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RESEARCH

Molecular Cloning, Co-expression, and Characterization of Glycerol Dehydratase and 1,3-Propanediol Dehydrogenase from *Citrobacter freundii*

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Abstract 1,3-Propanediol (1,3-PD), an important material for chemical industry, is biologically synthesized by glycerol dehydratase (GDHt) and 1,3-propanediol dehydrogenase (PDOR). In present study, the *dhaBCE* and *dhaT* genes encoding glycerol dehydratase and 1,3-propanediol dehydrogenase respectively were cloned from *Citrobacter freundii* and co-expressed in *E. coli*. Sequence analysis revealed that the cloned genes were 85 and 77 % identical to corresponding gene of *C. freundii* DSM 30040 (GenBank No. U09771), respectively. The over-expressed recombinant enzymes were purified by nickel-chelate chromatography combined with gel filtration, and recombinant GDHt and PDOR were characterized by activity assay, kinetic analysis, pH, and temperature optimization. This research may form a basis for the future work on biological synthesis of 1,3-PD.

Keywords *Citrobacter freundii* · Co-expression · Characterization · Glycerol dehydratase · 1,3-Propanediol dehydrogenase

Introduction

The bacterial conversion of glycerol to 1,3-propanediol (1,3-PD) has attracted significant interest because of the recent development of a new polyester called poly (propylene terephthalate). The polymer is essentially made by substituting 1,3-PD for ethylene glycol in producing polyester and results in a new fiber with superior properties [1–3]. In the biological systems, glycerol dehydratase (GDHt, EC 4.2.1.30) catalyzes the conversion of glycerol to 3-hydroxypropionaldehyde, which is further converted to the final fermentation product 1,3-PD by 1,3-propanediol dehydrogenase (PDOR, EC 1.1.1.202) [4]. GDHt and PDOR of *Citrobacter freundii* are encoded by *dhaBCE* and *dhaT* genes, respectively, which are naturally controlled by two different promoters and are transcribed in different directions (Fig. 1) [5]. GDHt is the key and rate-limited enzyme of 1,3-PD synthesis, and coenzyme B_{12} is an essential cofactor for the activation of GHDt. PDOR is an octamer of which a monomer has a molecular mass of 42 kDa. Molecular cloning and expression of genes encoding GDHt and PDOR from *C. freundii* had been reported [6, 7]. However, studies on co-expression and characterization of GDHt and PDOR are still limited. In present study, the *dhaBCE* and *dhaT* genes encoding GDHt and PDOR, respectively, were cloned from the *dha* regulon of *C. freundii* and co-expressed in 1,3-PD non-producible *E. coli* [8]. The characterization of GDHt and PDOR were also determined.

Materials and Methods

Bacterial Strains, Plasmids, Culture Media and Growth Conditions

Citrobacter freundii AS1.1732 was obtained from CGMCC (Beijing, China). pSE380 (Invitrogen, USA, trc promoter) was used for over-expression of the genes encoding GDHt and PDOR from *C. freundii*. *E. coli* JM109 (Promega,

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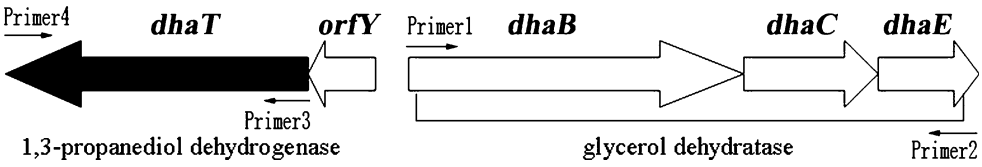

Fig. 1 *dha* Regulon containing the genes encoding 1,3-PD dehydrogenase and glycerol dehydratase

USA, 1,3-PD non-productive) was used as host. *C. freundii* was grown under aerobic condition in a pH 7.0 medium containing peptone (10 g/l); meat extract (3 g/l), and NaCl (5 g/l). Medium LB containing glycerol (1 %, w/v), coenzyme B_{12} (15 nM), and ampicillin (100 μg/ml) was used to produce 1,3-PD. *E. coli* and recombinant *E. coli* strains were routinely grown at 37 °C and 220 rpm in LB medium and LB with ampicillin (100 μg/ml), respectively.

Cloning of *dhaBCE* and *dhaT* Genes

dhaBCE and *dhaT* genes were amplified using primer1 and primer2, primer3, and primer4 (Table 1), respectively. The amplification conditions included initial melting at 95 °C for 2 min, 30 cycles of melting at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 3 min (*dhaBCE*), 1 min (*dhaT*), or 4 min (splicing by overlapping extension PCR), and a final extension at 72 °C for 10 min. PCR amplified *dhaBCE* and *dhaT* genes were identified by 0.8 % agarose electrophoresis and spliced by overlapping extension PCR (SOE-PCR). His-tagged PCR products of *dhaBCE*, *dhaT*, and *dhaBCET* (connected fragment) were treated with restriction enzyme *Nco*I or *Pag*I (isocaudarner of *Nco*I) and *Hin*d III, then ligated with pSE380 digested by *Nco*I and *Hin*d III, respectively. DNA sequence fidelity of these inserts of pSE380 was confirmed by sequencing (TaKaRa).

Primer1: upstream primer of *dhaBCE*; Primer2: downstream primer of *dhaBCE*; Primer3: upstream primer of *dhaT*; Primer4: downstream primer of *dhaT*. The underlined letters denote the synthetic restriction endonuclease recognition sites; the framed letters of primer2 and primer3 represent the overlapped sequences, primer2 and primer3 contain SD region (Italic); primer1 and primer3 for PCR contain sequences encoding 6 × His-tag (bold), and *Nde* I recognition site for recombinant plasmid analysis.

Table 1 Primer sequences used in PCR for *dhaBCE* and *dhaT* from *C. freundii*

Primer	Sequence
Primer1	5′-TGCCCATGGTT**CACCACCATCACCAT CAT**ATGGGAAGATCAAAACGATT-3′
Primer2	5′-AATAAGCTTATACAA*CCTCC*GTCACTG-3′
Primer3	5′-AGTGAC*GGAGG*TTGTCATGATT**CACCAC CATCACCATCAT**ATGAGCTATCGTATGT-3′
Primer4	5′-AAGAAGCTTATCAGAATGCCTGACGGA-3′

Co-expression of *dhaBCE* and *dhaT* Genes and Preparation of Cell Extracts

Constructed recombinant plasmids were transformed into the competent cells of *E. coli* JM109. When the OD_{600} of cell suspension reached 0.6, IPTG was added to induce gene expression. After 8 h induction, cells were harvested by centrifugation at 8,000×*g* for 5 min, washed twice with 100 mM potassium phosphate buffer at pH 8.0 and re-suspended in the relative buffer. The re-suspended cells were disrupted by sonication on ice. Cell debris was removed by centrifugation at 12,000×*g* for 5 min.

Purification and Identification of GDHt and PDOR

Recombinant GDHt and PDOR were purified by Ni-nitrilotriacetate affinity chromatography followed by Sephacryl S-300 gel filtration. The extract containing His-tagged GDHt was loaded onto Ni-nitrilotriacetate agarose gel column which had previously been equilibrated with Buffer A (100 mM potassium phosphate and 150 mM KCl) containing 5 mM imidazole. After washing the column with buffer A containing 40 mM imidazole, the enzyme was eluted with buffer A containing 200 mM imidazole. Then, the enzyme solution was loaded onto Sephacryl S-300 gel filtration column to remove unbound subunits by eluting with 100 mM potassium phosphate buffer. The purification of PDOR were similar to that of GDHt, except that the washing buffer for Ni-nitrilotriacetate agarose gel column was replaced with Buffer A containing 80 mM imidazole. All operations were carried out at 4 °C. Recombinant GHDt and PDOR were identified by 12.5 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Optimal Temperature and pH

Different buffers with pH values ranging from 6.0 to 10.8 were used to determine the optimal pH on the activity of recombinant GHDt and PDOR as described by Toraya et al. [9] and Daniel et al. [6]. Na_2HPO_4–KH_2PO_4 and K_2CO_3–$KHCO_3$ buffers (both at 100 mM) were prepared to cover the pH ranges of 6.0–9.0 and 9.0–10.8. Recombinant GDHt and PDOR activities were assayed with temperature ranging

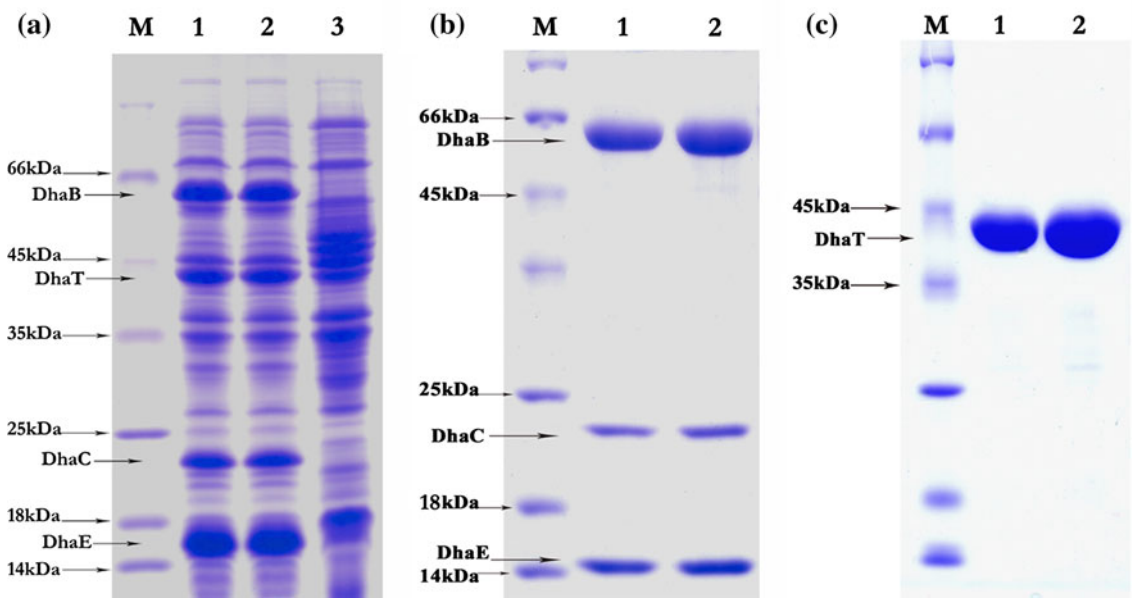

Fig. 2 **a** SDS-PAGE analysis of the crude extract from recombinant bacteria. *Lane M* protein molecular weight marker (the same in panel **b** and **c**); *Lanes 1 and 2 E. coli* JM109 with plasmid pSE-*dhaBCET*; *Lane 3*: *E. coli* JM109 with plasmid pSE380. **b** SDS-PAGE analysis of the purified GDHt. *Lane 1* purified GDHt by Ni-nitrilotriacetate and Sephacryl S-300; *Lane 2* purified GDHt by Ni-nitrilotriacetate. **b** SDS-PAGE analysis of the purified PDOR. *Lane 1* purified PDOR by Ni-nitrilotriacetate and Sephacryl S-300; *Lane 2* purified PDOR by Ni-nitrilotriacetate

from 20 to 55 °C and 20 to 45 °C, respectively, at the corresponding optimal pH using appropriate substrates.

Activity Assay and Kinetic Analyses

Activities of GDHt and PDOR were assayed according to Toraya's [9] and Daniel's [6] protocols. Protein concentrations were determined by the method of Bradford with bovine serum albumin as the standard. K_m and V_{max} values of enzymes at optimal temperature and pH were measured corresponding to standard Lineweaver–Burk plots. One-minute assay was employed for measurement of GDHt's K_m and V_{max} for glycerol and 1,2-propanediol (1,2-PD) [10].

Sequence Analyses

BLAST, DIALIGN (http://www.genomatix.de/cgi-bin/dialign/dialign.pl), ClustalX and Vector NTI were used to analyze the similarity of GDHt and PDOR.

Results

Molecular Cloning and Co-expression of *dhaBCE* and *dhaT* Genes

dhaBCE and *dhaT* genes were amplified from genomic DNA of *C. freundii* AS1.1732. DNA sequencing showed that *dhaBCE* and *dhaT* genes were 2,695 bp and 1,164 bp long, respectively. The sequence data of *dhaBCE* and *dhaT* genes have been deposited into GenBank under No. DQ152921 and DQ416747, respectively. Sequence analysis showed that GDHt was comprised of α subunit (556 amino acid residues), β subunit (195 amino acid residues), and γ subunit (145 amino acid residues), whereas each monomer of PDOR contained 388 amino acid residues.

In order to obtain high level expression of both genes, *dhaBCE* followed by *dhaT* was reinserted into operon controlled by a single promoter (trc promoter of pSE380). Then, the recombinant plasmid was transformed into *E. coli.* Homogenate of the recombinant *E. coli* cell was analyzed by SDS-PAGE to confirm the expression of GDHt and PDOR. The result showed three protein bands corresponding to α, β, and γ subunits of GDHt and one protein band corresponding to PDOR which had apparent molecular mass of about 61, 22, 16, and 42 kDa, respectively (Fig. 2a). These data are in excellent agreement with those derived from DNA sequencing. Protein bands of purified GDHt and PDOR were shown in Fig. 2b, c, respectively.

Analysis of Sequence Homology

The sequences of recombinant GDHt and PDOR were compared with corresponding data available in GenBank database. Recombinant GDHt exhibited 85 and 96 % similarities at nucleotide and amino acid levels, respectively, to that of *C. freundii* DSM30040 deposited in

GenBank (U09771). In addition, our GDHt was also similar to corresponding sequence from uncultured bacterium (GenBank No. AY205336), having 86 and 97 % sequence identity at nucleotide and amino acid level, respectively. Analysis result showed that our GDHt belonged to class II of coenzyme B_{12}-containing enzymes [11]. At the same time, our GDHt was highly similar to the same enzyme from *Klebsiella pneumoniae* (GenBank No. U60992) with amino acid sequence identity of 91 %. The conserved feature of the B_{12}-GDH interaction was also present in our GDHt, and about seven amino acid residues from α (E206, T223, and M374) and β (K102, T104, N117, and A124) subunits composed the conserved feature. Seven amino acid residues from α subunit (Q142, H144, E171, E222, Q297, D336, and S363) are also highly conserved, which are supposed to be the substrate preference residues [10, 12].

The similarity at amino acid level of PDOR obtained in this study to corresponding sequence from *C. freundii* DSM30040 was 90 % (77 % at nucleotide level), and 95 % to corresponding sequence from *K. pneumoniae* (GenBank No. U30903). Our PDOR also showed high similarity with member of a novel family (type III) of alcohol dehydrogenases (ADH), and analysis showed that there are two iron-containing alcohol dehydrogenases motif in our PDOR: one in amino acids 178–206 (SINDPLLMIGKPAALTAAT GMDALTHAVE), and the other in amino acids 265–285 (GYVHAMAHQLGGLYDMPHGVA) (Fig. 3). Amino acid residues Asp199, His203, and His282 were thought to be involved in Fe^{2+} binding. PDOR requires NAD(H) as a cofactor, but the highly conserved NAD(H) binding fingerprint pattern G–X–G–X–X–G was not present in the amino acid sequence. This is also characteristic of most type III alcohol dehydrogenases [13].

Optimal Temperature and pH

The effect of pH ranging from 6.0 to 10.8 on activities of GDHt and PDOR was investigated. As shown in Fig. 4a,

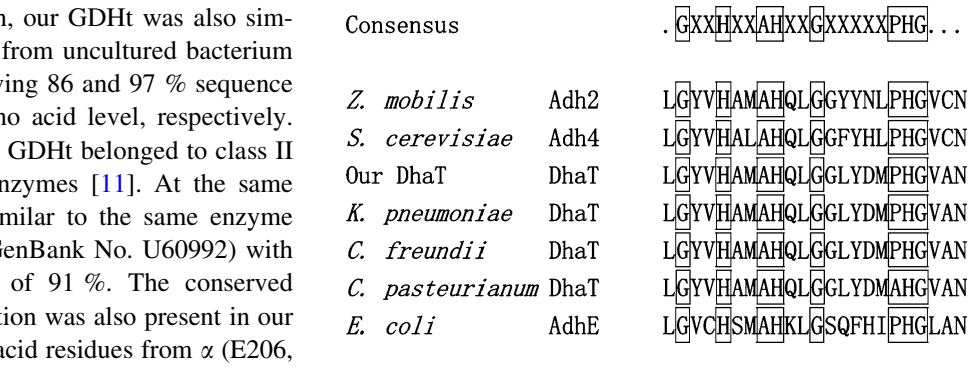

Fig. 3 Amino acid alignment of the protein regions of different alcohol dehydrogenases containing the putative iron-binding motif proposed as a typical feature of class III alcohol dehydrogenases. The consensus of the motif was compared with alcohol dehydrogenase (Adh2) of *Zymomonas mobilis* [16], alcohol dehydrogenase (Adh4) of *Saccharomyces cerevisiae* [17], 1,3-propanediol dehydrogenase (*dhaT*) of *K. pneumoniae* [12], 1,3-propanediol dehydrogenase (*dhaT*) of *C. freundii* [13], 1,3-propanediol dehydrogenase (*dhaT*) of *Clostridium pasteurianum* [18], and alcohol dehydrogenase (AdhE) of *E. coli* [19]

GDHt's maximal activity exhibited at pH 8.5, and the relative activity of GDHt on the condition of pH values ranging from 7.0 to 9.5 was over 80 %. However, the activities dropped dramatically when the pH value was higher than 9.5. GDHt's optimal temperature was 45.0 °C, and GDHt's activity lost dramatically at the temperatures above the optimal temperature (Fig. 4b).

In present study, PDOR's optimal pH of reduction and oxidation activity was 8.0 and 10.5, respectively. PDOR's activity (especially the oxidation activity) decreased quickly when the pH of reaction buffer was lower or higher than corresponding optimal values (Fig. 4a). The relative activity of PDOR's oxidation dropped below 10 % at pH 7.0. On the contrary, the relative activities of PDOR's reduction in the pH buffer ranging from 7.0 to 8.5 were above 80 %. As shown in Fig. 4b, the temperature effect of reduction and oxidation activity of PDOR were maximal at 37.0 and 25.0 °C, respectively, which dropped sharply near the corresponding optimal temperature values.

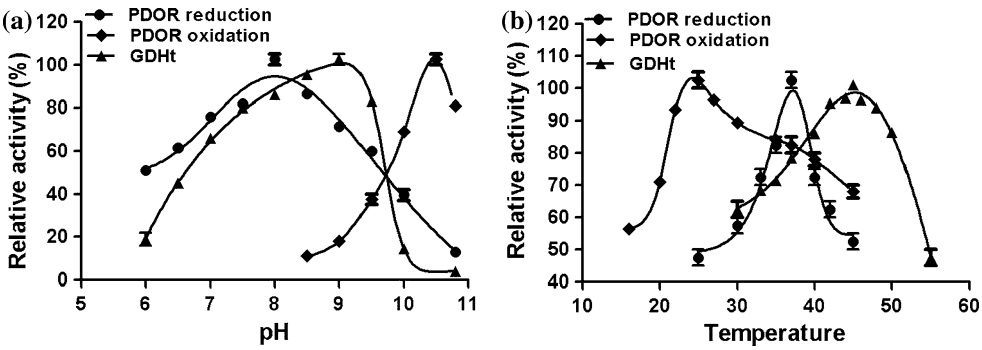

Fig. 4 Effect of pH (**a**) and temperature (**b**) on the activities of GDHt and PDOR. The activity under optimal pH and temperature was defined as 100 %

Table 2 Purification and kinetic properties of recombinant GDHt (for 1,2-PD) and PDOR (for propionaldehyde)

Purification step		Total protein (mg)	Total activity (units)	Specific activity (U/mg)	Purification fold	Enzyme yield (%)
GDHt	Crude extract	29	631	21.2	1.0	100
	Ni-nitrilotriacetic	5.2	436	83.8	4.0	69
	Sephacryl S-300	3.6	329	91.9	4.3	52
PDOR	Crude extract	32	202	6.3	1.0	100
	Ni-nitrilotriacetic	3.2	143	45.1	7.1	71
	Sephacryl S-300	2.4	119	49.5	7.8	59

Specific Activities and Kinetic Analysis

GDHt and PDOR from *C. freundii* and the recombinant enzyme produced in *E. coli* are capable of catalyzing the coenzyme B_{12}-dependent conversion of glycerol to 1,3-PD, as is typical of all characterized corresponding enzymes from other organisms [7]. In this experiment, recombinant GDHt from *C. freundii* AS1.1732 showed higher activity toward substrate glycerol than toward 1,2-PD. Specific activity of GDHt for 1,2-PD was 91.9 U/mg (Table 2). The reduction and oxidation activities of PDOR were surveyed respectively using propionaldehyde and 1,3-PD as substrates. It was found that reduction of PDOR for propionaldehyde was 49.5 U/mg and oxidation of PDOR for 1,3-PD was 77.9 U/mg.

GDHt displayed classical Michaelis–Menten kinetics, with apparent K_m values of 0.25 mM (for 1,2-PD) and 0.59 mM (for glycerol), and V_{max} values of 73.5 U/mg (for 1,2-PD) and 142.9 U/mg (for glycerol). The K_m and V_{max} values of PDOR for propionaldehyde were 10.05 mM and 37.3 U/mg, and those for 1,3-PD were 1.28 mM and 25.6 U/mg, respectively.

Discussion

Our study was the first report to demonstrate the co-expression of GDHt and PDOR genes and characterization of recombinant enzymes obtained from *C. freundii.* Sequence homology studies showed that our GDHt is very similar to corresponding enzymes from *C. freundii* DSM30040 and *K. pneumoniae*, and key amino acids residues are highly conserved. This slight difference in sequence might be due to the long time evolution and regional differences. Since the recombinant glycerol dehydratase of *C. freundii* AS1.1732 has been characterized in this study, more precise figures can now be given for this type of enzyme.

The K_m values of the PDOR were in reasonable agreement with the corresponding data of *C. freundii* DSM30040 (11 mM for propionaldehyde and 1.25 mM for 1,3-PD) [6], but were not in agreement with the K_m values for 1,3-PD of PDOR from *K. pneumoniae* ECL2103 (GenBank No. U30903, 18 mM) [14] and *K. pneumoniae* DSM2026 (8.5 mM) [15]. In addition, the optimal pH (10.5) and temperature (25.0°) about the oxidation activity of PDOR reported here were not in consistence with those of PDOR from *K. pneumoniae* DSM2026 (9.5 and 30°) [15]. However, it is very interesting that recombinant PDOR did not have the highest similarity with PDOR from *C. freundii* DSM30040, but had the highest similarity with those enzymes from *K. pneumoniae*. This could be the results of horizontal gene transfer and evolution.

The characterization of GDHt and PDOR reported in this study led us to suggest that the different properties of GDHt and PDOR is probably associated with the changes in their amino acid sequences apart from conserved residues, which further result in various catalytic abilities. As we knew, the production of 1,3-PD was dependent on the reduction of PDOR but not oxidation of PDOR [6]. Thus, optimal pH and temperature of GDHt and PDOR suggested that the conditions of pH 7.0–8.5 and 37 °C could maintain the highest activities of GDHt and PDOR's reduction, which might be a benefit to improve the production of 1,3-PD during fermentation.

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References

1. Ringel, A. K., Wilkens, E., Hortig, D., Willke, T., & Vorlop, K. D. (2012). An improved screening method for microorganisms able to convert crude glycerol to 1,3-propanediol and to tolerate high product concentrations. *Applied Microbiology Biotechnology, 93*(3), 1049–1056.
2. Demain, A. L., & Adrio, J. L. (2008). Contributions of microorganisms to industrial biology. *Molecular Biotechnology, 38*(1), 41–55.

3. Wang, F., Qu, H., Zhang, D., Tian, P., & Tan, T. (2007). Production of 1,3-propanediol from glycerol by recombinant *E. coli* using incompatible plasmids system. *Molecular Biotechnology, 37*(2), 112–119.
4. Liu, H., Xu, Y., Zheng, Z., & Liu, D. (2011). 1,3-Propanediol and its copolymers: Research, development and industrialization. *Biotechnology Journal, 5*(11), 1137–1148.
5. Seifert, C., Bowien, S., Gottschalk, G., & Daniel, R. (2001). Identification and expression of the genes and purification and characterization of the gene products involved in reactivation of coenzyme B_{12}-dependent glycerol dehydratase of *Citrobacter freundii*. *European Journal of Biochemistry, 268*(8), 2369–2378.
6. Daniel, R., Stuertz, K., & Gottschalk, G. (1995). Biochemical and molecular characterization of the oxidative branch of glycerol utilization by *Citrobacter freundii*. *Journal of Bacteriology, 177*(15), 4392–4401.
7. Seyfried, M., Daniel, R., & Gottschalk, G. (1996). Cloning, sequencing, and overexpression of the genes encoding coenzyme B12-dependent glycerol dehydratase of *Citrobacter freundii*. *Journal of Bacteriology, 178*(19), 5793–5796.
8. Nakamura, C. E., & Whited, G. M. (2003). Metabolic engineering for the microbial production of 1,3-propanediol. *Current Opinion in Biotechnology, 14*(5), 454–459.
9. Toraya, T., Ushio, K., Fukui, S., & Hogenkamp, P. C. (1977). Studies on the mechanism of the adenosylcobalamin-dependent diol dehydrase reaction by the use of analogs of the coenzyme. *Journal of Biological Chemistry, 252*(3), 963–970.
10. Yamanishi, M., Yunoki, M., Tobimatsu, T., Sato, H., Matsui, J., Dokiya, A., et al. (2002). The crystal structure of coenzyme B_{12}-dependent glycerol dehydratase in complex with cobalamin and propane-1,2-diol. *European Journal of Biochemistry, 269*(18), 4484–4494.
11. Raynaud, C., Sarcabal, P., Meynial, S. I., Croux, C., & Soucaille, P. (2003). Molecular characterization of the 1,3-propanediol (1,3-PD) operon of *Clostridium butyricum*. *Proceedings of the National Academy of Sciences of the United States of America, 100*(9), 5010–5015.
12. Liao, D. I., Dotson, G., Turner, I., Reiss, L., & Emptage, M. (2003). Crystal structure of substrate free form of glycerol dehydratase. *Journal of Inorganic Biochemistry, 93*(1–2), 84–91.
13. Daniel, R., Boenigk, R., & Gottschalk, G. (1995). Purification of 1,3-propanediol dehydrogenase from *Citrobacter freundii* and cloning, sequencing, and overexpression of the corresponding gene in *Escherichia coli*. *Journal of Bacteriology, 177*(8), 2151–2156.
14. Johnson, E. A., & Lin, E. C. (1987). *Klebsiella pneumoniae* 1,3-propanediol: NAD^+ oxidoreductase. *Journal of Bacteriology, 169*(5), 2050–2054.
15. Fenghuan, W., Huijin, Q., He, H., & Tan, T. (2005). High-level expression of the 1,3-propanediol oxidoreductase from *Klebsiella pneumoniae* in *Escherichia coli*. *Molecular Biotechnology, 31*(3), 211–219.
16. Conway, T., Sewell, G. W., Osman, Y. A., & Ingram, L. O. (1987). Cloning and sequencing of the alcohol dehydrogenase II gene from *Zymomonas mobilis*. *Journal of Bacteriology, 169*(6), 2591–2597.
17. Williamson, V. M., & Paquin, C. E. (1987). Homology of *Saccharomyces cerevisiae* ADH4 to an iron-activated alcohol dehydrogenase from *Zymomonas mobilis*. *Molecular and General Genetics, 209*(2), 374–381.
18. Luers, F., Seyfried, M., Daniel, R., & Gottschalk, G. (1997). Glycerol conversion to 1,3-propanediol by *Clostridium pasteurianum*: Cloning and expression of the gene encoding 1,3-propanediol dehydrogenase. *FEMS Microbiology Letters, 154*, 337–345.
19. Goodlove, P. E., Cunningham, P. R., Parker, J., & Clark, D. P. (1989). Cloning and sequence analysis of the fermentative alcohol-dehydrogenase-encoding gene of *Escherichia coli*. *Gene, 85*(1), 209–214.