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Screening of environmental samples for bacteria producing 1,3-propanediol from glycerol

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Twenty nine environmental samples were screened for the presence of anaerobic microorganisms fermenting glycerol with 1,3-propanediol as a final product. Seven samples were then selected for the next step of our research and eight bacteria strains were cultured anaerobically. Seven of them produced 1,3-propanediol with a yield of 0.47–0.58. Six of the the isolated microorganisms were then classified as *Clostridium butyricum* (four strains), C. *lituseburense* (one strain), and *C. sartagoforme* (one strain). We suggest that of all these strains *C. butyricum* 2CR371.5 is the best 1,3-propanediol producer as producing no lactate as a by-product and growing well on a glycerol-containing medium.

Key words: 1,3-propanediol, anaerobic fermentation, biodiesel, *Clostridium*, glycerol, screening

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INTRODUCTION

The global biodiesel production was over 15 billion litres in 2009 and it is still increasing. The forecast for the worldwide production is over 45 billion litres in 2020 (GlobalData, 2010). As a by-product, 5–10% of crude glycerol is produced (Yazdani & Gonzalez, 2007). The conversion of glycerol to higher-value products could be a way to decrease the cost of biofuel production.

1,3-propanediol (1,3-PDO) is one of the products obtainable from crude glycerol. The main application of 1,3-PDO is as a substrate in polymerization of polytrimethylene terephthalate (PTT), a type of polyester used in engineering thermoplastics and in the production of carpets and textile fibers (Liu *et al*., 2010).

Biological production of 1,3-propanediol is an alternative to chemical methods. Several bacterial species microorganisms able to ferment glycerol with 1,3-PDO as final product are known e.g. *Lactobacillus hilgardii, Citrobacter freundii, Clostridium saccharobutylicum, Clostridium butyricum, Clostridium diolis and Klebsiella pneumoniae* (Boenigk *et al*., 1993; Mu *et al*., 2006; Burkhard *et al*., 2009; Pasteris *et al*., 2009; Gungormusler *et al*., 2010; Chatzifragkou *et al*., 2011). Moreover, genetically modified *E. coli* strains can also be used (Emptage *et al*., 2009; Eliot *et al*., 2011).

Glycerol fermentation by the glycerol-fermenting microorganisms is a two-branched pathway. Production of 1,3-PDO is the reductive branch catalysed by two enzymes, (i) glycerol dehydratase and (ii) 1,3-PDO oxidoreductase, with 3-hydroxypropionaldehyde as an intermediate (Fig. 1). In the oxidative branch glycerol is dehydrogenated by glycerol dehydrogenase to dihydroxyacetone (DHA). DHA is then phosphorylated by ATP or phosphoenolpyruvate to dihydroxyacetone phosphate (DHAP) which is an intermediate in pyruvate synthesis (Gupta *et al*., 2009).

Microorganisms belonging to the genus *Clostridium* may also produce acetate, butyrate, lactate, propionate, ethanol and butanol as by-products of glycerol fermentation. The presence and amount of these compounds differs depending on the fermentation conditions and the *Clostridium* species strain (Dabrock *et al*., 1992; Biebl *et al*., 2001; Taconi *et al*., 2010).

The aim of our research was to screen different environmental samples for the presence of microorganisms fermenting glycerol with 1,3-PDO as a final product.

MATERIALS AND METHODS

Environmental samples and bacterial strains. The environmental samples were the waste containing crude glycerol from biogas plants from Poland (PWiK Gdynia, Przechlewo), Denmark (Lintrup, Blahoj, Hashoj, Filskow, Vegger) or from the A&A Biotechnology collection of environmental samples.

As the reference strains, *Clostridium butyricum* (DSMZ, DSM-2478), *Clostridium lituseburense* (DSMZ, DSM-797), and *Clostridium sartagoforme* (DSMZ, DSM-1292) were used.

Media and culture conditions. Mixed bacterial cultures, the newly isolated strains as well as the reference *Clostridium* sp. strains were cultured anaerobically at 37°C, 53°C or 60°C, for 4–7 days. Media used in this study contained: Yeast Nitrogen Base without amino acids and without ammonium sulphate (Formedium) — 6.9 g $\cdot l^{-1}$, $NaHCO_3$ — 2.6 g $\cdot l^{-1}$, yeast extract — 2 g $\cdot l^{-1}$, glycerol — 10–30 g $\cdot l^{-1}$, cysteine — 0.5 g $\cdot l^{-1}$, resazurin — 0.5 mg $\cdot l^{-1}$, $MgCl_2 \cdot 6H_2O$ — 0.9 g $\cdot l^{-1}$, $ZnCl_2$ — 50 ng $\cdot l^{-1}$, $MnCl_2 \cdot H_2O$ — 38 g $\cdot l^{-1}$, $CoCl_2 \cdot 2H_2O$ — 50 ng $\cdot l^{-1}$, $NiCl_2 \cdot 6H_2O$ — 92 ng $\cdot l^{-1}$, $FeCl_2 \cdot 2H_2O$ — 2.3 mg $\cdot l^{-1}$, $(NH)_4Mo_7O_{24} \cdot 4H_2O$ — 50 ng $\cdot l^{-1}$, EDTA — 0.5 mg $\cdot l^{-1}$. The solid media on Petri plates for the growth at 37°C were solidified with 20 g $\cdot l^{-1}$ of agar. An anaerobic atmosphere was generated by using Biogon® C-20 (80% N_2, 20% CO_2). All experiments in anaerobic conditions were performed in a MiniMACS anaerobic workstation (Don Whitley Scientific).

Screening for 1,3-PDO-producing microorganisms. The sediments were mixed with the liquid medium containing 10 g $\cdot l^{-1}$ glycerol in a 1:1 ratio in a total vol-

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Accession numbers (GenBank): JQ248565 — strain 2ER371.1; JQ248566 — strain 2MR375.1; JQ248567 — strain 2NR375.1; JQ248568 — strain 2CR371.5; JQ248569 — strain 2DR37.1; JQ248570 — strain 2DR37.2.

Abbreviations: 1,3-PDO- 1,3-propanediol; DSMZ- Leibnitz-Institut Deutsche Sammlung von Mikroorganizmen und Zellkulturen GmbH

Table 1. Glycerol fermentation by bacterial strains isolated from environmental samples.

Strain name	Origin	16S rDNA similarity	Yield [g per 1 g glycerol consumed]					
			1,3-PDO	Acetate	Butyrate	Lactate	Propionate	Ethanol
2CR371.5	A&A	Cb	0.570	0.019	0.160	0.000	0.045	0.000
2NR371.5	A&A	Cb	0.580	0.002	0.134	0.047	0.046	0.000
2DR37.1	A&A	Cb	0.500	0.000	0.142	0.008	0.049	0.000
2DR37.2	A&A	Cb	0.480	0.000	0.136	0.008	0.050	0.000
2HS37.2	Vegger	Cb	0.470	0.010	0.105	0.020	0.050	0.000
2ER371.1	Hashoj	Cl	0.540	0.110	0.000	0.038	0.049	0.117
2MS37.4	Przechlewo	Cb	0.580	0.007	0.000	0.000	0.052	0.008
2MR375.1	Przechlewo	Cs	0.000	0.000	0.000	0.000	0.560	0.120
Cb DSM 2478	DSMZ		0.368	0.000	0.090	0.107	0.208	0.000
Cl DSM 797	DSMZ		0.342	0.100	0.000	0.015	0.030	0.005
Cs DSM 1292	DSMZ		0.000	0.000	0.000	0.010	0.000	0.005

Cb, *Clostridium butyricum; Cl, Clostridium lituseburense; Cs, Clostridium sartagoforme.*

ume of 100 ml. They were cultivated at 37°C, 53°C or 60°C for 4–7 days and then were analysed using HPLC. Samples in which 1,3-PDO production was observed were then used for inoculation (in a ratio of 1:10) of the liquid medium containing 30 g $\cdot l^{-1}$ glycerol and cultured in the total volume of 1 l as described above. The mixed cultures were then cultivated on solid medium and single colonies were used for culturing on liquid medium with glycerol. To ensure that single strain cultures were obtained culturing on solid and liquid medium was repeated. At each step a HPLC analysis was performed for the presence of 1,3-PDO and glycerol. The yield of 1,3-PDO production was calculated as g 1,3-PDO per 1 g of consumed glycerol.

Phylogenetic analysis. Phylogenetic analyses were performed by alignment of 16S rDNA sequence fragments. To amplify the 16S rDNA fragments universal DNA primers FD1F: 5'-GAGTTTGATCCTGGCTCAG-3' and RP2: 5'-ACGGCTACCTTGTTACGACTT-3' (Weisburg *et al.*, 1991) were used. The PCR products were then purified with Clean-up AX kit (A&A Biotechnology), sequenced (Macrogen), and compared with the sequences deposited in the GenBank using BLAST program. A phylogenetic tree was constructed using the sequence distance method and the neighbour joining algorithm (Saitou & Nei, 1987) by use of Vector NTI software (InforMax).

HPLC analysis. HPLC analyses were performed using a HPLC Agilent 1200 Series system with RID and DAD detectors, a Phenomenex Rezex ROA column (300 × 7.80 mm; 8 microns) with a 3 mM H_2SO_4 as the eluent (0.6 ml $\cdot min^{-1}$) and the column temperature of 60°C. Glycerol, 1,3-PDO, butyrate, acetate, lactate, propionate, formic acid, ethanol, 1-butanol, and acetone in culture media were analysed.

GC analysis. GC analysis was performed using a ML-GC82, MicrolabAarhus. Fermentation gases, CO_2 and H_2, were separated on Hayesep Q column, 80/100 mesh, 1.5 m × 1.4 Cu med, with N_2 as the carrier gas.

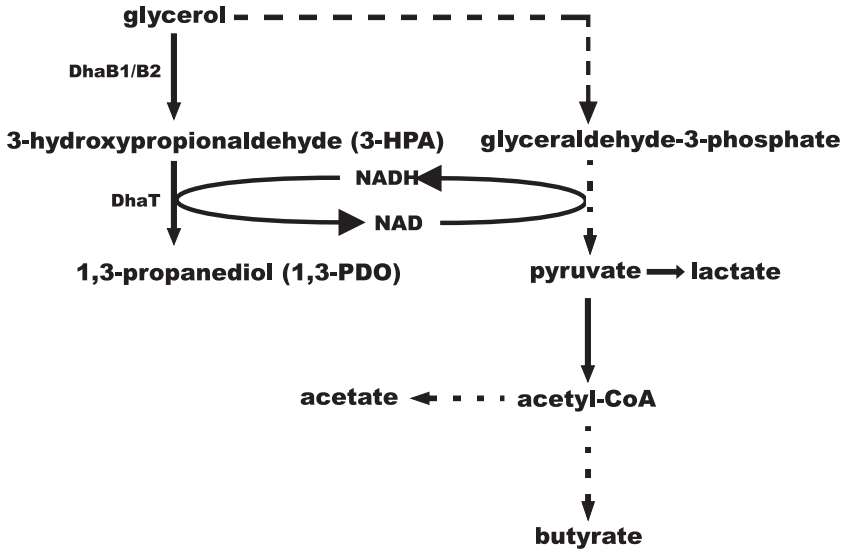

Figure 1. Glycerol fermentation pathway in anaerobic microorganisms.
Dashed lines indicate multiple steps. DhaB1, glycerol dehydratase; DhaB2, glycerol dehydratase activator; DhaT, NADH-dependent 1,3-propanediol oxidoreductase.

RESULTS

At the first step of our research 29 biological samples from biogas plants and A&A Biotechnology collection of environmental samples were tested. After 4–7 days at 37°C bacterial growth was observed for all the analysed samples. At 53°C and 60°C only few samples contained microorganisms able to grow anaerobically at these conditions (data not shown). The anaerobic cultures of these samples were then analysed by HPLC for the presence and concentrations of glycerol, 1,3-PDO, lactate, butyrate, and acetate. We were looking for samples fermenting glycerol to 1,3-PDO with only low concentration of the other analysed chemicals. The best results were obtained for the samples cultured at 37°C (Fig. 2).

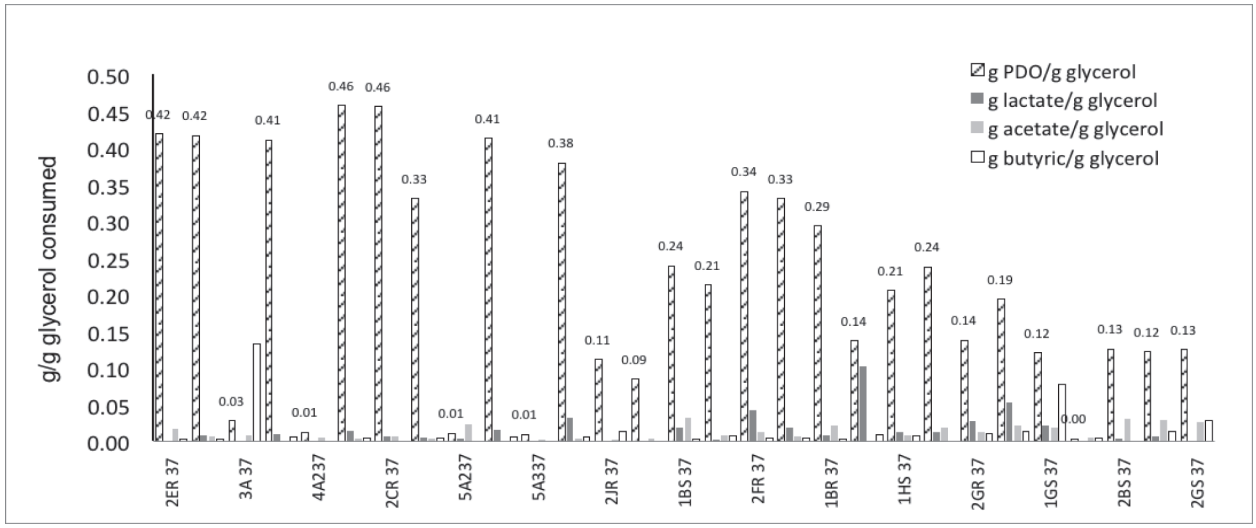

Figure 2. HPLC analysis of mixed cultures grown at 37°C.
The yield of analysed fermentation products was calculated as g analysed product per 1 g of glycerol consumed.

Samples producing the highest amount of 1,3-PDO in the range of 0.38–0.46 (g per g of glycerol consumed) were selected for the next step of the study.

Seven mixed cultures named 2NR37, 2CR37, 2HS37, 2ER37, 2MS37, 2DR37, and 2MR37 were then cultured in 1 litre of the liquid medium containing 30 g l^{-1} glycerol. Single bacterial strains were isolated by consecutive culturing on solid, liquid and solid medium with glycerol. Flasks with fresh liquid medium containing glycerol were then inoculated with single colonies and after 4-7 days of growth the concentration of glycerol, 1,3-PDO and other metabolites was analysed (Table 1).

One of the eight isolated strains, 2MR375.1, did not ferment glycerol to 1,3-PDO, produced 0.56 g of propionate per g of glycerol which was much more than the other strains.

The highest 1,3-PDO production, in the range of 0.57–0.58 g per 1 g of glycerol consumed was obtained for strains 2CR371.5, 2NR371.5 and 2MS37.4. The strain 2NR371.5 produced 0.047 g of lactate whereas 2MS37.4 produced neither acetate nor butyrate. The three strains, 2CR371.5, 2NR371.5 and 2MS37.4 produced propionate in the range of 0.045–0.052 g (Table 1). None of the bacterial strains tested produced formic acid, 1-butanol or acetone. The strains 2MR375.1, 2ER371.1 and 2MS37.4 also produced up to 0.12 g of ethanol (Table 1). They produced fermenting gas which was a mixture of CO_2 (98.5-99%) and H_2 (1–1.5%).

Phylogenetic analysis was performed for six of the eight new isolates. Four of them were classified as *C.butyricum*, whereas 2ER371.1 showed the highest similarity to *C. lituseburense* and 2MR375.1 was classified as *C. sartagoforme* (Fig. 2). The analysis was not performed for strains 2MS37.4 and 2HS37.2. The sequenced fragments of 16S rDNA of these six strains were submitted to the GenBank with the accession numbers JQ248565 to JQ248570.

DISCUSSION

Crude glycerol from biodiesel plants may be a good source of glycerol-fermenting microorganisms. It was found that supplementation with crude glycerol improves biogas production (Kolesárová *et al.*, 2011). Biogas is produced in reactors under anaerobic conditions by a consortium of microorganisms including bacteria of the genus *Clostridium* (Dohrmann *et al.*, 2011). All the bacterial strains we found in the environmental samples from biogas plants belong to this genus (Fig. 3).

The phylogenetic analysis was not performed for the 2MS37.4 and 2HS37.2 strains. However, comparing results of the fermentation with the other isolates and reference strains (Table 1) we can suppose that they are also *C. butyricum* strains.

The highest 1,3-PDO production in the range of 0.47–0.58 g per 1 g of glycerol consumed was observed for the *C. butyricum* strains. The yield of 1,3-PDO production was similar to the previously published results which were 0.52–0.55 g per 1 g of glycerol consumed (Chatzifragkou *et al.*, 2011). It was higher but comparable to 0.368 g of the reference *C. butyricum* strain DSM-2478 (Table 1). The higher yield to 1,3-PDO production by the newly isolated *C. butyricum* strains comparing with the reference DSM-2478 is probably connected with lactate level. In both these cases NADH is required so lactate production from glycerol is an alternative to 1,3-PDO production (Fig. 1). The reference *C. butyricum* strain produces the highest amount of lactate and conse-

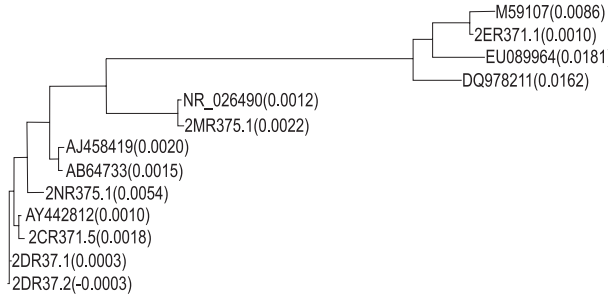

Figure 3. Phylogenetic analysis of newly isolated bacterial strains.
The analysis was based on the alignment of fragments of 16S rDNA sequences. Values in parentheses following the molecule name represent the calculated distance between strains. M59107 — *C. lituseburense*; EU089964 — *C. ruminatium* strain LA1; DQ978211 — *C. bifermentans* strain HT2; NR_026490 — *C. sartagoforme* strain DSM 1292; AY442812 — *C. butyricum*; AJ458419 — *C. diolis* strain E5.

quently the 1,3-PDO level is lower comparing with the new *Clostridium* strains.

Two of the strains, 2CR371.5 and 2MS37.4, did not produced lactate at all (Table 1).

Five of the six new *C. butyricum* strains produced butyrate as a by-product, similarly to the reference strain. The only *C. butyricum* strain which did not produce butyrate was 2MS37.4 (Table 1).

In contrast to the reference *C. butyricum strain* DSM-2478, the newly isolated *C. butyricum* strains produced acetate as a by-product with the yield of 0.002–0.019 g per g glycerol. On the other hand, they produced less propionate (Table 1).

The *C. lituseburense* strain 2ER371.1 which was isolated from the Hashoj biogas plant produced 1,3-PDO with a yield comparable to that of *C. butyricum* strains, but more acetate was present as a by-product (Table 1). Moreover, its growth was not as good as that of *C. butyricum* (data not shown).

C. sartagoforme 2MR37.5 as well as the reference strain *C. sartagoforme* DSM-1292 did not produced 1,3-PDO. In contrast to the reference *C. sartagoforme* strain, 2MR37.5 produced quite a large amount of propionate, an alternative of glycerol fermentation (Fig. 1, Table 1). We suggest that the glycerol fermentation to 1,3-PDO observed for the mixed culture 2MR37 (Fig. 2) was caused by other bacterial strain or strains we were unable to separate and cultivate.

According to our results, of the newly isolated bacterial strains 2NR371.5 is probably the best producer of 1,3-PDO. Just as 2MS37.4, it did not produce lactate, but it grew better than 2NR371.5. The anaerobic fermentation of glycerol by *C. butyricum* 2CR371.5 should now be optimized and performed in a larger scale. As the substrate for the fermentation we intend to test crude glycerol, a by-product of biodiesel production.

In the accompanying article (Dąbrowski *et al.*, 2012) we described construction of a recombinant *E. coli* strain producing 1,3-PDO from glycerol by introducing genes of the *dha* operon from *C. butyricum* 2CR371.5.

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