycerol, and acid-pretreated raw glycerol using the bacterial strains *C. butyricum* and *K. pneumoniae*.

Materials and Methods

Bacterial Strains and Culture Media

C. butyricum DSM 2477, *C. butyricum* DSM 2478, *C. butyricum* DSM 15410, *K. pneumoniae* DSM 2026, and *K. pneumoniae* DSM 4799 were purchased from the German Collection of Microorganisms and Cell Culture (DSMZ, Branunschweig, Germany). *C. butyricum* DSM 2477, *C. butyricum* DSM 2478, and *C. butyricum* DSM 15410 were grown in modified CAB (M-CAB) medium, which contained the following components per liter of distilled water: 1.5 g of K_2HPO_4, 1.5 g of KH_2PO_4, 0.1 g of $MnSO_4{\cdot}4H_2O$, 0.1 g of $MgSO_4{\cdot}7H_2O$, 0.1 g of NaCl, 4 g of yeast extract, 1 g of tryptone, 0.5 g of asparagine, and 15 mg of $FeSO_4{\cdot}7H_2O$. To prevent possible over-acidification, 100 mM of 2-(*N*-morpholino)ethanesulfonic acid (MES) [16] was added into the M-CAB medium [17]. *K. pneumoniae* DSM 2026 and *K. pneumoniae* DSM 4799 were grown in production medium, which contained the following components per liter of distilled water: 5 g of K_2HPO_4, 3 g of KH_2PO_4, 2 g of $(NH_4)_2SO_4$, 0.4 g of $MgSO_4{\cdot}7H_2O$, 0.1 g of $CaCl_2{\cdot}2H_2O$, 2 g of yeast extract, 0.5 g of peptone, 0.3 g of beef extract, and 1 mL of trace elements [18].

Pretreatment of Raw Glycerol

Raw glycerol was obtained from three biodiesel manufacturers (designated as S, N, and E), which produce biodiesel from soybean oil or waste vegetable oil using an alkali-catalyzed transesterification process. Raw glycerol from each manufacture was pretreated according to the method of Chi et al. [14] with minor modifications: (1) the raw glycerol was mixed

Table 1 Types of glycerol and their designations.

Pure glycerol	Glycerol from biodiesel process			
	Soybean-oil-based		Waste vegetable-oil-based	
	W/o pretreatment	W/pretreatment	W/o pretreatment	W/pretreatment
PG	N-RSO	N-TSO	S-RWO	S-TWO
	E-RSO	E-TSO	E-RWO	E-TWO

N-, E-, and S- indicate the biodiesel manufacturers from which the raw glycerol was obtained

RSO raw glycerol from soybean-based biodiesel production, *TSO* pretreatment of RSO, *RWO* raw glycerol from waste vegetable-oil-based biodiesel production, *TWO* pretreatment of RWO

with distilled water at a ratio of 1:4 (*v*/*v*) to reduce the viscosity of the raw glycerol; (2) the pH was adjusted to pH 3–4 with 5 N HCl; (3) the acid-treated raw glycerol was left for 10 min; (4) the lower phase was extracted with a syringe and centrifuged at 5,000 rpm for 10 min; and (5) the lower phase was again extracted with a syringe and used as the carbon source.

Batch Fermentation

Batch experiments were carried out in 150-mL serum bottles containing 50 mL of M-CAB media for *Clostridium* strains and production media for *Klebsiella* strains. To each serum

Table 2 Composition of raw and pretreated glycerol.

Item	Method	Results							
		E-RSO	E-TSO	E-RWO	E-TWO	N-RSO	N-TSO	S-RWO	S-TWO
Glycerol content, wt.%	KSM 1979	75.56	20.09	63.7	13.66	79.71	23.07	70.72	13.84
Water content, wt.%	ASTM E203-01	12.6	73.38	0.07	76.44	0.05	73.3	12.49	80.9
Methanol content, wt.%	GC	0.2	0.06	2.66	0.58	0.27	0.06	6.67	0.08
MONG, wt.%	ISO 2464	7.91	5.47	33.96	9.3	16.96	2.82	9.69	4.63
Ash, wt.%	ASTM D482-07	3.93	1.06	2.27	0.6	3.28	0.81	7.1	0.63
Sodium, mg/kg	ICP-AES	12,743	4,416.7	7,357.9	2,540.6	13,660	3,865	5,300	4,326.9
Magnesium, mg/kg	ICP-AES	101.8	33.5	2.3	1	1.9	0.6	47.5	1.5
Potassium, mg/kg	ICP-AES	242	94.2	49.8	15.9	72	2.8	27,400	15.2

Analyzed by SGS Testing, Korea

MONG matter organic non-glycerol

bottle, raw glycerol was added with or without pretreatment at a concentration of 15~25 g/L. Commercial glycerol (99.0% Junsei Chemical Co.) was also added to other serum bottles for comparison. Table 1 shows the various types of glycerol used in this study and their designations. The media were purged by argon gas to remove dissolved oxygen, and then the serum bottles were sealed with septa and aluminum crimp seals. After autoclaving the serum bottles, each serum bottle was inoculated (5% *v*/*v*) with microorganisms grown overnight in M-CAB media (*Clostridium* strains) and DSMZ media no. 427 containing 20 g/L of glycerol instead of glucose (*Klebsiella* strains), and then incubated at 37°C with shaking (200 rpm). All batch experiments were done in duplicate. Sampling was done at intervals of 24 h until the concentration of 1,3-PD remained constant.

Analytical Methods

The cell concentration was estimated with a spectrophotometer (UV-1700, SHIMADZU) by measuring the optical density (OD) at 600 nm and the glycerol concentration was analyzed with Free Glycerol Reagent (Sigma, USA). The concentration of 1,3-PD was measured by gas chromatography (Shimadzu GC-2010, FID, Agilent HP-INNOWAX column [30 m× 0.32 mm×0.25 μm]) under the following conditions: oven temperature from 50–240°C, at the rate of 30°C/min; injector temperature of 240°C; detector temperature of 250°C; and carrier gas (N_2) flow rate of 30 mL/min.

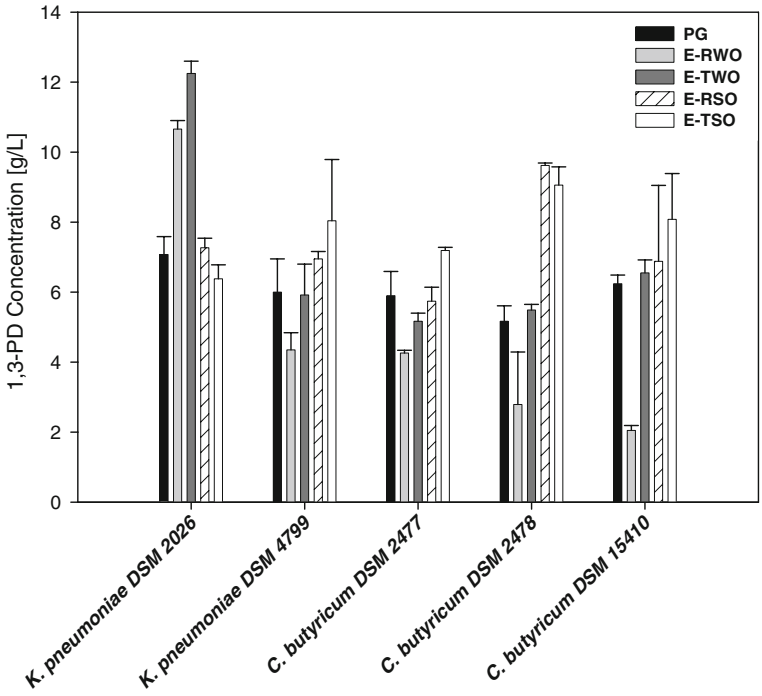

Fig. 1 1,3-PD production of various bacterial strains using glycerol obtained from manufacturer E. *Error bars* indicate the standard deviations of duplicates. *RSO* raw glycerol from soybean-based biodiesel production, *TSO* pretreatment of RSO, *RWO* raw glycerol from waste vegetable-oil-based biodiesel production, *TWO* pretreatment of RWO

Results and Discussion

Pretreatment of Raw Glycerol

Our first trial to remove the soap and MONG (matter organic non-glycerol) was to use hexane for extraction, which failed because hexane and raw glycerol turned into a gel when mixed. Therefore, raw glycerol was treated with 5 N HCl to remove the soap and free fatty acid components [14]. Table 2 shows the composition of the various types of raw and pretreated glycerol.

Effect of Various Types of Raw and Pretreated Glycerol on 1,3-PD Production

Figure 1 shows 1,3-PD production of five bacterial strains using glycerol obtained from manufacturer E. Production of 1,3-PD of all strains used in this study was not inhibited by raw glycerol from soybean-based biodiesel production (E-RSO). This result was unexpected because *C. butyricum* DSM 5431, one of the 1,3-PD-producing *Clostridium* strains, was reported not to grow in the presence of raw glycerol at all. However, Asad-ur-Rehman et al. reported an efficient pretreatment process to use raw glycerol for *C. butyricum* DSM 5431 [1]. Our results demonstrate that *C. butyricum* strains can grow and produce 1,3-PD on raw glycerol E-RSO from manufacturer E without any pretreatment.

In contrast to the results with E-RSO, *K. pneumoniae* DSM 4799 and all *Clostridium* strains were inhibited by raw glycerol E-RWO derived from waste vegetable-oil-based biodiesel

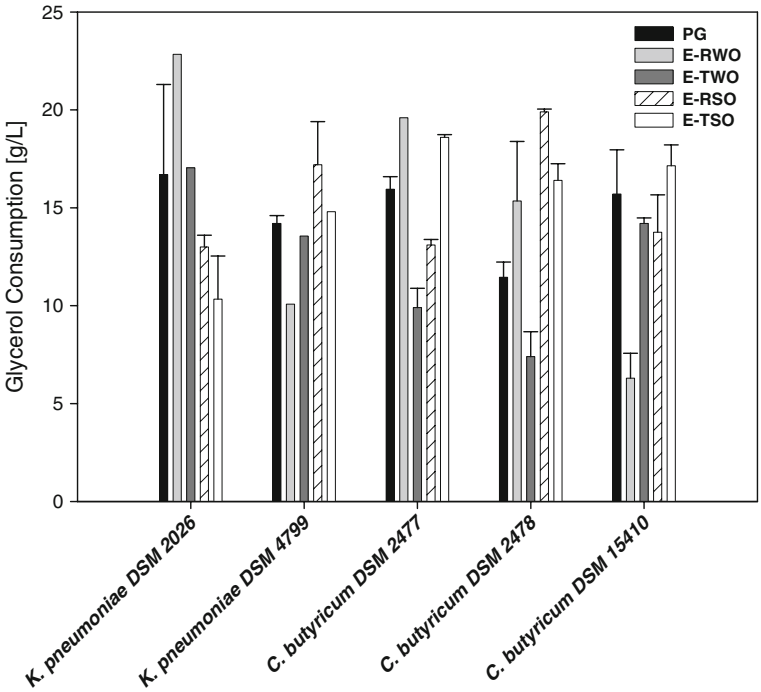

Fig. 2 Glycerol consumption of various bacterial strains using glycerol obtained from manufacturer E. *Error bars* indicate the standard deviations of duplicates

production (Fig. 1). Among them, *C. butyricum* DSM 15410 produced the lowest amount of 1,3-PD (2.1 g/L) and showed the lowest consumption of glycerol (6.3 g/L; Fig. 2) and a productivity of 0.04 g/L·h using E-RWO (Fig. 1). When pretreated E-RWO (i.e., E-TWO) was used for the *Clostridium* strains, the concentration of 1,3-PD and productivity were improved to the range of 5.2~6.6 g/L and 0.12~0.15 g/L·h, respectively, which were similar values obtained with pure glycerol (Fig. 1). Asad-ur-Rehman et al. [19] also reported similar 1,3-PD production using pretreated raw glycerol compared to that with pure glycerol by *C. butyricum* DSM 5431. Unlike other strains, the production of 1,3-PD by *K. pneumoniae* DSM 2026 was not inhibited by E-RWO and even increased compared to that with pure glycerol.

The effect of raw glycerol obtained from manufacturers S and N (i.e., S-RWO and N-RSO) on 1,3-PD production was more dramatic compared to that obtained from manufacturer E (Fig. 3). *C. butyricum* DSM 2477 produced only 1.9 and 2.9 g/L of 1,3-PD with productivities of 0.04 and 0.06 g/L·h using S-RWO and N-RSO, respectively. Those 1,3-PD concentrations correspond to 24% and 37% of 1,3-PD production using pure glycerol. The glycerol consumption was also only about 11 g/L, which corresponds to 55% of the pure glycerol consumption by *C. butyricum* DSM 2477 (Fig. 4). However, when pretreated S-RWO and N-RSO (i.e., S-TWO and N-TSO) were used, the 1,3-PD production (5.8 and 6.2 g/L) and productivity (0.12 and 0.13 g/L·h) of *C. butyricum* DSM 2477 were improved up to 75–80% of that with pure glycerol. The production of 1,3-PD by *C. butyricum* DSM 2478 was not inhibited when raw glycerol N-RSO was used. However, raw glycerol S-RWO was not consumed at all, so there was consequently no 1,3-PD production. The glycerol consumption of *C. butyricum* DSM 2478 was only 0.9 g/L with S-

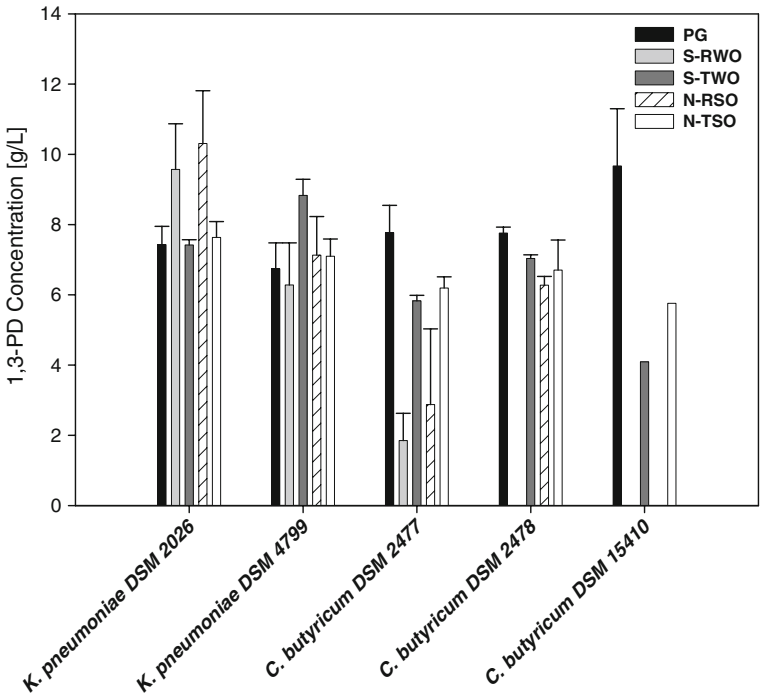

Fig. 3 1,3-PD production of various bacterial strains using glycerol obtained from manufacturers S and N. *Error bars* indicate the standard deviations of duplicates

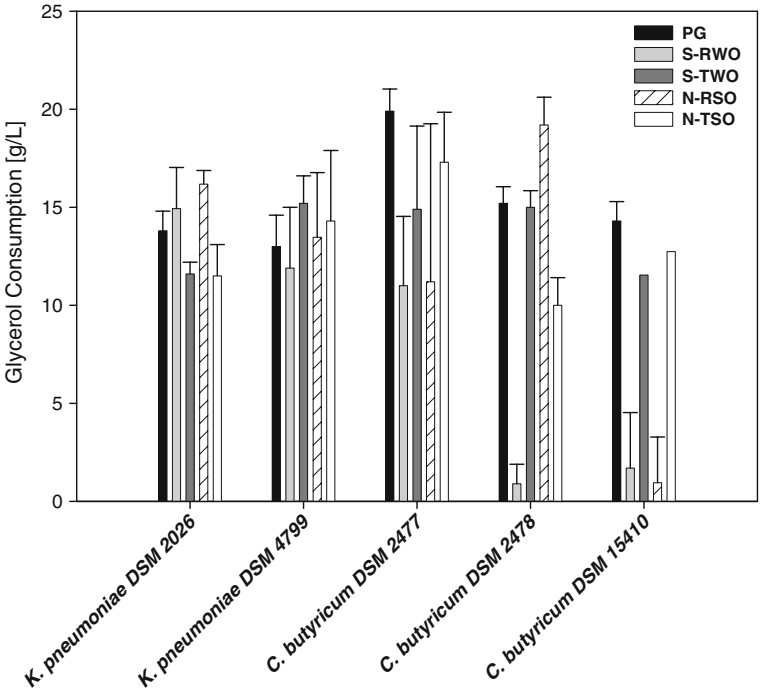

Fig. 4 Glycerol consumption of various bacterial strains using glycerol obtained from manufacturers S and N. *Error bars* indicate the standard deviations of duplicates

RWO (Fig. 4), while, using pretreated S-RWO (i.e., S-TWO), 1,3-PD production (7.0 g/L) and productivity (0.15 g/L·h) were significantly improved up to 90% of that with pure glycerol. When N-RSO and S-RWO were used, *C. butyricum* DSM 15410 did not grow and 1,3-PD was not produced at all. The glycerol consumptions of *C. butyricum* DSM 15410 were also only 0.95 and 1.7 g/L with N-RSO and S-RWO, respectively. Even after pretreatment of N-RSO and S-RWO, only 40% and 60% of 1,3-PD (4.1 g/L and 5.8 g/L) were produced, respectively, compared to that obtained with pure glycerol (9.7 g/L). Several components contained in raw glycerol have been suggested to cause growth inhibition. It was recently reported that raw glycerol from biodiesel production contains methyl/ethyl esters, soups, unreacted fatty acids, glycerides, and other natural compounds, such as phenolic antioxidants. The antioxidants may inhibit cell growth [20], and methanol, soap [1], sodium, and heavy metals can inhibit cell division [21]. In this study, when S-RWO containing the highest concentration of methanol among the tested glycerol types was used (Table 2), 1,3-PD production was significantly inhibited for the *Clostridium* strains (Fig. 3). Thus, methanol is likely to be an inhibitor of 1,3-PD production in this study. On the other hand, the production of 1,3-PD by two *Klebsiella* strains was not affected by either N-RSO or S-RWO.

Comparison of 1,3-PD Production Using Raw Glycerol from Different Manufacturers

There was a difference in 1,3-PD production among the biodiesel manufacturers, although the same type of oil was used in biodiesel production (E-RSO and N-RSO in Figs. 1 and 3,

respectively). While all strains used in this study showed no inhibition when E-RSO was used as the carbon source (Fig. 1), *C. butyricum* DSM 2477 produced only 2.9 g/L of 1,3-PD when N-RSO was used (Fig. 3), which is 40% of the 1,3-PD production obtained with pure glycerol. The production of 1,3-PD was improved to 80% of that obtained with pure glycerol after using the pretreated N-RSO (i.e., N-TSO) with *C. butyricum* DSM 2477. *C. butyricum* DSM 15410 produced no 1,3-PD when N-RSO was used without pretreatment (Fig. 3), which is a completely different result from that using E-RSO (6.9 g/L of 1,3-PD) (Fig. 1).

The production of 1,3-PD with raw glycerol derived from the waste vegetable-oil-based biodiesel process was also different between the manufacturers. The *Clostridium* strains produced 0–1.9 g/L of 1,3-PD using S-RWO, while 2.1-4.3 g/L were produced with E-RWO. For *C. butyricum* DSM 15410, the pretreatment of S-RWO (i.e., S-TWO) improved the production of 1,3-PD only to 50% compared to that with pure glycerol (Fig. 3). However, 1,3-PD production with pretreated E-RWO (i.e., E-TWO) was 105% of that with pure glycerol (Fig. 1). Overall, the glycerol obtained from manufacturer E exhibited less inhibition than that from manufacturers N and S.

In conclusion, *K. pneumoniae* DSM 2026 and *K. pneumoniae* DSM 4799 were more resistant to raw glycerol than the *C. butyricum* strains tested in this study. However, the 1,3-PD production of the *C. butyricum* strains could be improved by using acid-pretreated raw glycerol. The inhibitory effect of raw glycerol from soybean-oil-based biodiesel production was generally less than that of raw glycerol from waste vegetable-oil-based biodiesel production probably due to the presence of different concentrations of various impurities. There was a difference in 1,3-PD production among the glycerol types obtained from various manufacturers, even though the same oil type was used in the biodiesel process. Based on our results, the raw glycerol from manufacturer E showed the least inhibition on 1,3-PD production among the three manufacturers, suggesting that raw glycerol can be effectively used for 1,3-PD production by various microorganisms.

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