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1,3-propanediol production by *Escherichia coli* expressing genes of *dha* operon from *Clostridium butyricum* 2CR371.5

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1,3-propanediol is used as a monomer in the production of some polymers e.g. polytrimethylene terephthalate used in the production of carpets and textile fibers and in the thermoplastics engineering. However, the traditional chemical synthesis is expensive, generates some toxic intermediates and requires a reduction step under high hydrogen pressure. Biological production of 1,3-propanediol could be an attractive alternative to the traditional chemical methods. Moreover, crude glycerol which is a by-product of biodiesel production, can be used. We constructed a recombinant *Escherichia coli* strain producing 1,3-propanediol from glycerol by introducing genes of the *dha* operon from *Clostridium butyricum* 2CR371.5, a strain from our collection of environmental samples and strains. The *E. coli* strain produced 3.7 g of 1,3-propanediol per one litre of culture with the yield of 0.3 g per 1 g of glycerol consumed.

Key words: 1,3-propanediol, anaerobic fermentation, biodiesel, *Clostridium butyricum*, *dha* operon, *Escherichia coli*, glycerol

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INTRODUCTION

1,3-propanediol (1,3-PDO) is used as a monomer in the production of several types of polymers, one of which is polytrimethylene terephthalate (PTT), produced by polycondensation of 1,3-PDO with terephtalic acid or its esters. PTT is a type of polyester used in the production of carpets and textile fibers and in thermoplastics engineering (Liu *et al.*, 2010).

1,3-PDO can be produced in many ways i.a. from ethylene oxide over a catalyst in the presence of phosphine, water, carbon monoxide, hydrogen and an acid. However, the traditional chemical synthesis is expensive, generates some toxic intermediates and requires a reduction step under high hydrogen pressure (Raynaud *et al.*, 2003; Emptage *et al.*, 2009). Biological production of 1,3-PDO could be a useful alternative to the traditional chemical methods because of its numerous advantages, including a lower environmental impact. Moreover, crude glycerol, which is a by-product of biodiesel production, can be used as precursor. The conversion of glycerol to higher-value products, such as 1,3-PDO should decrease the cost of biofuel production (Zeng & Biebl, 2002; Yazdani & Gonzalez, 2007).

Several bacterial species fermenting glycerol to 1,3-PDO are known e.g., *Lactobacillus* sp., *Citrobacter freundii, Clostridium* sp., *Klebsiella* sp. (Boenigk *et al.*, 1993; Luo *et al.*, 2011; Mu *et al.*, 2006; Burkhard *et al.*, 2009; Pasteris *et al.*, 2009; Gungormusler *et al.*, 2010; Chatzifragkovet *et al.*, 2011; Zheng *et al.*, 2008). Additionally, genetically modified *E. coli, Saccharomyces cerevisiae* and *Pichia pastoris* strains can also be used (Burch *et al.*, 1997; Nagarajan & Nakamura, 1997; Nevoigt, 2008). However, traditional methods of 1,3-PDO production are still much more effective than the biological one.

Glycerol fermentation by glycerol-fermenting microorganisms is a two-branched pathway. The 1,3-PDO production by *C. butyricum* is the reductive branch catalysed by two enzymes, (i) glycerol dehydratase encoded by *dhaB1* and (ii) NADH-dependent 1,3-PDO oxidoreductase (encoded by *dhaT*), with 3-hydroxypropionaldehyde as an intermediate (Fig. 1). Glycerol dehydratase requires for activity the presence of a protein encoded by the *dhaB2* gene. All three genes are located in the *dha* operon, the expression of which is induced in the presence of dihydroxyacetone or glycerol. On the other hand, in the oxidative branch, glycerol is dehydrogenated by NAD-dependent glycerol dehydrogenase to dihydroxyacetone (DHA). DHA is then converted sequentially to glyceraldehyde-3-phosphate, phosphoenolpyruvate, and pyruvate (Saint-Amans *et al.*, 2001; Raynaud *et al.*, 2003; Daniel *et al.*, 2005; Gupta *et al.*, 2009; Marçal *et al.*, 2009).

Due to the lack of the *dha* operon *E. coli* cannot ferment glycerol to 1,3-PDO in the absence of an external electron acceptor. Instead 1,2-PDO and ethanol are produced (Tong *et al.*, 1991; Gupta *et al.*, 2009). However, genetically modified *E. coli* strains fermenting glycerol to 1,3-PDO have been constructed (Daniel *et al.*, 1995; Emptage *et al.*, 2009; Eliot *et al.*, 2011).

Here we report construction of a recombinant *E. coli* strain producing 1,3-PDO from glycerol by introducing *dhaB1*, *dhaB2*, and *dhaT* genes from the *dha* operon from *C. butyricum* 2CR371.5, a strain from our collection of environmental samples and strain. The strain was previously isolated and tested as a 1,3-PDO producer and the results were described in the accompanying article (Dąbrowski *et. al,* 2012).

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. butyricum* 2CR371.5 from our collection was used as the source of *dha* operon containing *dhaB* and *dhaT* genes. It was cultured anaerobically at 37°C for 3 days in a medium containing: 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

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Accession number (GenBank): JQ346526
Abbreviations: 1,3-PDO, 1,3-propanediol; 3-HPA, 3-hydroxypropionaldehyde; DSMZ, Leibnitz-Institut Deutsche Sammlung von Mikroorganizmen und Zellkulturen GmbH

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 $\cdot l^{-1}$, glycerol — 10 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 $\cdot l^{-1}$. 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).

E. coli TOP10F' (Invitrogen) was used as the host for cloning and the resulting transformants were grown at 37°C for 16–18 h in Luria-Bertani (LB) broth containing ampicillin (50 μg $\cdot ml^{-1}$).

Strains *E. coli* BL21(DE3) and Rosetta2(DE3) (Novagen) were used as hosts for expression of the *dhaB1, dhaB2* and *dhaT* genes. They were grown at 37°C in LB medium containing glycerol (10 g $\cdot l^{-1}$) and eventually also glucose (10 g $\cdot l^{-1}$). The solid medium and liquid medium for non-induced overnight culture did not contain glucose or glycerol. LB medium for *E. coli* Rosetta2(DE3) was additionally supplemented with 50 μg $\cdot ml^{-1}$ of chloramphenicol and media for culturing transformants were also supplemented with 50 μg $\cdot ml^{-1}$ ampicillin.

DNA manipulation. DNA manipulations were carried out according to standard procedures (Sambrook & Russel, 2001) or manufacturer's recommendations. Restriction endonucleases were purchased from Fermentas, T4 DNA ligase was from A&A Biotechnology. To purify DNA after enzymatic reactions DNA Clean-up AX and DNA Gel-out kits (A&A Biotechnology) were used. To isolate plasmid and genomic DNA Plasmid Mini AX and Genomic Mini AX Bacteria Spin (A&A Biotechnology), respectively, were used.

Sequencing and ORF analysis. Genomic DNA of *C. butyricum* 2CR371.5 was partially sequenced (Macrogen). The obtained genomic sequence fragments were analysed using the CLC Genomics Workbench software. To identify the DNA fragment encoding 1,3-propanediol operon components, the obtained *C. butyricum* 2CR371.5 sequence was compared with the nucleotide sequence of the 1,3-propanediol operon of *C. butyricum* DSM 2478 (GenBank: DQ901407.2).

Sequence similarity analysis was done with the BLASTN and BLASTX programs using the server at the National Center of Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Construction of expression plasmid. Plasmid pUC57 (Fermentas) was used for construction of the expression vector.

For amplification of the d*haB1* and *dhaB2* genes as a single fragment from *C. butyricum* 2CR371.5, DNA primers ForDhaB1SphI 5'-GCG GCG GCA TGC GTG ATT GAA GGA GTA AAA ATG ATA AG-3' and RevDhaB2SalI 5'-GCG GCG GTC GAC GTA AAG CTA CTA TTA CTC AGC TCC-3' were used. The obtained PCR product of 3360 bp was cloned into *Sph*I and *Sal*I sites of pUC57 vector which resulted in pUCdhaB1B2 plasmid.

To clone the *dhaT* gene, primers ForDhaB2BamHI 5'-GCG GCG GGA TCC AAG GAG ATA AAA GTA ATG AGT AAG G-3' and RevDhaTKpnI 5'-GCG GCG GGT ACC TTT TAC TTT GAA TCC TTT AAA TAG-3' were used. The PCR product of 2201 bp was then digested with *Afl*II and *Kpn*I and cloned into pUCdhaB1B2. The resulting plasmid was named pUCdhaB1B2T (Fig. 2). Sequence correctness of the constructed plasmid was confirmed by sequencing (Macrogen) with the use of universal primers M13F-pUC and M13R-pUC (Macrogen). Sequences of specific recognition sites in the primers used are underlined.

The PCR reaction profile was: (i) initial denaturation 94°C for 2 min; (ii) 30 repeats: 94°C for 30 s, 65°C for 30 s, 72°C for 2 min; (iii) final extension 72°C for 5 min. The reaction was performed with the use of 2×PCR Master Mix Plus High GC (A&A Biotechnology).

Expression in *E. coli*. *E. coli* BL21(DE3) or Rosetta2(DE3) cells were transformed with pUCdhaB1B2T.

Cells were grown aerobically for 16 h at 37°C in LB medium containing antibiotics. The preculture was then inoculated (1%) into 100 ml of fresh LB medium with antibiotics, glycerol or glycerol plus glucose. The cultivation was at 37°C until optical density at 600 nm of 0.5 was reached. The culture was then supplemented with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and grown without agitation for 2 weeks. To limit the oxygen concentration, the induced cultures were grown in anaerobic flasks.

As references, non-induced cultures and *E. coli* BL21(DE3) and Rosetta2(DE3) transformed with pUC57 were used.

To test the influence of iron ion on the 1,3-PDO production the liquid medium was supplemented with $FeCl_2$ up to 100 μM.

Analytical methods. 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.

For quantitative determination of 3-HPA a colorimetric method described by Krauter *et al.* (2012) was used. The absorbance was measured at 650 nm (Perkin Elmer Lambda EZ 150).

RESULTS

Analysis of the *dha* operon

A genomic DNA fragment containing the 1,3-propanediol operon *(dha* operon) *of C. butyricum* 2CR371.5 was identified using the CLC Genomic Workbench. It was submitted to GenBank with the accession number JQ346526. The nucleotide sequence of this fragment shows the identity with 99% to the corresponding operon of *C. butyricum* DSM 2478. We found three distinct open reading frames encoding DhaB1, DhaB2 and DhaT, components of the *dha* operon of *C. butyricum* 2CR371.5. DhaB1, comprising of 2,361 nucleotides, encodes a protein of 787 amino acids. The deduced amino acid sequence shows the highest identity (100%) with the amino acid sequence of B_{12}-independent glycerol dehydratase from *C. butyricum*. DhaB2, of 912 nucleotides, encodes a protein of 304 amino acids. The deduced amino acid sequence shows 100% identity with the amino acid sequence of glycerol dehydratase activator from *C. butyricum*. DhaT, of 1,155 nucleotides, encodes a protein of 385 amino acids. The deduced amino acid sequence shows 100% identity with the amino acid sequence of 1,3-propanediol dehydrogenase from *C. butyricum*.

Production of 1,3-PDO by *E. coli*

The growth of all the *E. coli* strains harbouring the pUCdhaB1B2BT plasmid was much slower comparing

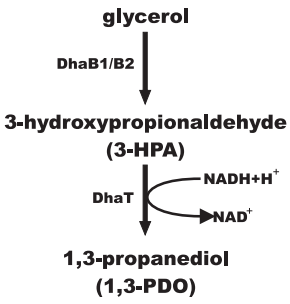

Figure 1. Glycerol fermentation pathway in anaerobic bacteria.
DhaB1, glycerol dehydratase; DhaB2, glycerol dehydratase activating protein; DhaT, 1,3-propanediol oxidoreductase.

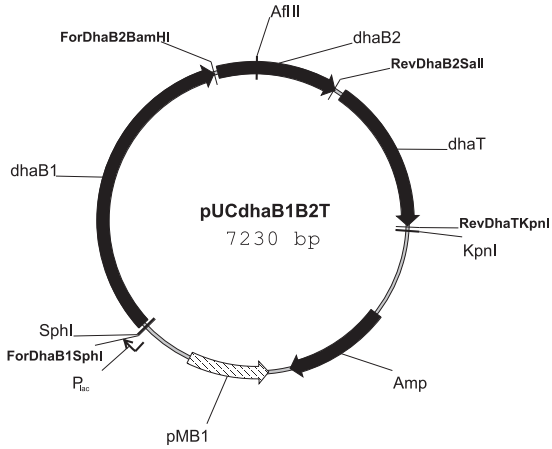

Figure 2. A map of the pUCdhaB1B2T plasmid.
Amp, ampicilin resistance gene; P_{lac}, lactose promoter; pMB1, replicon; *Afl*II, *Kph*I, *Sph*I, restriction endonuclease sites.

with control *E. coli* strains transformed with pUC57 plasmid.

Seven days after expression induction with IPTG the *E. coli* strains BL21(DE3) and Rosetta2(DE3) transformed with pUCdhaB1B2T produced 1,3-PDO in the concentration of 0.1 g ·l^{-1} (not shown).

The HPLC analysis performed two weeks after IPTG induction showed 1,3-PDO concentration of 0.34 g ·l^{-1} in the case of *E. coli* BL21(DE3) and 3.7 g ·l^{-1} in the case of *E. coli* Rosetta2(DE3) when glycerol was used as the sole carbon source (Fig. 3).

When a medium containing both glycerol and glucose was used, the concentration of 1,3-PDO two weeks after IPTG induction did not exceed 0.3 g ·l^{-1} for either of the two *E. coli* strains (Fig. 3).

The yield of the 1,3-PDO production relative to the amount of glycerol consumed was up to 0.028 g per one gram of glycerol for e*E. coli* BL21(DE3) and up to 0.3 g for Rosetta2(DE3) (Fig. 4).

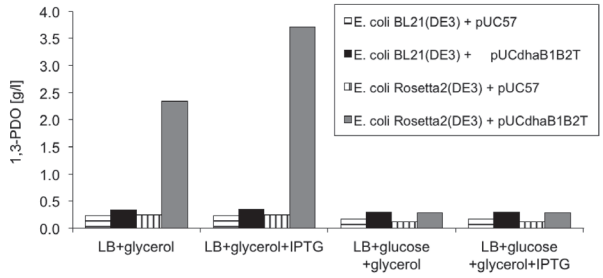

Figure 3. 1,3-propanediol concentration after two weeks of expression expressing *dha* genes of 2CR371.5 in *E. coli*.

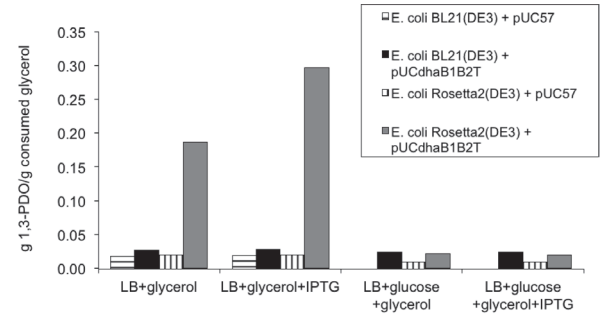

Figure 4. 1,3-propanediol production per consumed glycerol, after two weeks of expression *dha* genes of 2CR371.5 in *E. coli*.

Simultaneously with 1,3-PDO the amount of 3-HPA was also measured, following induction with IPTG its production increased more than that of 1,3-PDO (Table 1).

Iron ions at concentration up to 100 μM had no effect on 1,3-PDO production (not shown).

DISCUSSION

There are several microorganisms naturally fermenting glycerol to 1,3-PDO, but their large-scale application is limited due to some difficulties. The main problems are (i) accumulation of by-products which are toxic and can inhibit 1,3-PDO production; (ii) some of these microorganisms are human pathogens; (ii) most of the 1,3-PDO-producing bacteria need B_{12} vitamin as a cofactor for glycerol dehydratase (DhaB) which increases costs (Nakamura & Whited, 2003; O'Brien *et al.*, 2004*)*. Construction of genetically modified *E. coli* strains express B_{12}-independent glycerol dehydratase gene could overcome these difficulties.

We constructed a system for heterologous expression in *E. coli* genes of the glycerol of metabolic pathway from *C. butyricum* 2CR371.5, from the A&A Biotechnology collection. The cloned genes *dhaB1, dhaB2* and *dhaT* encode a vitamin B_{12}-independent glycerol dehydratase, its activating factor and an NADH-dependent 1,3-PDO oxidoreductase, respectively.

The first problem we came across was the poor growth of the *E. coli* strains harbouring the pUCdhaB1B2T plasmid. It can be

Table 1. 3-hydroxypropionaldehyde and 1,3-propanediol production by *E. coli Rosetta*2(DE3) cells expressing genes of *dha* operon of *C. butyricum* 2CR371.5 in LB medium supplemented with glycerol.
The increase of 1,3-PDO and 3-HPA was calculated as the ratio of the product amount at a given time to that at the previous time analysed.

Days after induction with IPTG	1,3-PDO [g·l^{-1}]	1,3-PDO increase	3-HPA [g·l^{-1}]	3-HPA increase
3	0.21	–	0.12	–
7	0.74	3.52	0.62	5,17
10	2.21	2.99	2.71	4,37
14	3.72	1.68	5.24	1,93

explained by the fact that the LB medium could contain a small amount of lactose to induce expression of the cloned genes under the lac promoter at a low level. That in turn would in accumulation of an intermediate (3-HPA), final product (1,3-PDO), or heterologous protein/-s (DhaB1, DhaB2 or DhaT). We suppose the agent which caused the growth inhibition was 3-HPA, because it was proved previously to be toxic to bacterial cells (Hao *et al*., 2008b).

The observed 1,3-PDO production by *E. coli* Rosetta2(DE3) was about 11-fold higher comparing with *E. coli* BL21(DE3) (Fig. 3). It can be explained by the fact that *E. coli* Rosetta2(DE3) contains a plasmid conferring chloramphenicol resistance that supplies rare tRNAs. Consequently, translation is not limited by the codon usage of *E. coli* (Kane, 1995). Analysis of the predicted amino acid sequences of DhaB1, DhaB2 and DhaT revealed that they contain 14, 12 and 14% of rare codons, respectively.

The low amount of 1,3-PDO produced in our expression system could be correlated with 3-HPA concentration. 3-HPA, an intermediate of 1,3-PDO production, is an inhibitor of DhaB1 (Barbirato *et al*., 1996a; Hao *et al*., 2008a; 2008b). The 3-HPA accumulation is correlated with a higher NAD/NADH ratio and it is known that NAD behaves as a competitive inhibitor of glycerol dehydratase (Barbirato *et al*., 1996b). The level of 3-HPA in culture medium could be controlled by the substrate (glycerol) concentration, and a lower level of glycerol could prevent 3-HPA accumulating to a high, lethal concentration (Hao *et al*., 2008). 3-HPA accumulates when the first step of glycerol fermentation to 1,3-PDO is faster than the second one, e.g. when the activity of DhaT is lower than that of DhaB1. Such a situation often occurs in polycistronic expression systems where the mRNA lacks internal ribosome entry sites for the next cistrons (Nishizawa *et al*., 2010).That was not the case in our expression system, as we cloned the whole *dha* operon with all the sequences between individual genes. However, in a heterologous expression system the expression of individual genes may differ from that in the natural host (*C. butyricum*). An analysis of DNA sequences upstream of the *dha* genes has revealed some departures from the consensus Shine-Dalgarno sequence of *E. coli*. Thus, the recognition of the ribosome binding sites of the *C. butyricum dha* genes by *E. coli* translation machinery could be less effective (Makrides, 1996; Mironova *et al*., 1999; Shultzaberger *et al*., 2001). However, above the difficulty could probably be overcome by cloning a gene encoding a DhaT isoenzyme more active than DhaT from *C. butyricum* e.g. YqhD from *E. coli,* encoding a NADPH-dependent 1,3-PDO dehydrogenase (Emptage *et al*., 2009). YqhD utilizes NADPH rather than NADH and it is likely that the differences in the cofactor reduced/oxidized ratios contribute to the higher production of 1,3-PDO by this enzyme (Nakamura & Whited, 2003).

Media containing glycerol or glycerol-glucose mixture as the carbon source were tested. *E. coli* cells used glucose preferentially (not shown), which resulted in no or lower 1,3-PDO production (Fig. 3). Moreover, the *dha* operon is repressed by glucose (Sprenger *et al*., 1989). Therfore, when glycerol was used as sole carbon source, the obtained 1,3-PDO amount was 1.1 and 13.2-times higher than in glycerol–glucose medium for *E. coli* BL21(DE3) and *E. coli* Rosetta2(DE3), respectively (Fig. 3).

Fermentation by recombinant *E. coli* strains was performed in anaerobic flasks, without shaking, but the media used were not reduced before. Thus, it is possible that, in such conditions a part of glycerol is dehydrogenated by the glycerol dehydrogenase (DhaD) of *E. coli* (Sprenger *et al*., 1989). Consequently, low 1,3-PDO concentration and yield was observed — 3.7 g $\cdot l^{-1}$ and 0.3 g per 1 g of glycerol consumed, respectively, for induced expression in *E. coli* Rosetta2(DE3) (Fig. 3, 4). On the other hand, culturing of the recombinant *E. coli* in strict anaerobic conditions failed to produce satisfying results because the strain was not able to grow (not shown).

One way to improve 1,3-PDO production in *E. coli* could be construction of a double-induced expression system, with a delayed dhaB1 and B2 induction. DhaT may be also cloned under control of a stronger promotor than that of the *dhaB1B2* genes.

Is was observed that DhaT from *C. freundii* expressed in *E. coli* was more active in the presence of 50 μM Fe^{2+}(Daniel *et al*., 1995). However, there is no such information for its isoenzymes from Clostridium sp. On the other hand, iron limitation causes higher 1,3-PDO production by *Clostridium* sp., probably by inhibiton of other dehydrogenases involved in the formation of butanol and ethanol (Dabrock *et al*., 1992; Raynaud *et al*., 2003; O'Brien *et al*., 2004). We therefore checked if supplementing the medium with Fe^{2+} up to 100 μM could improve 1,3-PDO production, but no such effect was obtained. One of the reasons for this lack of improvement could be the absence of competing dehydrogenases in the heterologous system used.

Several papers describing construction of 1,3-PDO-producing *E. coli* strains have been published. Emptage *et al.* (2009) constructed an *E. coli* strain producing up to 130 g $\cdot l^{-1}$ 1,3-PDO. However, in there expression system glucose was used as the sole carbon and energy source. Moreover, the recombinant *E. coli* strain harboured genes of the *dha* operon of *K. pneumoniae* and so B_{12} vitamin had to be added to the medium to activate the glycerol dehydratase. Glycerol was used as the sole carbon source was obtained by others (Skraly *et al*., 1998) and 6.3 g $\cdot l^{-1}$ of 1,3-PDO was produced, again for *E. coli* expressing the *K. pneumoniae dha* genes.

For comparision, a single-stage culture of *C. butyricum* with raw glycerol produced up to 35–48 g of 1,3-PDO per litre with a yield of 0.55 g per one gram of glycerol (Papanikolaou *et al*., 2000; Chatzifragkou *et al*., 2011). Glycerol fermentation by the *C. butyricum* 2CR371.5, which was the source of the genes studied here, results in 0.57 g of 1,3-PDO per 1 g of glycerol consumed (Dąbrowski *et al*., 2012).

The recombinant *E. coli* strains expressing genes of the *dha* operon from *C. butyricum* 2CR371.5 did not give satisfying results due to the low 1,3-PDO production level and long time required for maximal production. We believe it should be possible to obtain much more efficient glycerol fermentation to 1,3-PDO by *E. coli* expressing genes of the 1,3-propanediol operon from *C. butyricum* 2CR371.5 if modifications discussed above are introduced. That effort should be worthwhile, because 1,3-PDO is a desired chemical and the costs of its synthesis are still high.

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