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1,3-Propanediol production by *Escherichia coli* using genes from *Citrobacter freundii* ATCC 8090

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Compared with chemical synthesis, fermentation has the advantage of mass production at low cost, and has been used in the production of various industrial chemicals. As a valuable organic compound, 1,3-propanediol (1,3-PDO) has numerous applications in the production of polymers, lubricants, cosmetics and medicines. Here, conversion of glycerol (a renewable substrate and waste from biodiesel production) to 1,3-PDO by *E. coli* bacterial strain carrying altered glycerol metabolic pathway was investigated. Two gene constructs containing the 1,3-PDO operon from *Citrobacter freundii* (pCF1 and pCF2) were used to transform the bacteria. The pCF1 gene expression construct contained *dhaBCE* genes encoding the three subunits of glycerol dehydratase, *dhaF* encoding the large subunit of the glycerol dehydratase reactivation factor and *dhaG* encoding the small subunit of the glycerol dehydratase reactivating factor. The pCF2 gene expression construct contained the *dhaT* gene encoding the 1,3-propanediol dehydrogenase. Expression of the genes cloned in the above constructs was under regulation of the T7*lac* promoter. RT-PCR, SDS-PAGE analyses and functional tests confirmed that 1,3-PDO synthesis pathway genes were expressed at the RNA and protein levels, and worked flawlessly in the heterologous host. In a batch flask culture, in a short time applied just to identify the 1,3-PDO in a preliminary study, the recombinant *E. coli* bacteria produced 1.53 g/L of 1,3-PDO, using 21.2 g/L of glycerol in 72 h. In the Sartorius Biostat B Plus reactor, they produced 11.7 g/L of 1,3-PDO using 24.2 g/L of glycerol, attaining an efficiency of 0.58 [$mol_{1,3\text{-}PDO}/mol_{glycerol}$].

Key words: bio-based 1,3-propanediol, genetically modified bacteria, glycerol utilization, renewable resources, *Citrobacter freundii*, *Escherichia coli*

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INTRODUCTION

1,3-Propanediol (1,3-PDO), a trimethylene glycol, is an organic compound belonging to the diol group, with a molecular formula of $C_3H_8O_2$, molar mass of 76.09 g/mol and density of 1.0597 g/cm^3. Under normal conditions it is a colorless liquid, miscible with water. 1,3-PDO exhibits many valuable properties, thanks to which it has been applied for years in various branches of industry (Igari *et al*., 2000). 1,3-PDO is mainly used in the chemical industry as a monomer in the synthesis of biodegradable polyesters, polyurethanes and polyethers providing them with greater strength, while it is also contained in various products in the cosmetics, textile and medical industries. This compound provides specific properties to solvents, resins, laminates, and detergents (Menzel *et al*., 1997; Zeng & Biebl, 2002; Zhu *et al*., 2002; Cheng *et al*., 2007; Sauer *et al*., 2008). A breakthrough in the use of 1,3-PDO was connected with the British-Dutch petrochemical consortium (Shell) commercialization in 1995 of a novel aromatic polyester, Corterra® PTT polytrimethylene terephthalate, based on 1,3-PDO (Shell Chemical Company, Press release, 1995). This polymer has greater strength properties, greater tensile strength and elasticity than polyester based on ethylene or butylene glycol or 1,2-propanediol (1,2-PDO) (Chuah *et al*., 1995; Kurian 2005; Liu *et al*., 2010). It is used as fiber to produce yarn in the manufacturing of textiles and carpets (Chuah, 1996; Brown & Chuah, 1997). In 2000, DuPont commercialized polytrimethylene terephthalate Sorona®, based on 1,3-propanediol obtained in the fermentation process (Chotani *et al*., 2000; EMBO reports, 2003; Nakamura & Whited, 2003). It was one of the first global achievements on the biotech market which used a genetically modified *E. coli* strain K-12 and a renewable energy source, i.e. corn glucose. 1,3-PDO is produced mainly by chemical methods, although this compound may be produced using microorganisms in the fermentation of various carbon sources, e.g. glycerol. Pure glycerol does not pose a hazard and it is used in various branches of industry, while glycerol from the production of biofuel, containing numerous contaminants, is a noxious waste (Dharmadi *et al*., 2006). Purification of waste glycerol is unprofitable, while its potential conversion to a valuable compound solves the problem of its troublesome management. Production of 1,3-PDO from waste glycerol is a cheaper method than chemical production of this compound (Rehman *et al*., 2008). The findings of studies on the effect of waste glycerol on bacterial growth and the production of 1,3-PDO prove that there is no significant difference in comparison to pure glycerol (Papanikolaou *et al*., 2004). It has been known for over 120 years that 1,3-PDO is one of the products formed during microbial fermentation of glycerol. This molecule was identified for the first time in 1881 by the chemist August Freund as a product of glycerol fermentation run by *Clostridium*

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Abbreviations: DHA, dihydroxyacetone; DHAK, dihydroxyacetone kinase; DHAP, dihydroxyacetone phosphate; GDH, glycerol dehydrogenase; GDHt, glycerol dehydratase; 3-HPA, 3-hydroxypropionaldehyde; IPTG, isopropyl β-D-thiogalactopyranoside; PEP, phosphoenolpyruvate; 1,3-PDDH, 1,3-propanediol dehydrogenase; 1,3-PDO, 1,3-propanediol; RBS, ribosome binding site

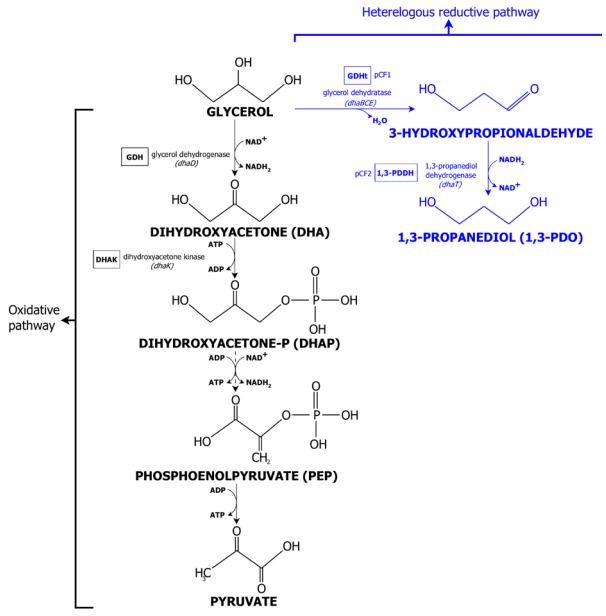

Figure 1. Catabolism of glycerol in natural 1,3-PDO producers and *Escherichia coli* transformed with both plasmids, pCF1 and pCF2. Enzymes of the *dha* regulon are marked by rectangles. Pyruvate is reduced to different organic compounds depending on the microorganism. GDHt, glycerol dehydratase; 1,3-PDDH, 1,3-propandiol dehydrogenase; GDH, glycerol dehydrogenase; DHAK, dihydroxyacetone kinase. The figure was prepared based on (Biebl, 2001).

pasteurianum (Nakas *et al*., 1983; Biebl *et al*., 1999). In the last two decades, advances have been observed in biological production methods of 1,3-PDO from cheap, renewable energy sources, applying newly developed strains to produce 1,3-PDO (Deckwer, 1995; Zhu *et al*., 2002). The physiological role of 1,3-PDO synthesis in glycerol metabolism concerns regeneration of the NAD^+ coenzyme from the reduced form obtained *via* the oxidative pathway, in which glycerol is first oxidized to dihydroxyacetone (DHA), which is followed by phosphorylation and formation of phosphodihydroxyacetone (DHAP), which is subsequently transformed into pyruvate (Forage & Lin, 1982; Zhu *et al*., 2002). There are natural producers of 1,3-PDO, as well as heterologous hosts of the 1,3-PDO synthesis pathway (Homann *et al*., 1990; Gonzalez-Pajuelo *et al*., 2006). Both, the former and the latter, are used in studies on the production of 1,3-PDO. Natural producers of 1,3-PDO include bacteria from the *Klebsiella* (Huang *et al*., 2002; Németh *et al*., 2003), *Citrobacter* (Boenigk *et al*., 1993; Seifert *et al*., 2001), *Clostridia* (Colin *et al*., 2001; Malaoui & Marczak, 2001), *Enterobacter* (Zhu *et al*., 2002) and *Lactobacilli* genera (Schutz & Radler, 1984). The species most often described in the literature on this subject include for e.g. *Citrobacter freundii* (Malinowski, 1999), *Klebsiella pneumoniae* (Biebl *et al*., 1998), *Clostridium pasteurianum* (Biebl, 2001), *Clostridium butyricum* (Papanikolaou *et al*., 2004), *Lactobacillus brevis* and *Lactobacillus buchnerii* (Schutz & Radler, 1984), whose glycerol metabolic pathway contains a branch in which 1,3-PDO is formed in the fermentation process. Most natural 1,3-PDO producers metabolize glycerol via two pathways: reductive and oxidative (Zhu *et al*., 2002) (Fig. 1). 1,3-PDO is formed in the reductive pathway. In the first stage of the reductive pathway, glycerol is dehydrated by glycerol dehydratase (GDHt, *dhaBCE* genes) in the presence of coenzyme B_{12}, with the formation of 3-hydroxypropionaldehyde (3-HPA) (Knietsch *et al*., 2003). Next, $NADH^+$ H^+-dependent 1,3-PDO dehydrogenase (1,3-PD DH, PDOR-oxidoreductase 1,3-PDO, *dhaT* gene) reduces 3-HPA to 1,3-PDO, regenerating NAD^+ (Ahrens *et al*., 1998; Skraly *et al*., 1998). In the oxidative pathway, NAD^+-dependent glycerol dehydrogenase (GDH) catalyzes oxidation of glycerol to dihydroxyacetone (DHA), which is then phosphorylated in the presence of the glycolytic enzyme ATP-dependent dihydroxyacetone kinase (DHAK) (Forage & Lin, 1982; Daniel *et al*., 1995a). Reduced coenzymes formed in the oxidative pathway are necessary for the execution of reductive pathways, in which 1,3-PDO is synthesized, as well as the other products of glycerol metabolism. In our work, a strain from the *Citrobacter* genera was a donor of heterologous genes. This microorganism belongs to a species efficiently producing 1,3-PDO from glycerol; however, it is classified as a microbial hazard group II, which limits its potential application in industrial processes. The application of *E. coli* (as a heterologous host) in biotechnological processes is becoming increasingly desirable (also in the 1,3-PDO production) as an alternative to using "natural producers". Molecular cloning and expression of genes encoding GDHt and 1,3-PD DH from *C. freundii* had been reported (Daniel & Gottschalk, 1992; Daniel *et al*., 1995a; Daniel *et al*., 1995b; Seyfried *et al*., 1996; Seifert *et al*., 2001; Qi *et al*., 2013), but studies on 1,3-PDO production by *E. coli* containing *C. freundii* 1,3-PDO operon genes are still limited. Production of 1,3-PDO both from glucose and glycerol, using a native strain of *E. coli*, is based on a completely heterologous pathway (Cameron *et al*., 1998). Due to the lack of the *dha* regulon (which is present in the 1,3-PDO producers) *E. coli* cannot ferment glycerol to 1,3-PDO. Instead, 1,2-PDO and ethanol are produced (Fig. 2). The aim of this study was to engineer a modified *E. coli* capable of producing 1,3-PDO from glycerol as a substrate. This aim was achieved by designing and making gene expression constructs carrying a total of six heterologous genes participating in glycerol catabolism and originating from *C. freundii*. The next stages in carrying out of this study included introduction of these constructs into *E. coli* cells and performing molecular and metabolic analyses of the recombinant strain, confirming the functioning of the incorporated heterologous genes in *E. coli*.

MATERIALS AND METHODS

Strains and plasmids. The genes for the production of 1,3-PDO in *E. coli* were cloned into the pET-22b(+) (Novagen) and pET-26b(+) (Novagen) expression vectors and sequenced. Genomic DNA from *Citrobacter freundii* ATCC 8090 (American Type Culture Collection, Manassas, VA 20108 USA) was the gene source for cloning experiments. *E. coli* BL21(DE3)pLysS and Rosetta 2(DE3)pLysS strains (Novagen) were used as hosts for gene expression experiments.

Media and growth conditions. *E. coli* was grown in the LB medium supplemented with ampicillin (100 μg/ml), chloramphenicol (34 μg/ml) and/or kanamycin (50 μg/ml). In the first (24-hour) and the second (72-hour) experiment, bacteria were cultured in six flasks, with flasks no. 1–3 initially containing 4 g/L of pure glycerol (anhydrous glycerol POCH S.A.), and flasks no. 4–6 containing 70 g/L. The culture medium in flasks no. 1, 2, 4, and 5 was inoculated with 3 ml of recombinant *E. coli* BL21(DE3)pLysS culture (with pCF1 and pCF2 gene constructs), while 3 ml of non-recombinant

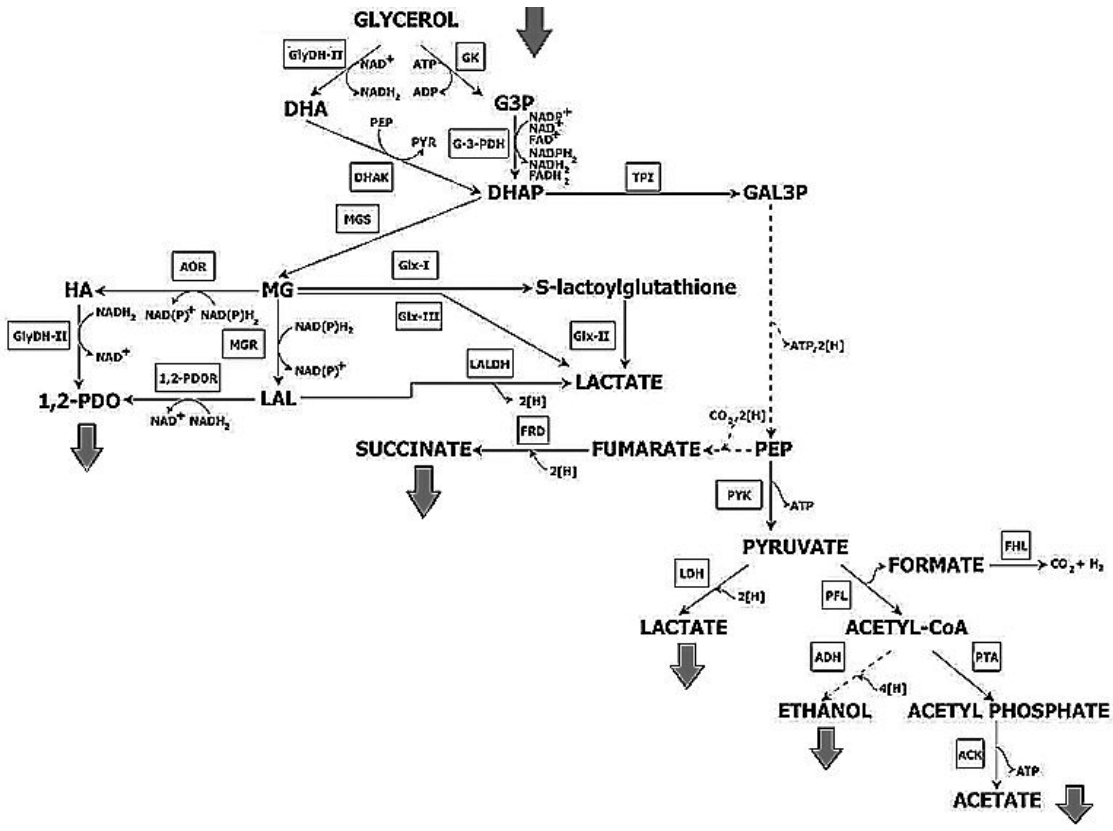

Figure 2. Glycerol metabolism in a wild-type *Escherichia coli* strain.
Branch from glycerol to DHAP *via* glycerol-3-phosphate occurs in the presence of external electron acceptors. GlyDH-II, glycerol dehydrogenase; DHAK, dihydroxyacetone kinase; GK, glycerol kinase; G-3-PDH, glycerol-3-phosphate dehydrogenase; TPI, triosephosphate isomerase; MGS, methylglyoxal synthase; AOR, aldehyde oxidoreductase; 1,2-PDOR, 1,2-propandiol reductase; MGR, methylglyoxal reductase; Glx-I, glyoxalase type I; Glx-II, glyoxalase type II; Glx-III, glyoxylase type III; LALDH, lactaldehyde dehydrogenase; FRD, fumarate reductase; PYK, pyruvate kinase; LDH, lactate dehydrogenase; PFL, pyruvate formate lyase; FHL, formate hydrogen lyase; ADH, alcohol dehydrogenase; PTA, phosphate acetyltransferase; ACK, acetate kinase; DHA, dihydroxyacetone; G3P, glycerol-3-phosphate; DHAP, dihydroxyacetone phosphate; GAL3P, glyceraldehyde-3-phosphate; HA, hydroxyacetone; MG, methylglyoxal; 1,2-PDO, 1,2-propandiol; LAL, lactaldehyde; PEP, phosphoenolpyruvate. The figure was prepared based on (Gonzalez *et al*., 2008; Clomburg & Gonzalez, 2010).

E. coli BL21(DE3)pLysS culture were transferred into flasks 3 and 6. Subsequently, in the logarithmic growth phase ($OD_{600} \approx 0.6–0.8$), the IPTG inducer was added to flasks no. 1 and 4, to a final concentration of 1mM. Cultures were maintained at 37°C, 160 rpm, without pH adjustment. Subsequently, after 24 and 72 hours, glycerol and 1,3-PDO concentrations in the production medium were determined by high-performance liquid chromatography (HPLC). Third batch fermentation was performed in a 5 L bioreactor (Sartorius Stedim Poland) integrated with the inoculum jar (Duran), a bottle of 20% NaOH and a distilled water bottle, which enables control of the correct gas flow through the system. A total of 0.4 vvm of air was sparged into the broth to maintain microaerobic conditions. The fermentation was carried out in two stages: the first was conducted to obtain high-cell density, the other to carry out the main production process. Primary inoculum (4 ml) was transferred to the jar (proliferation medium with a total volume of 200 ml, 5 g/L yeast extract, 0.5 g/L NaCl, 0.48 g/L $MgSO_4 \cdot 7H_2O$, 11.3 g/L $Na_2HPO_4 \cdot 7H_2O$, 3 g/L KH_2PO_4, 2 g/L NH_4Cl, 100 μg/ml ampicillin, 50 μg/ml kanamycin, 34 μg/ml chloramphenicol) integrated with the bioreactor. The jar was placed in a water bath (37°C), and incubated for 24 h. Following this, the contents of the jar were pumped into the bioreactor. Initially, the production medium contained no glycerol (final volume of 2 L, the medium composition differed from the composition of the proliferation medium by the absence of glucose, the presence of vitamin B_{12} in a concentration of 1.5 mM and IPTG added in the bacteria logarithmic growth phase to a final concentration of 1 mM). After 24 h, pure glycerol was added to a final concentration of 35 g/L. Fermentation proceeded for 168 h. The pH was controlled at 7.0, the growth temperature was 37°C, and agitation at 60 rpm was maintained.

Construction of recombinant plasmids. The pCF1 construct contained *dhaBCE*, *dhaF*, *dhaG* genes from *C. freundii* ATCC 8090 inserted under the control of the T7*lac* promoter in the pET-22b(+) expression vector (Fig. 3). This construct was prepared by the following steps: 1) The *dhaBCE* genes were amplified using genomic DNA of *C. freundii* ATCC 8090 as the template with primers F (5'-CGACATATGAGAAGATCAAAACGATTCGAA-3') and R (5'-CCCGTCGACTCACTGGCTGCCTTTACGCAGCTTATTTC-3'). The 5' end of the fragment was modified by the addition of a *Nde*I restriction site, while at the 3' end, a *Sal*I restriction site was introduced. PCR products were digested with restriction enzymes (*Nde*I and *Sal*I) and cloned into a pET-22b(+) expression vector within *Nde*I and *Sal*I restriction sites. 2) The sequence encoding *dhaF* gene was amplified by PCR using the same template and primers F (5'-TTTGTCGACCGGAGGCAGCATGCCGTTAATTGCAGGGATCGATATC-3') and R (5'-TTTAAGCTTCTATTTTTTTGCATCTCCGGCTAAAAC-

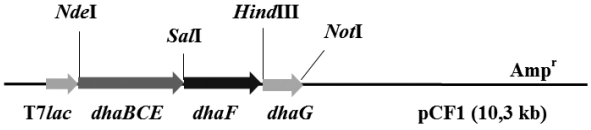

Figure 3. pCF1 genetic construct scheme.
The construct contains the *dhaBCE* genes encoding the three subunits of glycerol dehydratase, the *dhaF* gene encoding the major subunit of the glycerol dehydratase reactivation factor and the *dhaG* gene encoding the smaller subunit of the glycerol dehydratase reactivation factor derived from *C. freundii* ATCC 8090 in expression vector pET-22b(+).

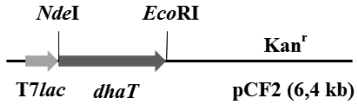

Figure 4. pCF2 genetic construct scheme.
The construct contains sequence encoding the 1,3-propanediol dehydrogenase (*dhaT*) derived from *C. freundii* ATCC 8090 in expression vector pET-26b(+).

CAG-3'). The 5' end of the gene was modified by the addition of a *Sal*I restriction site and the RBS sequence (underlined). The 3' end of the gene was modified by the addition of a *Hind*III restriction site. PCR products were digested with restriction enzymes and ligated into the pET22b(+) construct containing the *dhaBCE* gene and cleaved by *Sal*I and *Hind*III. 3) The fragment containing a *dhaG* gene was amplified using genomic DNA as the template and primers F (5'-TTTAAGCTTACAGGGGGCGCAATGTCACTTTCATCACCGGG-3') and R (5'-TTTGCGGCCGCTTATTTTATCTCGCTGAAGGGA-3'). The 5' end of the gene was modified by the addition of a *Hind*III restriction site and the RBS sequence (underlined), while at the 3' end a *Not*I restriction site was introduced. PCR products were digested with restriction enzymes *Hind*III and *Not*I and cloned into a plasmid containing *dhaBCE* and *dhaF* genes cleaved with the same enzymes.

The pCF2 construct contained a *dhaT* gene from *C. freundii* ATCC 8090 inserted under the control of T7*lac* promoter in the pET-26b(+) expression vector (Fig. 4). The *dhaT* gene was amplified using genomic DNA of *C. freundii* ATCC 8090 as the template with primers F (5'-CGACATATGAGCTATCGTATGTTTGATTA-3') and R (5'-TTCGAATTCAGAATGCCTGACGGAAGAT-3'). The 5' end of the gene was modified by the addition of a *Nde*I restriction site, while at the 3' end, an *Eco*RI restriction site was introduced. PCR products were digested with restriction enzymes (*Nde*I and *Eco*RI) and cloned into a pET-26b(+) expression vector within *Nde*I and *Eco*RI restriction sites. All genes were sequenced using automated genetic analyzers (Applied Biosystems Prism). General procedures for manipulating DNA were carried out according to Sambrook *et al.* (2001). Plasmid DNA was isolated by using StrataPrep Plasmid Miniprep Kit (Stratagene). PCR reagents, restriction enzymes and T4 DNA ligase were purchased from Sigma, Fermentas or New England Biolabs.

Overexpression, preparation of cell extracts and SDS/PAGE analysis. Single colonies of the *E. coli* BL21(DE3)pLysS and Rosetta 2(DE3)pLysS strains containing the expression plasmids pCF1 and pCF2 were inoculated into an LB broth containing ampicillin (100 μg/ml)/kanamycin (50 μg/ml) and chloramphenicol (34 μg/ml) and grown at 37°C to OD_{600} ~0.6–0.8.

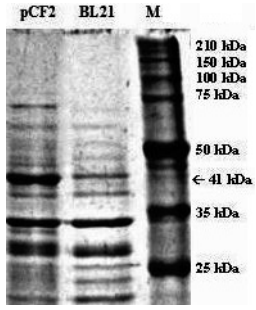

Figure 5. SDS/PAGE (16% w/v) analysis of the whole cell lysate sample from the *Escherichia coli* BL21(DE3)pLys cell culture containing plasmid pCF2.
Lane 1, cell lysate from IPTG-induced bacteria containing pCF2 construct; lane 2, negative control (without plasmid); lane 3, molecular mass marker (Promega). Recombinant 1,3-propanediol dehydrogenase (*dhaT*) (41 kDa) (lane 1) derived from *C. freundii* is indicated by an arrow.

IPTG was added to the final concentration of 1 mM. Cells were harvested after a 5-hour induction and resuspended in 0.1 vol. of 50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 8 M urea, and were then homogenized. Insoluble material was removed by centrifugation (10 000 rpm, 20 min at 4°C). Protein samples were separated by SDS-PAGE using 16% (w/v) SDS-polyacrylamide gel and identified by staining with Coomassie Brilliant Blue R-250 (Merck) (Fig. 5). SDS/PAGE analysis was carried out by the Laemmli method (Laemmli, 1970), with the use of a Mini-Protean3 Cell Electrophoresis System (BioRad). A protein molecular weight marker (Promega) was used to estimate the molecular masses of the proteins.

RNA isolation and RT-PCR analysis. Total RNA was prepared using a Total RNA Mini Plus kit as described by the manufacturer (A&A Biotechnology). The expression of the *dhaBCE*, *dhaF*, *dhaG* and *dhaT* genes was evaluated by RT-PCR. First strand cDNA synthesis was prepared by reverse transcription, using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Expression analysis was performed with the use of a PCR reaction with the total RNA matrix as a negative control to confirm the purity of RNA preparations. Gene constructs were used as a positive control.

Table 1. List of RT-PCR primers used in this study

Sequence of primers	Gene/Product size (bp)
F 5'-ATGAGAAGATCAAAACGATTCGA-3' R 5'-TTATTCGATCGAGCCGGGC-3'	*dhaB*/1668
F 5'-GTGGAATGCACAACTGAACG-3' R 5'-TCATTCCCTTACCAGTGCAAT-3'	*dhaC*/585
F 5'-ATGAACGACAACATCATGACTG-3' R 5'-TCACTGGCTGCCTTTACGC-3'	*dhaE*/429
F 5'-ATGCCGTTAATTGCAGGGATCGATATC-3' R 5'-CTATTTTTTTGCATCTCCGGCTAAAACCAG-3'	*dhaF*/1815
F 5'-ATGTCACTTTCATCACCGGG-3' R 5'-TTATTTTATCTCGCTGAAGGGA-3'	*dhaG*/354
F 5'- ATGAGCTATCGTATGTTTGATTA -3' R 5'- TCAGAATGCCTGACGGAAGAT -3'	*dhaT*/1164

Primers used in the RT-PCR analysis are listed in Table 1.

High-performance liquid chromatography analysis of metabolites. The presence of 1,3-propanediol and glycerol was analyzed using an Agilent Technologies 1200 series high-performance liquid chromatography system with a refractive index detector G1362A and a Rezex ROA — Organic Acid H^+ (8%), 300×7.8 mm column, while maintaining the temperature of the column oven at 40°C. The mobile phase was 0.0025 mM H_2SO_4. The samples were introduced through an injector valve (10 μl volume), while maintaining the flow rate of 0.6 ml/min using an isocratic pump system. The liquid chromatography system was also equipped with the Bin Pump G1312B, Autosampler G1329B, Column Thermostat G1316A and Degasser G1379B. The retention times of 1,3-PDO and glycerol were 16.4 and 12.9, respectively. The quantitative and qualitative identification was carried out by an external standard using peak areas (for measurement and integration, the ChemStation for LC 3D Systems, Agilent was used).

RESULTS

Sequence analysis

In total, 6897 bp of the 1,3-PDO operon in the *C. freundii* ATCC 8090 strain was sequenced (GenBank HM032027). The nucleotide sequence of the 1,3-PDO operon in the analyzed strain was shown to be 92% homologous with the DSM30040 strain sequence available in the GenBank database (U09771). For amino acid sequences of specific genes, the homology was between 92% and 99%.

RT-PCR analysis

RT-PCR analysis was used to determine the expression of heterologous genes in *E. coli* BL21(DE3)pLysS cells. This analysis was performed for bacteria that underwent cotransformation with two gene expression constructs, i.e. pCF1 and pCF2, containing genes from *C. freundii* ATCC 8090. RT-PCR analysis revealed the expression of all heterologous genes. No specific PCR products were observed in the negative control reactions.

Overexpression of pCF1 and pCF2 constructs

Genes *dhaBCE*, *dhaF* and *dhaG* from the pCF1 construct, as well as the *dhaT* gene from pCF2, were all transcribed in the same direction and were under control of the T7*lac* promoter. The *E. coli* BL21(DE3)pLysS bacteria were transformed using the pCF1 gene construct. Protein overexpression was observed in cell extracts obtained from the bacteria after overnight culture and induction of expression with IPTG added in the logarithmic growth phase ($OD_{600} \approx 0.6–0.8$), followed by homogenization in a glass homogenizer. Protein samples were separated by SDS-PAGE using 16% (w/v) polyacrylamide gel, and stained with Coomassie Blue (Fig. 5). An analogous procedure was followed for the pCF2 construct. For the latter, kanamycin (50 μg/ml) was used as a selection factor.

The results of the experiment (Fig. 5) indicated efficient overexpression of the *dhaT* gene from *C. freundii* in *E. coli*. In the gel, no products of the genes contained in the pCF1 construct, i.e. the three subunits of glycerol dehydratase (*dhaBCE*) and the two subunits of the glycerol dehydratase reactivation factor (*dhaF, dhaG*) were observed. Their presence was, however, confirmed by a functional test (the conversion of glycerol to 1,3-PDO in batch fermentation). The expression of the genes at the mRNA level in *E. coli* BL21(DE3)pLys was confirmed by RT-PCR. To assess the expression of the other heterologous genes through protein electrophoresis separation in the next stage of the study, the *E. coli* Rosetta 2(DE3)pLysS strain was used. SDS/PAGE analysis of the expression of products from genes inserted into the pCF1 construct indicated a higher level of the expressed protein in *E. coli* Rosetta 2(DE3)pLysS cells, compared to the *E. coli* BL21(DE3) pLysS strain. This was likely due to the presence of the plasmid equipping the bacterial cells with a set of tRNAs that are rare in *E. coli*.

Batch fermentation by the recombinant *E. coli* BL21(DE3)pLysS strain

After cotransformation of the *E. coli* BL21(DE3)pLysS strain cells with the pCF1 and pCF2 constructs, the presence of the introduced plasmids was verified *via* the PCR reaction with primers specific for the sequences encoding each enzyme. The *E. coli* BL21(DE3)pLysS colony, in which the presence of heterologous genes was confirmed, was used in culture tests. However, PCR vector stability analysis for *E. coli* BL21(DE3)pLysS confirmed that the bacteria could successfully be used after long-term storage at –80°C in 10% glycerol stock (Fig. 6).

In the first (24-hour) experiment, 1,3-PDO was identified in flask no. 4 at a concentration of 1.23 g/L. In the second (72-hour) experiment, which differed from the first one in terms of culture time, 1,3-PDO was identified in flask no. 4 at the concentration of 1.53 g/L. In both experiments, in flasks no. 3 and 6 containing the non-recombinant *E. coli* strain (negative control), and in flasks no. 2 and 5 containing the recombinant strain with no IPTG inducer, no 1,3-PDO was observed; in flask no. 1, small amounts of 1,3-PDO were found. All results are shown in Tables 2 and 3.

The purpose of the bioreactor culture was to determine 1,3-PDO yield and the efficiency of 1,3-PDO synthesis from glycerol in a pH-controlled, non-optimized culture. IPTG was added after 12 hours, and glycerol was added after 24 hours of culturing. In the Sartorius Biostat B Plus bioreactor, the recombinant strain containing gene constructs pCF1

Table 2. 1,3-propanediol production in flask cultures of the *E. coli* BL21(DE3) pLysS strain with the pCF1 and pCF2 gene constructs comprising *C. freundii* 1,3-PDO operon genes.
Results at 24 hours of culturing. Samples 1 and 4, recombinant *E. coli* BL21(DE3) pLysS strain induced with IPTG; 2 and 5, recombinant strain with no IPTG induction; 3 and 6, *E. coli* BL21(DE3)pLysS with no gene constructs (negative control). RT, retention time (min).

Sample No.	Glycerol RT	Glycerol [g/L]	1,3-PDO RT	1,3-PDO [g/L]	Initial glycerol [g/L]
1	13.0989	0.2468	16.4304	0.1333	
2	13.1140	0.1820	16.4088	0.0656	4
3	12.8414	3.7133	16.2720	0.0431	
4	12.8447	55.8650	16.3661	1.2279	
5	12.8438	55.5358	16.2950	0.5579	70
6	12.8339	63.9948	16.2756	0.4755	

Table 3. 1,3-propanediol production in flask cultures of the *E. coli* BL21(DE3) pLysS strain with the pCF1 and pCF2 gene constructs comprising *C. freundii* 1,3-PDO operon genes.
Results at 72 hours of culturing. Samples 1 and 4 — recombinant *E. coli* BL21(DE3) pLysS strain induced with IPTG; 2 and 5 — recombinant strain with no IPTG induction; 3 and 6 — *E. coli* BL21(DE3)pLysS with no gene constructs (negative control). RT – retention time (min).

Sample No.	Glycerol RT	Glycerol [g/L]	1,3-PDO RT	1,3-PDO [g/L]	Initial glycerol [g/L]
1	13.0380	0.2389	16.4450	0.1333	
2	13.0570	0.1805	16.4540	0.0656	4
3	12.8420	3.8244	16.1780	0.0431	
4	12.8500	48.8480	16.4110	1.5326	
5	12.8460	53.1630	16.1960	0.4456	70
6	12.8420	67.7660	16.1860	0.4168	

and pCF2 was shown to have used 24.2 g/L of glycerol to produce 11.7 g/L of 1,3-PDO, attaining the efficiency of 0.58 [$mol_{1,3\text{-PDO}}/mol_{glycerol}$] (Table 4).

DISCUSSION AND CONCLUSIONS

Demand for chemical raw materials, including 1,3-PDO, rises each year. Currently, 1,3-PDO (which is a substrate for polyurethane production) is obtained using chemical methods that have an adverse environmental impact and use non-renewable energy sources. PTT and polyurethanes produced from 1,3-PDO are widely used for e.g. in clothing and carpet manufacturing. Biotechnological synthesis of chemicals will gradually supersede the original method, as it is a novel approach enabling the use of renewable energy sources and waste substrates, taking advantage of the natural fermentation capabilities of bacterial strains.

Obtaining polyol from waste glycerol allows manufacturers to forgo the use of petroleum products for this purpose. Natural deposits are being gradually depleted, while demand from modern industry is growing exponentially. Hence, the importance of recuperation and recycling.

The purpose of the present study was to obtain, by means of genetic modification, *E. coli* bacteria capable of producing 1,3-PDO from glycerol. Native *E. coli* do not have a metabolic pathway for 1,3-PDO synthesis. Therefore, 1,3-PDO genes transferred from a donor (1,3-PDO producing) strain are fully heterologous for the microorganism. The main objectives of the study were to design and construct expression vectors and to insert them into the host cells, to enable 1,3-PDO production from glycerol.

The selection of *E. coli* bacteria for the 1,3-PDO production seems fully justified, since they are non-pathogenic, easy to culture and proliferate rapidly. *E. coli* bacteria are very convenient for these applications. For these reasons *E. coli* is one of the best prokaryotic systems for the production of valuable chemical compounds.

Table 4. 1,3-propanediol production in bioreactor Biostat B Plus (Sartorius) culture during the growth of *E. coli* BL21(DE3)pLysS strain containing constructs pCF1 and pCF2.

Fermentation time [h]	Glycerol [g/L] (final concentration)	1,3-PDO [g/L]	Yield $Y_{P/S}$ [mol/mol]
168	10.80	11.65	0.58

In this study, two gene expression constructs were generated, containing six heterologous genes of glycerol catabolism coming from *C. freundii* ATCC 8090. Selected genes belong to the reductive branch of glycerol dissimilation, which leads directly to the compound desired.

In *C. freundii*, 1,3-PDO operon genes, i.e. glycerol dehydratase (*dhaBCE*), the large subunit of the glycerol dehydratase reactivation factor, and 1,3-PDO dehydrogenase (*dhaT*), the small subunit of the glycerol dehydratase reactivation factor, are naturally controlled by two promoters and transcribed in opposite directions (Skraly *et al.*, 1998; Seifert *et al.*, 2001).

The literature available does not contain reports concerning the modification of *E. coli* strains using operon genes coming from *C. freundii* ATCC 8090 strain. In our work we have also sequenced a 1,3-PDO operon of this strain. Sequence data were submitted to the GenBank database and assigned accession number HM032027.1.

Our gene expression constructs were designed so that one vector incorporated genes responsible for the first stage of conversion, while the second vector contained genes connected with the final stage of conversion, all transcribed in the same direction and located under the control of T7*lac*, which is a strong promoter, allowing efficient expression of the inserted genes (Ikeda *et al.*, 1992). This approach allows gene constructs from various organisms to be inserted interchangeably into the *E. coli* cells, so that combinations yielding the highest efficiencies of 1,3-PDO production could be found.

This study incorporated a transformant selection based on genes encoding resistance to antibiotics (ampicillin and kanamycin), carried by the pET-22b(+) and pET-26b(+)vectors, respectively. Thanks to the use of vectors with different antibiotic resistance genes, it was possible to simultaneously maintain two gene constructs carrying the complete reductive pathway in the *E. coli* cells.

In the section concerning the molecular characteristics of the recombinant *E. coli* BL21(DE3)pLysS strain, the expression of incorporated genes was analyzed at the mRNA and protein levels. Electrophoretic separation of proteins confirmed efficient expression of the *dhaT* gene in the *E. coli* BL21(DE3)pLysS cells. RT-PCR analysis has confirmed the expression of all the incorporated genes at the mRNA level. Since one of the causes of problems with observation of pCF1 encoded proteins may be the presence of rare codons for *E. coli*, the pCF1 construct was incorporated into the Rosetta 2 (DE3) pLysS strain. Such an approach made it possible to observe several proteins which may correspond to products of genes contained in pCF1, although the results were difficult to interpret due to the presence of *E. coli* endogenous proteins (data not shown). Difficulties in identifying particular proteins in polyacrylamide gel notwithstanding, the results of the bacterial cultivation part of the study (glycerol-to-1,3-PDO conversion in batch fermentation) indicated that all the required proteins were produced in the recombinant *E. coli* BL21(DE3)pLysS bacteria.

Construct stability analysis for *E. coli* BL21(DE3) pLysS cells, performed using a colony PCR reaction with primers specific for the heterologous genes present in the bacterial colony (containing constructs pCF1 and pCF2) known to convert glycerol to 1,3-PDO, aimed

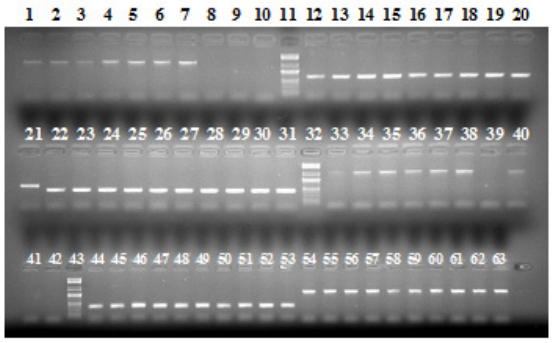

Figure 6. Analysis of *dhaB* (1668 bp), *dhaC* (585 bp), *dhaE* (429 bp), *dhaF* (1815 bp), *dhaG* (354 bp) and *dhaT* (1164 bp) gene presence, performed by using a colony PCR reaction with primers specific for the amplified genes from *C. freundii* ATCC 8090. PCR products were analyzed on a 1.2% agarose gel.
Lanes 1–10, *dhaB*; lanes 12–21, *dhaC*; lanes 22–31, *dhaE*; lanes 33–42, *dhaF*; lanes 44–53, *dhaG*; lanes 54–63, *dhaT*. Lanes no. 11, 32, and 43, size marker (KAPA Universal DNA Ladder).

at proving that both gene constructs are maintained in *E. coli* cells after long-term storage. The results confirmed that antibiotic selection is a sufficiently effective method to maintain both gene constructs in *E. coli* cells after a longer storage time (9 months) (Fig. 6).

A necessary stage in carrying out of the aim assumed in this study was connected with sequencing 1,3-PDO operon from the donor strain ATCC 8090. The differences observed between the sequences of the donor strain and the DSM30040 strain described in the GenBank database may affect the efficiency of 1,3-PDO production, which may vary for different strains.

Metabolic analysis of the recombinant *E. coli* BL21(DE3)pLysS strain involved flask cultures (24 h and 72 h) with no pH adjustments, and a more controlled bioreactor culture, aiming to identify 1,3-PDO in the production medium and determine its concentration via HPLC. The main purpose of the metabolic analysis was to confirm the expected functioning of the constructs introduced into *E. coli*. The 1,3-PDO yield established should be considered a preliminary result. Culture optimization, which was not a part of the present study, could significantly enhance the efficiency of glycerol-to-1,3-PDO conversion in the future.

In this study, batch fermentation was used, as the bacterial cultivation part of the study was designed to confirm the inserted genes' functionality. The results obtained (expression and cultures) indicate that the constructs were designed properly, as the expression of the heterologous genes enabled *E. coli* to produce 1,3-PDO, as opposed to the wild-type strain that has no such capabilities. This was confirmed with the flask experiments using a non-recombinant *E. coli* BL21(DE3)pLysS strain as a negative control.

In a batch flask culture, the recombinant *E. coli* bacteria produced 1.23 g/L of 1,3-PDO using 14.1 g/L of glycerol in 24 h, and 1.53 g/L of 1,3-PDO using 21.2 g/L of glycerol in 72 h. In the Sartorius Biostat B Plus reactor, they produced 11.7 g/L of 1,3-PDO using 24.2 g/L of glycerol, attaining an efficiency of 0.58 [$mol_{1,3\text{-}PDO}/mol_{glycerol}$] (Tables 2, 3, 4). Variants currently in use also include microorganisms naturally producing 1,3-PDO (despite their invasiveness), while the emphasis is placed on the use of recombinant microorganisms. Thanks to this study, input material for 1,3-propanediol production was obtained. The biotechnological process is highly sensitive to any change and sometimes even a slight difference in culture conditions (e.g. setting of mixer blades) influences the outcome.

More significant obstacles to efficient 1,3-PDO production include 3-HPA accumulation and the inactivation of glycerol dehydratase by glycerol (Tang *et al.*, 2009). The latter issue could be solved effectively by using a fed-batch or continuous culture, maintaining a constant low glycerol concentration. As to the accumulation of 3-HPA, which inhibits 1,3-PDO production, it could be limited in the future by overexpressing the *yqhD* gene present in native *E. coli*. The literature reports several examples of efficient 1,3-PDO production with this approach (Tang *et al.*, 2009). Additional overexpression of alcohol dehydrogenase from *E. coli* would likely decrease 3-HPA accumulation. *E. coli* has a single copy of the *yqhD* gene; the enzyme is not highly sensitive to oxygen and is capable of utilizing the NADH and NADPH coenzymes. This increases the number of cofactors available for reduction (Nakamura & Whited, 2003). In *E. coli*, it acts as a catalyst in reactions including the transformation of methylglyoxal to hydroxyacetone (Tang *et al.*, 2009; Clomburg *et al.*, 2010). The literature shows that with additional *yqhD* overexpression, the efficiency of 1,3-PDO production from glycerol is typically higher than in natural producers (Zhang *et al.*, 2005; Tang *et al.*, 2009; Seo *et al.*, 2010; Zhuge *et al.*, 2010). In regards to the concentration of methylglyoxal (which inhibits cellular growth and 1,3-PDO production), which rises significantly under anaerobic conditions, its amount could be reduced by the overexpression of glyoxalase 1 from *Pseudomonas putida*, as suggested by Zhu *et al.* (2001).

The literature also suggests adding fumarate to stimulate the activity of anaerobic G3P dehydrogenase, and consequently, to curb G3P accumulation (which inhibits cellular growth and 1,3-PDO production), or disable the gene encoding glycerol kinase responsible for G3P production (Zhu *et al.* 2002).

The final result is also affected by the cultivation temperature. Thus, a lower temperature may contribute to a more effective conversion of glycerol to 1,3-PDO, as it reduces the probability of producing heterologous enzymes in the form of inclusion bodies (Nuc *et al.*, 2006). Other good solutions for the industrial application of microbiological 1,3-PDO synthesis include immobilizing bacterial cells using polyurethane foams or microencapsulation. Such solutions facilitate the execution of continuous processes and the separation of the final product from biomass; they also increase cell stability and substrate tolerance, and enable the re-use of the microorganisms (Pflugmacher & Gottschalk, 1994; Zhao *et al.*, 2006). Data in the literature suggests that efficiency could be also increased by adding a specific mixture of ingredients enriching the growth medium, including thiamine, iron sulfate, zinc and calcium chlorides, cysteine hydrochloride (Tong *et al.*, 1991; Tong *et al.*, 1992), as well as citric acid, boric acid, and manganese, cobalt, copper and iron chlorides (Ma *et al.*, 2009; Ma *et al.*, 2010). These examples refer to cultures of recombinant *E. coli* bacteria comprising *K. pneumoniae* regulon genes, grown under aerobic conditions at pH 6.8.

In terms of potential changes in the method of inducing "foreign" gene expression, a glycerol-induced promoter merits consideration; this could reduce the cost, and expression would only occur during 1,3-PDO production. Another alternative is the use of a temperature-induced λ phage promoter.

Yet another solution is the use of the *E. coli rpoS* promoter, which is induced by a stress factor (low pH, high

or low temperature, nutrient deficiency, osmotic shock) (Liang *et al*., 2011).

The *E. coli* Rosetta 2 (DE3)pLysS strain with the expression constructs used in the present study could also be tested in the future.

So far, chemical technology has remained the most efficient and profitable method of 1,3-PDO production. However, the biotechnological product is indisputably more desirable on the market for numerous reasons, including its low environmental impact and the reduction in mined resource consumption. Currently, some available patents describe biotechnological processes for 1,3-PDO production, but mostly from glucose. So far, Emptage *et al*. have attained the highest efficiency of 1,3-PDO production from glucose by a recombinant *E. coli* strain containing genes from *S. cerevisiae* and *K. pneumoniae*.

The results of the present study give an optimistic view of the future for biotechnological production of valuable chemicals from renewable raw materials. Implementation will be possible under two conditions: optimizing the production stage and developing an efficient process for purifying 1,3-PDO from the production medium. These stages are vital to the development of an efficient biotechnological production method for this chemical, which is the first step towards it commercialization.

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