Screening for Natural Producers Capable of Producing 1,3-Propanediol from Glycerol

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Abstract

Glycerol is a renewable resource found as the main by-product in the transesterification of triglycerides and fat saponification. Due to the increased production of plant oils, especially palm oil in developing countries, and their larger use by the oleochemical industry, glycerol surpluses are on the world market and this may result in a decrease in glycerol price. As a consequence, biotechnological processes have been developed to convert this substrate into value-added products, such as 1,3-propanediol (1,3-PD). The microbial production of 1,3-PD could be competitive to chemical routes assuming that it is based on cheap raw material and an optimised process. In the screening for 1,3 PD–producing bacteria, raw glycerol as by-product from rapeseed oil processing unit was used as a carbon source compared with commercial glycerol. By using increasing concentration of both glycerols from 50 to 150 g/l, two potential bacteria were obtained from soil samples. BMP-1 was obtained from an enrichment culture using 50 g/l commercial glycerol, while BMR-1 was obtained from an enrichment culture using 100 g/l raw glycerol. The highest conversion yield obtained using the isolate BMP-1 was around 0.62 g 1,3-PD formed per mol glycerol consumed, and 0.73 mol 1,3-PD formed per mol glycerol using the isolate BMR-1. No bacteria were obtained from cultures using 150 g/l commercial and raw glycerol, respectively, which indicated that higher concentration of glycerol has inhibition effect.

Keywords: 1,3-propanediol, enrichment culture, glycerol, palm oil, screening

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Introduction

Due to the increased production of plant oils, especially palm oil, in developing countries, and their larger use by the oleochemical industry, glycerol surpluses are on the world market and this may result in a decrease in glycerol price. As the glycerol supplies increase and the cost decreases, the oleochemical unit operation will need to find ways to move this unavoidable coproduct to market. An alternative way to new use applications for glycerol is conversion to 1,3-propanediol (1,3-PD). 1,3-PD can be produced by either chemical synthesis or microbial fermentation. Current commercial routes to produce 1,3-PD are chemical synthetical methods from acrolein or ethylene oxide (Besson *et al.,* 2003; Knifton *et al.,* 2004). The microbial production of 1,3-PD was proved competitive to chemical routes assumed that it is based on cheap raw material and an optimised strategy. The production of 1,3-PD from glycerol is generally performed under anaerobic conditions in the absence of other exogenous reducing equivalent acceptors (Laffend *et al.,* 1997; Charles & Gregory, 2003). The microbial production of 1,3-PD from glycerol by different mesophilic bacterial strains like *Klebsiella pneumoniae, Clostridium butyricum,* and *Citrobacter freundii* has been studied over the past ten years (Deckwer, 1995; Biebl *et al.,* 1999; Wittlich *et al.,* 1999; Papanikolaou *et al.,* 2000; Wittlich *et al.,* 2001).

Over the past few decades, there has been growing interest in 1,3-PD as an industrial chemical. 1,3-PD can be formulated into a variety of industrial products including composites, adhesives, laminates, coatings,

solvents, antifreeze and other end uses (Zeng & Biebl, 2002). 1,3-PD is also known as a key raw material required for the synthesis of polytrimethylene terephthalate (PTT) and other polyester fibers (Jian *et al.,* 2008). The belief that improved chemistries, including both traditional petrochemical and biological routes, could enable the production of 1,3-PD with the economy required by these competitive markets has fueled large efforts in this arena (Tullo, 2002). As aspects of the natural process from glycerol have been reviewed recently, the subject of this work is screening the natural producers that able to utilize glycerol to produce 1,3-PD.

Here, we presents our recent work in the screening for natural producers that are able to produce 1,3-PD from glycerol. The isolates obtained in this work will be used in the production of 1,3-PD using raw glycerol from palm oil-based oleochemical unit operation.

Materials And Methods

Growth medium. The growth medium used in the screening process was M9-Medium containing (per liter): 9.09 g KH_2PO_4; 0.535 g NH_4Cl; 0.123 g $MgSO_4.7H_2O$; 0.017 g $CaSO_4.2H_2O$; 0.01 g $FeSO_4.7H_2O$; 2.0 g yeast extract; 1.0 ml resazurine 0.1%; 0.25 g L-cysteine HCl, and 10 ml of trace elements solution DSMZ 144. The pH-value of the medium was adjusted to pH 7.0 by the addition of 5 M NaOH. The carbon sources used were commercially available glycerol (Merck, purity 87% w/v) and raw glycerol (Glyctec GmbH, Schwarzheide, Germany) which was the main by-product of a rape-seed oil-based biodiesel production (purity 88% w/v) and consisted of glycerol, water, KH_2PO_4, H_3PO_4, methylester and others.

Enrichment culture. Soil samples were collected from two different locations at palm oil storage area in Belawan Harbour, Medan, Indonesia. For the enrichment cultures M9-Medium was used. Commercial pure and raw glycerol was added to the medium to favor the growth of glycerol utilizing microorganisms. About 1 g of soil sample was inoculated to a 50 ml vial containing 30 ml enrichment medium with increasing concentration of commercial pure and raw glycerol 50, 100 and 150 g/l. Cultures were incubated anaerobically at 37°C. After 5 days of incubation, 1.5 ml of the culture broth was transferred to another vial with the same enrichment medium. The enrichment was done in three rounds.

Isolation and Screening for 1,3-PD producers. Isolation of 1,3-PD producers was conducted anaerobically. After 3 rounds of enrichment cultures, 500 µl of the last enrichment culture were heat-shocked at 60°C for 30 min. 100 µl of diluted culture broth were streaked out on agar plate with M9-Medium containing 25 g/l of pure glycerol, and incubated anaerobically at 37°C. Anaerobic condition was obtained by placing Microbiologia Anaerocult® A in anaerobic jar, with Microbiologia Anaerotest® as indicator. Visual evaluation of the growing colonies was used for the selection of bacterial strain for further evaluation of glycerol utilization and 1,3-PD production.

Evaluation of glycerol utilization and 1,3-PD production. Each purified isolate was inoculated into 10 ml of M9-Medium containing 50 g/l glycerol, and incubated at 37°C for 5 days. One ml of the culture were reinoculated into a fresh M9-Medium with 50 g/l glycerol, and incubated at the same temperature for 5 days. After centrifugation, the supernatant was diluted with 5 mM sulfuric acid. The concentrations of glycerol and fermentation products were determined by using a HPLC system equipped with a refractive index detector and a cation type Aminex HPX87H column (Biorad, Munich, Germany). Analysis was carried out at 60ºC column temperature, with 5 mM sulfuric acid as eluent at a flow rate of 1 ml/min.

Identification of the isolate by 16S rDNA sequencing. Genomic DNA of the isolate was extracted by using Genomic DNA Mini Kit (Geneaid Biotech). A pair of primers specific towards bacterial 16S ribosomal DNA was used to amplify the 16S rDNA by means of PCR. Purification of PCR product was carried out using PCR Purification Kit (Qiagen). Purified PCR product was sequenced with *Taq* Dyedeoxy terminator cycle sequencing kits (Applied Biosystems) as directed in the manufacturer´s protocol. The Multiple Sequence Alignment Program ClustalX was employed to align the 16S rDNA sequence obtained in this experiment against the 16S

rDNA sequences of representatives of the main bacterial lineages available from NCBI GenBank.

Results and Discussion

The enrichment cultures were performed to favor the growth of glycerol fermenting bacteria. After three rounds of enrichment cultures and growing anaerobically on agar plates, ten different colonies were isolated and further purified on RCM and agar plates containing M9-Medium and 50 g/l glycerol. There was no colony that was able to grow on the solid media inoculated with the enrichment cultures containing commercial or raw glycerol at the concentration of 150 g/l as the carbon source. This indicated that higher concentration of glycerol showed inhibition effect on the bacterial growth. This phenomenon was consistent with previous report for glycerol fermentation, which may be explained by the fact that glycerol dehydratase, a key enzyme for conversion of glycerol to 1,3-PD, could undergo rapid suicidal inactivation by glycerol during catalysis (Zhang *et al.,* 2007). Thus, it was infeasible to only enhance initial glycerol concentration for the maximum 1,3-PD accumulation.

These ten isolates were further tested for their ability to produce 1,3-PD (Table 1). BMR isolates were obtained from enrichment cultures that used raw glycerol as carbon source, while BMP isolates were obtained from enrichment cultures using commercial glycerol (Figure 1). The isolates BMP-1, BMP-2, BMR-1 and BMR-2 were the most active microorganisms with regard to formation of 1,3-PD and the molar yields of 1,3-PD to glycerol. They are strictly anaerobic bacteria. The isolates BMP-1 and BMP-2 are Gram-positive, butyrate forming bacteria, and hence probably belong to the genus *Clostridium*. Some clostridial strains are described as butyrate producers, e.g. *C. thermobutyricum* (Wiegel *et al.,* 1989), *C. thermopapyrolyticum* or *C. thermosaccharolyticum* (Canganella & Wiegel, 1993) and *C. butyricum* (Biebl *et al.,* 1999). The highest molar yield obtained using isolate BMP-1 was around 0.62 mol 1,3-PD formed per mol glycerol consumed, which was comparable to the molar yields obtained by *C. butyricum* E5 (Petitdemange *et al.,* 1995), *C. butyricum* VPI 3266 (Saint-Amans *et al.,* 2001), and *C. butyricum* F2b (Papanikolau *et al.,* 2000).

The isolates BMR-1 and BMR-2, on the other hand, are Gram-negative bacteria and showed the best molar yields in the range of 0.70 to 0.73 mol 1,3-PD/mol glycerol. Of the known natural producers of 1,3-PD reported so far, only *Klebsiella pneumonia* reached under anaerobic conditions a molar yield of 0.72 mol 1,3-PD/mol glycerol (Chen *et al.,* 2003). Since *K. pneumoniae* is Gram-negative bacteria, it was suspected that the isolates BMR-1 and BMR-2 could also belong to *Klebsiella* strains. The utilization of these isolates should be verified further, since all strains of *Klebsiella* are classified as opportunistic pathogens. Special safety precautions are required to grow them, therefore fermentation at large scale using the strains is more difficult to be carried out (Biebl *et al.,* 1999). Both strains are able to grow in the M9 medium containing 80 g raw glycerol with a glycerol content of 87% (data not shown).

In order to test the consistency in glycerol utilization and 1,3-PD production, the isolates BMP-1 and BMR-1 were cultured in M9-Medium with the initial glycerol concentration of 50 g/l, and samples were taken after 5 and 10 days. Both isolates showed an increase in 1,3 PD production and glycerol utilization with the incubation time (Table 2), with the molar yield of BMP-1 similar to those for other strains of *C. butyricum*, and the molar yield of BMR-1 similar to those for other strains of *K. penumoniae*.

According to the 16S rDNA sequence alignments, the isolate BMP-1 shows as high as 99% sequence identity, especially with *C. butyricum* NCIMB 8082, DSM 2478, ATCC 43755, VPI 3266 dan DSM 523. The strains *C. butyricum* DSM 2478, VPI 3266 and DSM 523 belonged to the same group of glycerol fermenting *C. butyricum* strains (Biebl dan Spröer, 2002). The other group of glycerol fermenting *C. butyricum* strains, including *C. butyricum* DSM 5430, DSM 5431 and E5 was later assigned to a new species, *Clostridium diolis* (Biebl & Spröer, 2002), which is clearly separated from the first group as shown in the phylogenetic dendrogram (Figure 2). On the other hand, the isolate BMR-1 was identified by 16S rDNA sequencing as *Klebsiella pneumoniae* (data not shown)

In the present work, two bacterial strains have been isolated, which showed high potential in the bioconversion of glycerol to 1,3-PD. Especially the Gram-positive isolate BMP-1, which produced 1,3-PD and butyrate, and showed molar yield similar to those of other clostridial strains, is a potential candidate to be used in the fermentation of raw glycerol from palm oil-based biodiesel production to 1,3-PD. Based on its 16S rDNA sequence, the isolate BMP-1 was identified as *Clostridium butyricum*.

Table 1. Paterns of fermentation products obtained by the ten isolates (initial glycerol concentration: 50 g/l, temperature: 37°C)

Isolates	1,3-PD (g)	Glycerol used (g)	Yield 1,3-PD (g/g)	Yield 1,3-PD (mol/mol)	*n*-Butyrate (g)	Lactate (g)	Acetate (g)	Ethanol (g)	pH
BMP-1	12.23	23.71	0.52	0.62	2.01	0.00	1.56	1.39	5.34
BMP-2	12.95	26.42	0.49	0.59	2.52	0.63	1.48	1.33	5.23
BMP-3	1.41	3.60	0.39	0.47	0.00	0.15	0.94	1.40	6.97
BMP-4	1.54	3.83	0.40	0.49	0.00	0.16	0.94	1.29	6.94
BMP-5	1.82	4.48	0.41	0.49	0.00	0.20	0.96	1.32	6.88
BMP-6	1.60	3.60	0.45	0.54	0.00	0.19	0.95	1.34	6.94
BMR-1	23.43	38.46	0.60	0.73	0.00	1.83	0.84	1.94	5.69
BMR-2	22.55	38.79	0.58	0.70	0.00	1.66	0.83	2.05	5.71
BMR-3	6.61	12.27	0.54	0.61	0.00	3.78	1.17	1.33	5.68
BMR-4	5.2	10.38	0.50	0.61	0.00	2.31	1.20	1.33	6.22

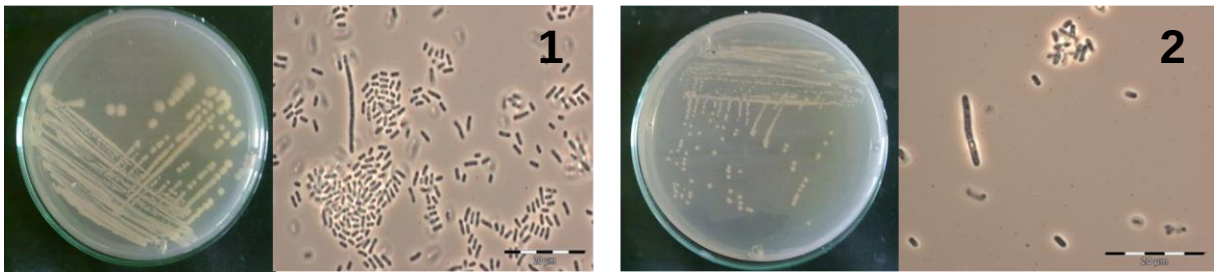

Figure 1. Two potential isolates capable of producing 1.3 PD from glycerol: (1) BMR-1 and (2) BMP-1

Table 2. Consistency of glycerol utilization and 1,3-PD production by BMP-1 and BMR-1 (Initial glycerol concentration 50 g/l, temperature 37°C)

Culture	BMP-1			BMR-1		
	1,3 PD (g/l)	Glycerol used (g/l)	Molar yield (mol/mol)	1,3 PD (g/l)	Glycerol used (g/l)	Molar yield (mol/mol)
5 days	12.23	23.71	0.62	23.43	38.46	0.73
10 days	13.45	26.95	0.61	24.93	38.67	0.78

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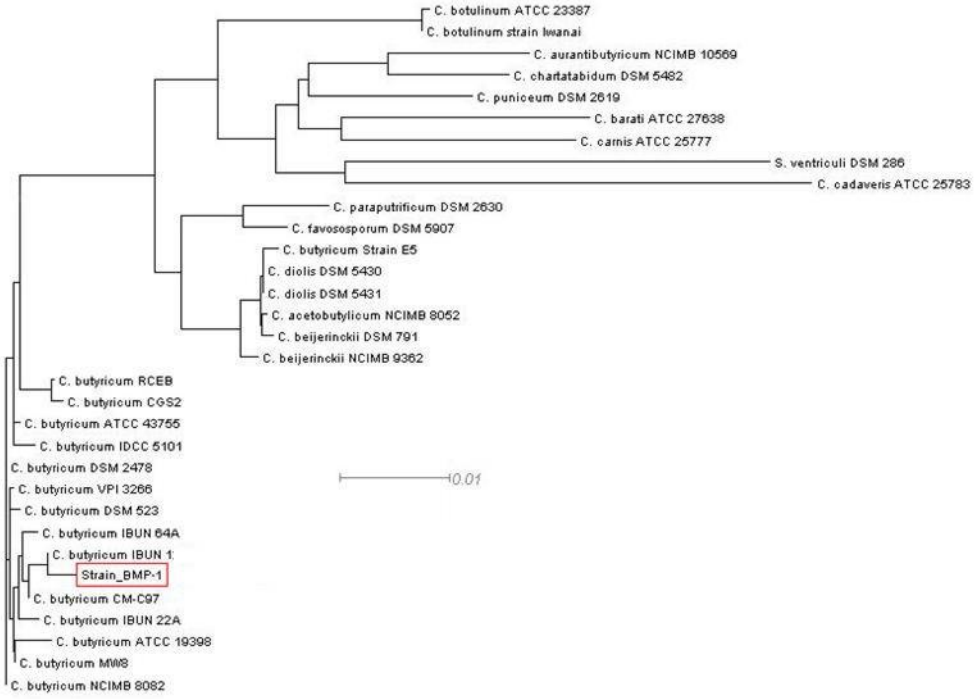

Figure 2. 16S rDNA based phylogenetic dendrogram showing the position of the glycerol fermenting and 1,3-PD producing strain BMP-1 among related clostridia

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