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Research Article

High tolerance to glycerol and high production of 1,3-propanediol in batch fermentations by microbial consortium from marine sludge

1,3-Propanediol (1,3-PD) is a versatile bulk chemical and widely used as a monomer to synthesis polymers, such as polyesters, polyethers and polyurethanes. 1,3-PD can be produced by microbial fermentation with the advantages of the environmental protection and sustainable development. Low substrate tolerance and wide by-product profile limit microbial production of 1,3-PD by *Klebsiella pneumonia* on industrial scale. In this study, microbial consortia were investigated to overcome some disadvantages of pure fermentation by single strain. Microbial consortium named DL38 from marine sludge gave the best performance. Its bacterial community composition was analyzed by 16S rRNA gene amplicon high-throughput sequencing and showed that *Enterobacteriaceae* was the most abundant family. Compared with three *K. pneumonia* strains isolated from DL38, the microbial consortium could grow well at an initial glycerol concentration of 200 g/L to produce 81.40 g/L of 1,3-PD with a yield of 0.63 mol/mol. This initial glycerol concentration is twice the highest concentration by single isolated strain and more than the critical value (188 g/L) extrapolated from the fermentation kinetics for *K. pneumonia*. On the other hand, a small amount of by-products were produced in batch fermentation of microbial consortium DL38, especially no 2,3-butanediol detected. The mixed culture of strain W3, Y5 and Y1 improved the tolerance to glycerol and changed the metabolite profile of single strain W3. The batch fermentation with the natural proportion (W3: Y5: Y1 = 208: 82: 17) was superior to that with other proportions and single strain. This study showed that microbial consortium DL38 possessed excellent substrate tolerance, narrow by-product profile and attractive potential for industrial production of 1,3-PD.

Keywords: 1,3-Propanediol / *Klebsiella pneumonia* / Metabolite profile/ Microbial consortium/ Substrate stress

Received: October 10, 2016; *revised:* December 10, 2016; *accepted:* December 22, 2016

DOI: 10.1002/elsc.201600215

1 Introduction

1,3-Propanediol (1,3-PD) is a versatile bulk chemical with numerous possible applications in cosmetics, foods, lubricants, adhesives, antifreezes and medicines [1]. 1,3-PD is widely used as a monomer to synthesis polymers, such as polyesters, polyethers and polyurethanes, which are mostly used for plastics, carpets,

textile fibres and films. Due to their unique properties, such as good resilience, inherent stain resistance, low static generation and biodegradation, the demand for 1,3-PD production has dramatically increased in recent years [2].

1,3-PD can be produced by either chemical synthesis or microbial fermentation. So far, 1,3-PD is mainly manufactured by chemical synthesis, which requires expensive catalysts, high temperature, high pressure and produces toxic intermediates [3, 4]. Considering the environmental protection and sustainable development of 1,3-PD, much attention has been paid to its microbial production, either based on glycerol or glucose [5–8]. In the last years, due to the tremendous expansion of biodiesel industries, there has been an excessively abundant supply of glycerol into the market [9, 10]. Conversion, therefore, of this material

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Abbreviations: 1,3-PD, 1,3-propanediol; 2,3-BD, 2,3-butanediol

to higher added-value compounds via fermentation currently attracts high and continuously increasing interest. Various microbial added-value metabolites (e.g. polyols, citric acid, lipid, ethanol, etc.) have been produced through utilization of this waste stream as microbial substrate [9–12]. Microbial conversion of glycerol to 1,3-PD, therefore, was paid more and more attention.

Up to date, most research work have focused on the fermentation of 1,3-PD from glycerol using single strain, e.g. *Klebsiella pneumoniae* or *Clostridium butyricum* [13–20]. In pure cultures of single strains, many by-products would be generated, such as alcohols and organic acids, leading to low conversion rate of substrate and much difficulty in the separation and purification of 1,3-PD [10, 21]. For example, 2,3-butandiol (2,3-BD) as a by-product is similar to the target product, 1,3-PD, in the fermentation broth by *K. pneumoniae*. The separation of 1,3-PD from 2,3-BD is difficult due to their similar properties, e.g. strong hydrophilicity and high boiling point. On the other hand, the tolerance of cells to substrate and product with high concentrations also limits to obtain high concentration of 1,3-PD. For example, the critical concentration of glycerol is 2039 mM (188 g/L) for *K. pneumoniae*, at which the cells would stop growing [22].

Microbial consortia are considered to be able to overcome some disadvantages in pure culture by single strain. For example, microbial consortia have wide substrate and special product spectra as well as no aseptic requirements [23, 24]. Microbial consortia exhibit strong superiority in the environmental remediation and energy production, such as wastewater treatment and biogas production, etc. [25, 26]. As the research on microbiomes would be consolidated [27, 28], industrial application of microbial consortia would undoubtedly increase. Some researchers already found the potentials of microbial consortia for production of bulk chemicals, especially the possibility of converting glycerol to 1,3-PD [24, 29–32]. However, the initial glycerol concentration used in batch fermentations was relatively low, resulting in lower concentration of 1,3-PD than pure cultures [26]. Additionally, to date, there are no reports on production of 1,3-PD by microbial consortia from marine sources.

In this study, microbial consortia producing 1,3-PD were screened from marine sludge. Their tolerance to glycerol was investigated by comparing with single strains involved in the microbial consortium. The metabolites was also analyzed and compared with pure fermentations. The experimental results revealed some advantages of microbial consortium over single strain, especially in substrate stress and by-product profile.

2 Materials and methods

2.1 Medium

Enrichment medium used for bacterial enrichment contained the following components: yeast extract 1.0 g/L, peptone 5.0 g/L, glycerol 10 g/L, NaCl 5.0 g/L, $K_2HPO_4 \cdot 7H_2O$ 5.8 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L, $CaCO_3$ 2.0 g/L.

Seed medium used for seed culture contained the following components: glycerol 20 g/L, $K_2HPO_4 \cdot 7H_2O$ 4.454 g/L,

KH_2PO_4 1.3 g/L, $(NH_4)_2SO_4$ 2.0 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L, $CaCO_3$ 2.0 g/L, yeast extract 1.0 g/L, 1 mL/L Fe^{2+} solution (HCl 4 mL/L, $FeSO_4 \cdot 7H_2O$ 5 g/L), 1 mL/L $CaCl_2$ solution (20 g/L), 2.0 mL/L trace element A solution (HCl 0.9 mL/L, $CuCl_2 \cdot 2H_2O$ 20 mg/L, $ZnCl_2$ 70 mg/L, $MnCl_2 \cdot 4H_2O$ 100 mg/L, H_3BO_3 60 mg/L, $CaCl_2 \cdot 6H_2O$ 200 mg/L, $NiCl_2 \cdot 6H_2O$ 25 mg/L, $NaMoO_4 \cdot 2H_2O$ 35 mg/L).

Fermentation medium used for 1,3-PD production contained the following components: KH_2PO_4 1.36 g/L, $(NH_4)_2SO_4$ 6.61 g/L, $MgCl_2 \cdot 6H_2O$ 0.26 g/L, $CaCl_2 \cdot 6H_2O$ 0.29 g/L, citric acid 0.42 g/L, yeast extract 2.0 g/L, 5 mL/L trace element B solution ($CuCl_2 \cdot 2H_2O$ 0.47 g/L, $ZnCl_2$ 0.68 g/L, $MnCl_2 \cdot 4H_2O$ 0.17 g/L, H_3BO_3 60 mg/L, $NaMoO_4 \cdot 2H_2O$ 5 mg/L, $FeCl_2 \cdot 4H_2O$ 3.97 g/L, $CoCl_2 \cdot 6H_2O$ 0.47 g/L, HCl 10 mL/L). Glycerol was used as sole carbon source. Analytical grade glycerol (99.0 %) used throughout experiments was obtained from Tianjin Guangfu Technology Development Co. Ltd, China.

2.2 Enrichment and isolation of microorganisms

Forty different samples were obtained from Dalian seashore, including barnacles, seaweeds, sponges, marine sludge. All the samples were numbered DL 01–40, respectively, and stored at $-20^\circ C$ for further studies. The samples of barnacles, seaweeds and sponges were grinded by mortar under aseptic condition with 1.5 % NaCl solution. The marine sludge was used without any pretreatment. About 2 g of sample was inoculated to a 250 mL flask sealed with cotton cap and kraft paper containing 50 mL enrichment medium and then incubated at $37^\circ C$ and 200 rpm. After 24 h incubation, 1 mL of the culture broth was transferred to another flask with the same enrichment medium and incubated 24 h. The same process was repeated for the third time and the enrichment broth obtained was diluted by 10^5 folds. 0.05 mL of the diluted broth was plated on agar plates at $37^\circ C$ for 24 h. Medium used for strains separation was the same as seed medium, and only 15 g/L of agar was added. Visual evaluation of colonies was used to select bacterial strains for further identification and characterization. The proportion of each single strain in selected microbial consortium was calculated by colony counting method.

2.3 Batch fermentations

The samples and screened strains were first cultured in shake flasks to identify whether 1,3-PD was produced in the broth. 1 mL of the enrichment culture broth was inoculated to a 250 mL flask sealed with cotton cap and kraft paper containing 100 mL seed medium and incubated at $37^\circ C$, 200 rpm. The seed broth was obtained after 12 h. Then, 1 mL of the seed broth was inoculated to a 250 mL flask sealed with cotton cap and kraft paper containing 100 mL fermentation medium then incubated at $37^\circ C$ and 200 rpm for 24 h. The initial pH values of the media were adjusted to 7.0. The fermentation medium was used with 40 g/L of the initial glycerol.

The seed cells were grown in a 250 mL shake flask sealed with cotton cap and kraft paper containing 100 mL seed medium at $37^\circ C$ for 12 h. 10 % (v/v) of seed broth was inoculated into a

5 L stirred tank bioreactor (Biotech-5BG, China) with a working volume of 2 L fermentation medium. The pH was controlled at 7.0 by automatic addition of 5 M sodium hydroxide, the temperature at 37°C and the agitation rate at 250 rpm. An anaerobic environment in the bioreactor was maintained by sparging nitrogen at 0.1 vvm during fermentation.

2.4 Composition analysis of fermentation broths

The biomass concentration was measured as optical density (OD) at 650 nm. Samples were withdrawn from the bioreactor and centrifuged at 10 000 rpm for 10 min. The supernatant was then filtered with 0.22 μ m filter and diluted before chromatography measurement. The concentrations of product and by-products were analyzed with HPLC (Agilent Instruments, USA) with a flow rate of 0.6 mL/min and a 5 mM sulfuric acid as eluent. An Aminex 300 mm $\times$ 7.8 mm HPX-87H column (Bio-Rad Laboratories, USA) was used for the separation with a column temperature of 65 °C. The column was coupled to a differential refractometer CTO-10vp detector (Agilent Instruments, USA). The mobile phase was 0.005 mol/L H₂SO₄. Glycerol was assayed by a modified titration [33]. All data presented are the average of two independent experiments performed under the same culture conditions.

2.5 Composition analysis of microbial consortium

The bacterial community composition of microbial consortium DL38 was studied by 16S rRNA gene amplicon high-throughput sequencing. Total DNA of selected microbial consortium was extracted by miniBEST bacterial genomic DNA extraction kit (Takara, Ver 3.0) and sequenced on Illumina HiSeq 2000 platform by Beijing Genome Institute (BGI) in Shenzhen, China. The sequences were connected into tags based on overlapping relationship by using FLASH v 1.2.11 [34]. Then the tags were assigned to operational taxonomic units (OTUs) by using USEARCH v 7.0.1090 [35]. Greengenes database available on May, 2013 was used for OTU picking. Sequences from the study were assigned to a reference sequence using the UPARSE with 97 % similarity threshold. Chimeric sequences were detected and removed by using the UCHIME v 4.2.40 [36]. Taxa summaries at the all taxonomic level were performed using the RDP classifier trained on the May, 2013 Greengenes. The sequencing raw data of microbial consortium DL38 have been submitted to the sequence read archive database of National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>) (NCBI) and assigned the accession number SRP066989.

16S rRNA genes of isolated strains were sequenced by Takara Bio Corporation in Dalian, China. Standard methods were used for DNA manipulations and cloning. The obtained 16S rRNA gene sequences were first compared to sequences stored in GenBank using the BLAST algorithm from NCBI. The 16S rDNA sequences of isolated strains W3, Y1, Y5 have been submitted to GenBank under accession numbers KR259311, KR259312, KR259313, respectively.

3 Results

3.1 Selection of microbial consortia

Forty different microbial consortia were chosen to determine their ability to convert glycerol to 1,3-PD. Figure 1 showed the 1,3-PD concentrations in the shake flask cultures of eight microbial consortia, which were able to produce relatively high quantities of 1,3-PD. The highest 1,3-PD concentration (16.95 g/L) was obtained by microbial consortium DL38 from marine sludge. It was thus chosen as the microbial consortium for subsequent investigation.

3.2 Taxonomic classifications of microbial consortium DL38 and isolated strains

16S rRNA gene amplicon high-throughput sequencing was performed to investigate the bacterial composition of microbial consortium DL38. A total of 28 865 good quality sequence reads were generated and the read utilization ration was 90.10%. These reads were connected into 28 728 tags based on the overlapping relationship. Clustering of all tags with a 97% pairwise sequence identity resulted in a total of 16 OTUs (operational taxonomic units). All tags with less than 97% similarity with the known organisms in Greengenes database were not considered for further analysis. Representative sequences for each OUT were assigned to different bacterial taxonomic levels by using Greengenes database. Four different bacterial families were identified. The most abundant family type found in microbial consortium DL38 belonged to *Enterobacteriaceae* (95.57 %), followed by *Enterococcaceae* (2.10 %), *Moraxellaceae* (1.21 %) and *Streptococcaceae* (0.64 %).

The screening and isolation of microorganisms from microbial consortium DL38 were performed after incubated in solid seed medium for 24 h, and three different types of colonies were isolated and further purified on solid seed medium plates. A colony named as W3 is smooth, creamy-white, and without bubble. Two colonies with bubbles are different in the size of bubble, large one named Y1 and small one Y5. All three isolated strains (named W3, Y5 and Y1) were identified based on partial sequencing of 16S rRNA genes as *Klebsiella pneumoniae* (100% of confidence for strain W3 and 99% of confidence for strains Y5 and Y1). The isolated strains all belonged to *Enterobacteriaceae*.

3.3 Batch fermentations by microbial consortium DL38 and isolated strains

Batch fermentations by microbial consortium DL38 and isolated strains were performed at an initial glycerol concentration of 40 g/L in order to evaluate their ability to produce 1,3-PD from glycerol. As shown in Table 1, the duration of the fermentation process with isolated strain W3 (5.30 h) was the shortest, following by microbial consortium DL38 (6.00 h), strain Y5 (9.50 h) and strain Y1 (23.00 h). The highest concentration of 1,3-PD (20.96 g/L) was achieved by microbial consortium DL38 with the maximum yield 0.70 mol/mol and productivity

Figure 1. Concentration of 1,3-PD in flask culture with eight microbial consortia.

Table 1. Compositions of product and by-products of batch fermentation with selected microbial consortium and isolated strains at an initial glycerol concentration of 40 g/L

Inoculum	Duration (h)	Concentration (g/L)					Yield (mol/mol)	Productivity (g/L·h)
		1,3-PD	Acetate	Lactate	Ethanol	2,3-BD		
DL38	6.00	20.96	1.95	0.84	1.77	0	0.67	3.49
W3	5.30	17.84	4.04	0.66	0.77	0.30	0.62	3.37
Y5	9.50	9.63	0.72	0.43	7.60	0	0.32	1.01
Y1	23.00	2.05	0	2.93	12.69	0	0.07	0.09

3.49 g/L·h. Only a small amount of by-products, such as acetate (1.95 g/L), lactate (0.84 g/L) and ethanol (1.77 g/L), were secreted in the broth, especially no 2,3-BD. The metabolites produced by isolated strains (W3, Y5, Y1) were significantly different from microbial consortium DL38. The primary product of isolated strain W3 was 1,3-PD (17.84 g/L), however, strain Y5 produced 1,3-PD (9.63 g/L) and ethanol (7.60 g/L), strain Y1 produced ethanol (12.69 g/L) as the main product, respectively. The highest concentration of acetate (4.04 g/L) was produced by strain W3, and a small amount of 2,3-BD (0.30 g/L) was produced, however, which was not detected in the fermentation broths of isolated strains (Y5, Y1) and microbial consortium DL38. Furthermore, strain Y1 did not produce acetate.

3.4 Glycerol tolerance of microbial consortium DL38 in batch fermentations

Table 2 showed the effect of the initial glycerol concentration on 1,3-PD production in batch fermentations by microbial consortium DL38. The main product in all fermentations was 1,3-PD and its concentration gradually increased with the increase of initial glycerol concentration. The maximum concentration of 1,3-PD was 81.40 g/L with yield of 0.63 mol/mol at an initial glycerol concentration of 200 g/L. Correspondingly, the

fermentation time also enhanced as the initial glycerol concentration increased from 40 to 200 g/L. At an initial glycerol concentration of 180 g/L, the substrate can still be completely consumed in 66 h. When the fermentation medium contained 200 g/L of glycerol, glycerol was consumed only to a certain extent about 87 % (w/w) of the initial concentration. From another angle as shown in Fig. 2, the glycerol consumption rate kept almost stable (6.50 g/L·h) up to an initial concentration of 120 g/L of glycerol. When the initial glycerol concentration was above this value, glycerol consumption rate decreased significantly to 2.13 g/L·h at 200 g/L of the initial glycerol concentration.

Taking into consideration that relatively high 1,3-PD concentration (59.74 g/L), yield (0.66 mol/mol glycerol), and productivity (3.20 g/L/h) were obtained in batch fermentation at the initial glycerol concentration of 120 g/L, this glycerol concentration was thus chosen as the optimum concentration for microbial consortium DL38 (Figs. 3 and 4). The main product was 1,3-PD with acetate (10.83 g/L), formate (6.00 g/L), lactate (3.04 g/L), ethanol (5.00 g/L) and succinate (3.05 g/L) as major by-products. It is also worth pointing out that the microbial culture with DL38 generated a small amount of by-products, especially no 2,3-BD.

An interesting phenomenon is that the bacterial composition of the consortium DL38 is not constant, but varied during batch fermentation. For example, the ratio of W3 to Y1+Y5

Table 2. Effect of the initial glycerol concentration on products in batch fermentations by microbial consortium DL38

Initial glycerol (g/L)	Duration (h)	1,3-PD (g/L)	Yield (mol/mol)	Productivity (g/L·h)	Acetate (g/L)	Ethanol (g/L)	Lactate (g/L)	Succinate (g/L)
40	6.00	20.96	0.67	3.49	1.95	1.77	0.84	0.81
60	9.00	26.08	0.61	2.90	7.71	1.94	1.57	1.38
80	12.00	34.21	0.57	2.85	8.85	2.48	3.58	3.75
100	15.00	47.95	0.65	3.19	9.47	4.52	2.46	2.87
120	18.75	59.74	0.66	3.20	10.83	5.00	3.04	3.05
140	35.00	63.12	0.59	1.80	7.29	4.04	4.06	3.03
160	51.29	73.29	0.66	1.43	7.18	5.91	5.71	3.26
180	66.00	77.31	0.64	1.17	7.39	5.62	6.62	3.53
200	82.26	81.40	0.63	0.99	7.64	5.35	7.73	3.99

Figure 2. Glycerol consumption rate in batch fermentations with microbial consortium DL38 at different initial glycerol concentrations.

varied from 1:2 to 8.8:1 and 1:1 at the primary, middle, and final phase in batch fermentation at an initial glycerol concentration of 140 g/L.

3.5 Effect of bacterial composition on batch fermentations

The proportion of the isolated *K. pneumoniae* W3, Y5 and Y1 in the consortium DL38 was about 208: 82: 17. To investigate the effect of bacterial composition, batch fermentations were conducted at initial glycerol concentration of 100 or 120 g/L as shown in Table 3. Comparing with pure fermentation by the strain W3 (the first row in Table 3), the mixed cultures with Y5 or/and Y1 could consume more glycerol (from 76.95 to 116.78 g/L) and produce more 1,3-PD (from 36.25 g/L to 59.74 g/L) within shorter fermentation time (from 47.0 h to 18.75 h). The best result was obtained at a ratio of W3/Y5/Y1 = 208: 82: 17, i.e. the proportion in the consortium DL38. At the same or similar ratio of W3 to Y5 or Y1, e.g. 1:1, 208: 28 or 208: 17, the mixed culture of W3 and Y1 seemed to be better than that of W3 and Y5, although the strain Y1 grew slower and

produced less 1,3-PD than Y5. The mixed culture improved the tolerance to glycerol and changed the metabolite profile. When three strains were cultured together, batch fermentation with the natural proportion (208: 82: 17) of strain W3, Y5 and Y1 was superior to that with the proportion (1: 1: 1). The duration of fermentation (18.75 h) was shorter, the concentration of 1,3-PD (59.74 g/L) was higher and the by-products such as lactate and succinate were less under the natural proportion (W3: Y5: Y1 = 208: 82: 17).

4 Discussion

Mixed culture with microbial consortia is a promising biotechnology for the production of bioenergy and chemicals due to the specific characteristic of no sterilization requirements, no risk of degradation, high substrate tolerance and broad substrate spectrum [25, 26]. Microbial consortia originated from wastewater treatment plants (compost and sludge) [32], potato starch processing acidification tank (sludge) [37], greenhouse (tomato soil) [31], farms (wheat soil and organic soil) [38] and

Figure 3. Time course of glycerol consumption, 1,3-propanediol production and OD for batch fermentation with microbial consortium DL38 at the initial glycerol concentration of 120 g/L.

Figure 4. Time courses of by-products for batch fermentation with microbial consortium DL38 at the initial glycerol concentration of 120 g/L.

different biogas plants (sludge) [24] have been proved to possess the ability of converting glycerol to 1,3-PD.

In this study, for the first time, barnacles, seaweeds, sponges and marine sludge near the wastewater treatment plants were selected as the potential sources for obtaining 1,3-PD producers. Recent researches in our lab have proved that seashore could be considered as a precious source of the natural inoculum. Different from traditional strain screening, samples from seashore were cultured directly in enrichment medium to enrich the 1,3-PD producers. This kind of natural screening method could obtain the 1,3-PD producers as much as possible, i.e. eight microbial consortia from forty samples, and also keep the microbial communities in the samples. The prokaryotic community

composition of microbial consortium DL38 revealed that *Enterobacteriaceae* was the most abundant family. Common 1,3-PD producing bacteria genera in nature include *Klebsilla* [39, 40], *Enterobacter* [41], *Citrobacter* [42], *Clostridium* [17] and *Lactobacillus* [43]. Among them, the genera of *Klebsilla*, *Enterobacter* and *Citrobacter* all belong to *Enterobacteriaceae*. The isolated strains were all identified as *K. pneumoniae* by 16S rDNA sequencing analysis. At the same time, the main product in all the fermentations was 1,3-PD, with acetic acid, lactic acid, ethanol as major by-products, as exemplified in Table 2 for fermentation at an initial glycerol concentration of 40 g/L. The product spectrum of microbial consortium DL38 was similar to that of conventional fermentations with a pure culture of

Table 3. Compositions of product and by-products of batch fermentation with different proportion of isolated strains

The ratio of W3: Y5: Y1	Cs0 (g/L)	Cs (g/L)	Duration (h)	Concentration (g/L)						
				1,3-PD	Acetate	Lactate	Ethanol	Formate	Succinate	2,3-BD
1: 0: 0	100	23.05	47.00	36.25	7.68	5.23	2.35	1.20	1.89	0.45
208: 82: 0	100	16.88	30.59	40.38	7.67	7.48	0	2.86	4.72	0
1: 1: 0	100	1.98	23.00	43.08	6.08	7.04	4.24	3.55	5.28	0
208: 0: 17	100	2.49	24.00	39.67	9.10	5.17	1.89	3.43	5.81	0
1: 0: 1	100	1.86	23.43	40.35	8.25	7.15	2.97	4.48	5.95	0
208: 82: 17	120	3.22	18.75	59.74	10.83	3.04	5.00	5.98	3.05	0
1: 1: 1	120	4.24	32.15	40.58	8.43	13.84	5.13	1.98	6.54	0

a) Cs0, the initial concentration of glycerol.

b) Cs, the residual concentration of glycerol.

K. pneumoniae. Preliminary characterization of the culture showed that the dominating 1,3-PD producer in our selected microbial consortium DL38 is probably *Klebsilla* species like *K. pneumoniae*. It indicated that *K. pneumoniae* was the predominant population in microbial consortium DL38. The good ability of microbial consortium DL38 converting glycerol to 1,3-PD may owe to the abundant 1,3-PD producers existing in the microbial community.

Glycerol is assimilated by several prokaryotic microorganisms under anaerobic condition via two parallel metabolic pathways encoded by the *dha* regulon: through the reductive pathway, glycerol is dehydrated to 3-hydroxypropionaldehyde by the enzyme glycerol dehydratase, which is then reduced to 1,3-PD by the NADH₂-dependent enzyme 1,3-propanediol oxidoreductase. Through the oxidative pathway, glycerol is dehydrogenated to dihydroxyacetone (DHA) by the enzyme glycerol dehydrogenase, which after phosphorylation is converted to DHA-phosphate. Further, via pyruvate it can be converted to various by-products, such as 2,3-BD, acetate, lactate, succinate and ethanol etc., in order to balance reducing equivalents in the fermentation process [44,45]. These by-products also shunts the carbon flux and subsequently decreases 1,3-PD yield. The separation of 1,3-PD plays an important role in its microbial production and the cost of downstream processing can make a very high portion in the total production cost, mounting up to about 50–70%. Although the by-products of the fermentation, such as ethanol or acetic acid can be easily separated from 1,3-PD, it is a challenge to efficiently separate 1,3-PD from 2,3-BD, which is a dominant by-product in *K. pneumoniae* and similar to 1,3-PD in many aspects, such as boiling points and molecular weight [10]. So far, several attempts have been made to decrease or prevent by-products production, especially 2,3-BD by using genetically modified organisms [46,47]. However, results were not desirable because the abolishment of one by-product tended to increase other by-products and further inhibited cell growth and 1,3-PD production. For example, Cui et al. [46] engineered a 2,3-BD-deficient *K. pneumoniae* mutants by knockout of the 2,3-BD pathway and found that the metabolic flux tended to increase lactate production, which inhibited cell growth and 1,3-PD production. In this study, we interestingly found that in batch fermentations with microbial consortium DL38, high quantities of 1,3-PD and less by-products were produced, especially no 2,3-BD (Figs. 3 and 4). Thus, 1,3-PD production with

microbial consortium DL38 was a beneficial biotechnology for industrial production, due to the high yield of substrate and the low concentration of by-products, which reducing the cost of purification products.

In fermentation broth of strain W3, a small amount of 2,3-BD (0.30 g/L) was detected, but no 2,3-BD was found in the broths of microbial consortium DL38, strain Y5 and Y1. The production of 2,3-BD is probably related to the concentration of acetate. As shown in Table 1, strain W3 produced the highest concentration of acetate (4.04 g/L); however, microbial consortium DL38 and strain Y5 produced less acetate 1.95 and 0.72 g/L, respectively. The strain Y1 had no acetate formation, but produced ethanol as the main product with a yield of 0.61 mol/mol, which had a good application potential in the conversion of glycerol into ethanol [12]. Acetic acid stimulates the expression of operon in 2,3-BD metabolic pathway and in 2,3-BD fermentation some of the acetate in the medium may be converted to 2,3-BD through conversion to acetaldehyde, condensation to form acetoin, and subsequent reduction to 2,3-BD [48]. So the low concentration of acetic acid may be lead to no 2,3-BD production with microbial consortium DL38 or a higher concentration of acetic acid was needed to trigger 2,3-BD production. When the strain W3 was cultured with strain Y5 and (or) Y1, no 2,3-BD was also secreted (Table 3). It indicated that strain Y5 and Y1 inhibited the 2,3-BD production by strain W3. The mixed culture with strain W3 and Y5 or Y1 was better than that with single strain W3, it may be due to the synergy relationship between the strains which improved the substrate tolerance and 1,3-PD production. The preferable performance with the natural proportion (W3: Y5: Y1 = 208: 82: 17) also demonstrated that microbial consortium under the natural state had the superiority and rationality. The lower concentration by-products and the higher ability of producing 1,3-PD with microbial consortium DL38 than isolated strains could be attributed to the diversity of microorganisms in microbial consortium DL38 and the by-products may be inhibited or degraded. Microbial consortium DL38 contained many unidentified strains, which made the relationship among the strains more complex.

In the batch fermentations with increased initial glycerol concentration, results verified the ability of selected microbial consortium DL38 to tolerate high concentration of glycerol (up to 200 g/L) and to produce significant quantities of 1,3-PD. Recently, some newly isolated wild-type strains belonging to the

Table 4. Comparison of 1,3-propanediol production by pure and mixed cultures

Inoculum	Fermentation type	1,3-PD (g/L)	Yield (mol/mol)	References
Pure culture				
<i>Klebsiella pneumoniae</i> DSM 4799	Fed-batch	80.20	0.54	Jun et al. [39]
<i>Klebsiella oxytoca</i> M5al	Fed-batch	83.56	0.62	Yang et al. [6]
<i>Citrobacter freundii</i> FMCC-B 294	Fed-batch	68.10	0.48	Metsoviti et al. [42]
<i>Clostridium butyricum</i> AKR102a	Fed-batch	93.70	0.63	Wilkens et al. [19]
<i>Lactobacillus reuteri</i> ATCC 55730	Fed-batch	65.30	0.81	Jolly et al. [43]
Mixed culture				
Organic soil	Batch	3.76	0.65	Liu et al. [38]
Wheat soil	Batch	1.71	0.69	Selembo et al. [31]
Sludge	Batch	15.21	0.51–0.76	Dietz and Zeng [24]
	Fed-batch	70.00	0.52–0.56	
Granular sludge	Continuous	10.74	0.52	Gallardo et al. [37]
Marine sludge	Batch	81.40	0.63	This work

species *K. oxytoca* and *Citrobacter freundii* can also tolerate very high initial glycerol concentrations (i.e. up to approximate 170 g/L) in batch experiments [40, 42]. But the durations and concentrations of 1,3-PD in batch fermentations with above two strains were 171 h, 22.6 g/L and 96 h, 35.1 g/L, respectively, at initial glycerol concentration of 170 g/L, which were much longer and lower than that in batch fermentation with microbial consortium DL38 at initial glycerol concentration of 180 g/L (66 h, 77.31 g/L) (Table 2). The substrate tolerance of single strains (W3, Y5, Y1) was more lower than microbial consortium DL38 (Tables 1 and 3). In this study, the results shown that elevating the initial glycerol concentration may have an inhibitory effect on glycerol assimilation, and this effect has been attributed to several reasons, such as the increased osmotic pressure caused by glycerol itself, the generation of toxic by-products or the depletion of nutrients in the medium [18, 22]. In theory, the critical concentration of glycerol is 2039 mM (188 g/L) for pure culture of *K. pneumoniae* [22]. These all indicated that microbial consortium DL38 had better substrate tolerance than pure culture. To the best of our knowledge, the selected microbial consortium DL38 had the best substrate tolerance in microbial consortia reported so far. This may be attributed to marine strains existing in the microbial consortium which has excellent properties of salt and pressure tolerance [49]. Dietz and Zeng [24] selected microbial consortia from wastewater treatment plants to convert glycerol to 1,3-PD. The initial glycerol concentration was up to 95 g/L, but only about one half of substrate was consumed in 24 h, and no more consumed after 24 h. This may be due to the fact that crude glycerol was used and that nutrient limitations occurred. The authors pointed out that the dominating 1,3-PD producer in their selected microbial consortium was probably a *Clostridium* species. Liu et al. [38] used organic soil as inoculum to produce hydrogen and 1,3-PD from glycerol. The initial glycerol concentration was very low (3–7 g/L), the highest 1,3-PD (0.65 mol/mol) was obtained at 7 g/L of glycerol. In any cases, the maximum 1,3-PD concentration obtained in this study was rather high and comparable to those achieved by pure cultures or mixed cultures with microbial consortia documented in literatures so far (Table 4). In recent years, many efforts were made to further reduce the cost of 1,3-PD production by utilizing biodiesel-derived glycerol directly [20, 40, 42, 50]. These researches showed that the effects of crude glycerol on microbial

fermentation were different with the sources of glycerol and the species of strains. Microbial consortium should have advantages over single strain on the utilization of crude glycerol. The experiments are being conducted by microbial consortium DL38 in our lab.

In this study, we successfully achieved high production of 1,3-PD and high tolerance of glycerol with a selected microbial consortium DL38. In batch fermentations, the highest 1,3-PD concentration of 81.40 g/L was obtained at the initial glycerol concentration of 200 g/L, especially no 2,3-butanediol production. Results showed that mixed culture with strain W3, Y5 and Y1 was better than pure culture with strain W3. And microbial consortia DL38 overcame the shortcomings of poor substrate tolerance in pure culture, improved production efficiency, simplified the product purification. The reasons for why the microbial consortium DL38 possessed excellent substrate tolerance and narrow product spectrum need to be studied in the future. Microbial consortium is expected to provide a simple and economical fermentation process for the industrialization of 1,3-propanediol production.

Practical application

Industrial 1,3-propanediol (1,3-PD) production has attracted attention as an important monomer to synthesize a new type of polyester, poly(trimethylene terephthalate) (PTT), and the market demand is increasing year by year. The traditional microbial fermentation to produce 1,3-PD is pure culture by single strain. But low substrate tolerance and wide by-product profile limit pure culture of 1,3-PD on industrial scale. In this study, the ability of microbial consortium from marine sludge to convert glycerol into 1,3-PD was evaluated. In batch fermentations, the microbial consortium exhibited higher tolerance to glycerol and production of 1,3-PD than single strain. This work overcame the poor substrate tolerance in pure culture, improved production efficiency, simplified the product purification. This method is expected to provide a simple and economical fermentation process for the industrialization of 1,3-PD production.

The authors acknowledge the China National Natural Science Foundation (grant no. 21476042), and Open Fund of Key Laboratory of Biotechnology and Bioresources Utilization (Dalian Minzu University), State Ethnic Affairs Commission & Ministry of Education, China.

The authors have declared no conflicts of interest.

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