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Full Length Research Paper

Production of 1,3-propanediol from glycerol by engineered *Escherichia coli* using a novel co-expression vector

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1,3-Propanediol (1,3-PD) has versatile applications in polymers, cosmetics, foods and medicines. In order to consolidate the functions of glycerol dehydratase gene *dha*B and 1,3-propanediol oxidoreductase gene *dha*T and produce 1,3-PD from glycerol, the genes *dha*B and *dha*T from *Klebsiella pneumoniae* were inserted into a co-expression vector pACYCDuet-1 synchronously and the recombinant strain *E. coli*/pACYCDuet-*dha*B-*dha*T was obtained. Both enzymes were functionally co-expressed in *E. coli* at the presence of the selective pressure and the addition of the IPTG. The specific enzyme activity of DHAB and DHAT were 8.3 and 6.2 U/mg, respectively. When cultivated at 37°C for 30 h, the recombinant microorganisms produced 1,3-PD of 11.3 g with the consumption of 40 g glycerol per liter. The production of 1,3-PD by the strain *E. coli*/pACYCDuet-*dha*B-*dha*T was about 13-fold higher than the recombinant *E. coli* harboring the gene *dha*B.

Key words: 1,3-propanediol, *Klebsiella pneumoniae*, co-expression system, T_7 RNA polymerase promoter.

INTRODUCTION

1,3-Propanediol (1,3-PD) is one of the most interested raw materials for chemical industries due to its wide use in the different fields such as inks, polymers and medical applications. Annually over one million ton of 1,3-PD is produced by chemical method, which requires high cost and is non-friendship to the environment (Biebl et al., 1999). More interests have now been focused on the production of 1, 3-PD from the renewable resources through economical and "green" processes such as microbial fermentation (Biebl et al., 1999; Huang et al., 2002). Glycerol becomes a good renewable resource because it is formed as a byproduct during biodiesel production with a large volume. So it is necessary to develop a new technology to convert glycerol into the other products of high value such as 1,3-PD. Dupont and Genencor Corporations (USA) constructed a few of recombinant microorganisms to produce 1,3-PD (Emptage et al., 2003). DuPont was also granted patent on a series of processes to convert glycerol or sugar to 1, 3-PD with the various organisms expressing the target genes from *Klebsiella pneumoniae* (Laffend et al., 1997).

In nature, 1,3-PD is produced by a few microorganisms including *Klebsiella* (Huang et al., 2002), *Citrobacters* (Boenigk et al., 1993) and *Closyridia* (Saint-amans et al., 2001). *K. pneumoniae* is one of the microorganisms which converts to 1, 3-PD with glycerol as the substrate (Menzel et al., 1997) by a dismutation process involving oxidative and reductive branch pathways. In the oxidative branch pathway, glycerol is dehydrogenated by a NAD-linked glycerol dehydrogenase to dihydroxyacetone, which is then phosphorylated and funneled to central metabolism by dihydroxyacetone kinase. In the reductive one, glycerol is dehydrated by coenzyme B_{12}-dependent glycerol dehydratase to form 3-hydroxypropionaldehyde (3-HPA), which is then reduced to 1, 3-PD by the NADH-linked 1,3-propanediol oxidoreductase.

Escherichia coli has been used as a host to express

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Table 1. Strains, plasmids and primers used in this study.

Strains/plasmids/primers	Description	Source or reference
Strains		
K. pneumoniae	the source of *dha*B and *dha*T	Our lab
E. coli JM109	host for gene cloning	(Yanish-Perron et al., 1985)
E. coli BL21	host for gene expression	Our lab
E. coli /pACT	*E. coli* BL21 harboring plasmid pACT	This work
E. coli/ pACB	*E .coli* BL21 harboring plasmid pACB	This work
E. coli /pACTB	*E .coli* BL21 harboring plasmid pACTB	This work
Plasmids		
pGEM-T-Easy vector	cloning vector	Promega
T-*dha*T	pGEM-T-Easy vector containing gene *dha*T	This work
T-*dha*B	pGEM-T-Easy vector containing gene *dha*B	This work
pACYCDuet	a vector for the coexpression of two target genes	Novage
pACT	pACYCDuet containing gene *dha*T	This work
pACB	pACYCDuet containing gene *dha*B	This work
pACTB	pACYCDuet containing genes *dha*T and *dha*B	This work
Primers		
*dha*B_F	5'-ACCG*GAATTC*ATGAAAAGATCAAAACGATTTGCAGTAC-3'	This work
*dha*B_R	5'-ACCG*AAGCTT*TTAATTCGCCTGACCGGCCAGTA-3'	This work
*dha*T_F	5'-ACCG*AGATCT*ATGAGCTATCGTATGTTTG -3'	This work
*dha*T_R	5'-ACCG*CTCGAG*TCAGAATGCCTGGCG-3'	This work

the genes of the *dha* regulon from *K. pneumoniiae* in *E. coli* AG1 (Skraly et al., 1998) or those from *C. freumdii dha* regulon in *E. coli* ELC707 (Daniel and Gottschalk, 1992). Wang et al. (2007) recently reported to produce 1, 3-PD from glycerol with the recombinant *E. coli* containing the genes *dha*B and *dha*T under control of the same T_7 RNA polymerase promoter in parallel plasmid respectively.

In this work, in order to realize a new process to produce 1,3-PD from glycerol, we developed the novel system in *E. coli* by co-expressing the glycerol dehydratase gene *dha*B and 1,3-propanediol oxidoreductase gene *dha*T on a vector pACYCDuet-1 with two multiple cloning sites. The results showed that both enzymes were functionally expressed in *E. coli*. The recombinant strain was confirmed to produce 1,3-PD by fermentation of glycerol. Therefore, we successfully developed the novel co-expression system to transform the product 1,3-PD from glycerol .

MATERIALS AND METHODS

Materials

PCR reagents and restriction enzymes were purchased from TaKaRa Biotechnology Co. Ltd. The Miniprep kit and Gel Extraction kit were purchased from Promega, USA. All other chemicals were of the highest grade that could be obtained commercially.

Microorganisms and cultivation

K. pneumoniae was grown in the Luria-Bertani (LB) medium. *E. coli* was cultivated in LB medium supplemented with 100 μg /ml ampicillin or 25 μg /ml chloramphenicol (CM) when necessary.

Construction of expression plasmids

Bacterial strains, plasmids and oligonucleotide primers were used in Table 1. The gene *dha*B encoding the glycerol dehydratase was amplified by PCR technique using *dha*B_F and *dha*B_R as the primers (Table 1) and *K. pneumoniae* genome as the template. The purified PCR product was ligated to the vector pGEM-T-Easy. The positive plasmid T-*dha*B was confirmed by digestion with restriction enzymes *Eco*RI/*Hin*dIII and DNA sequencing. The recombinant plasmid T-*dha*T was constructed in the similar way to T-*dha*B and confirmed by digestion with *Bgl*IIand XhoI and DNA sequencing. The positive plasmids T-*dha*B and T-*dha*T were transformed into *E. coli* JM 109 by the calcium chloride method (Ausubel et al., 1987), respectively.

The *Bgl*II and *Xho*I site of *dha*T released from T-*dha*T was ligated into the corresponding site of pACYCDuet-1. The positive plasmid pACT was confirmed by digestion with restriction enzymes. The construction of pACB was similar to that of pACT. The *Eco*R I/*Hin*dIII fragment of *dha*B from pACB was inserted to the other multiple clone site of pACT. After confirmation by digestion with *Eco*RI/*Hin*dIII, the recombinant pACTB was obtained. The positive plasmids pACT, pACB and pACTB were transformed into the competent *E. coli* BL21 (DE3) cells by the calcium chloride method, respectively. The recombinant strains *E. coli*/ pACT, *E. coli*/ pACB and *E. coli*/ pACTB were obtained by confirmed by with restriction

enzymes (*Eco*R I/*Hin*dIII and/or *Bgl* I/*Xho* I) and DNA sequencing.

Expression of DHAB and DHAT

The recombinant strains *E. coli*/ pACT, *E. coli*/ pACB and *E. coli*/ pACTB were grown on the LB liquid medium containing the 25 μg /ml CM at 37℃, respectively. The protein expression was induced with addition of 0.1 mM isopropyl-β-D- thiogalactopyranoside (IPTG) when the OD_{600} value of culture reached 0.4 - 0.6. After continuous cultivation for 5-6 h, the cells were harvested by centrifugation at 10,000×g for 15 min.

Preparation of the cell-free extract

The collected cells were washed twice with 20 mM Tris buffer (pH 8.0) and disrupted with an ultrasonic oscillator (Sonic Materials Co., Danbury, CT, USA). The cell debris was removed by centrifugation (15 min at 15 000 g) at 4℃ and the supernatant was used as the cell-free extract.

Enzyme assays

The enzyme activity of DHAB was estimated by the 3-methyl-2-benzothiazolinon hydrazone (MBTH) method described by Toraya et al. (1977). It was based on the ability of aldehydes to react with MBTH and form the azine derivatives, whose concentration could be determined spectrophotometrically. The assay mixture contained 0.2 mM glycerol, 0.05 mM KCl, 100 mM potassium phosphate buffer (pH 8.0) and 15 μM coenzyme B_{12}. The action was carried out at 37℃, then terminated by adding 1 ml 100 mM potassium citrate buffer (pH 3.6) and 0.5 ml 0.1% MBTH hydrochloride. The 3-hydroxypropionaldehyde (3-HPA) formed was determined by the absorbance at 305 nm relative to a standard curve.

The activity of DHAT was measured in the oxidative direction as described by Johnson and Lin, (1987). The enzyme assay was carried out in a reaction mixture in 1 mL containing 2 mM NAD^+, 30 mM $(NH_4)_2SO_4$, 100 mM 1,3-propanediol and 100 mM potassium bicarbonate (pH 9.0) with 2 mM DTT. The reduction of NAD^+ was recorded spectrophotometrically at 340 nm. One unit of enzyme activity is defined as the amount of enzyme catalyzing the reduction/oxidation of 1 mM of NAD(H) per min under the assay conditions.

Glycerol fermentation

The fermentation medium in one liter consisted of yeast extract 5 g, KH_2PO_4 7.5 g, $MgSO_4 \cdot 7H_2O$ 2 g, citric acid 2 g, $CaCl_2 \cdot 2H_2O$0.5 g, $(NH_4)_2SO_4$ 0.2 g, $FeSO_4 \cdot 7H_2O$ 0.005 g, glycerol 40 g, 1.5 μM coenzyme B_{12} and 5 ml trace element solution (0.68 g $ZnCl_2$, 2.0 g $MnCl_2 \cdot 4H_2O$, 60 mg H_3BO_3, 0.47 g $CoCl_2 \cdot 6H_2O$, 5 mg $Na_2MoO_4 \cdot 2H_2O$, 17 g $CuCl_2 \cdot 2H_2O$, 5.4 g $FeCl_3 \cdot 6H_2O$, 10 ml 37%HCl). The fermentations were carried out in a rotary shaker incubator under aerobic conditions at 150 rpm, initial pH 6.8 and 37℃. Five percent (v/v) of the seed culture was inoculated. All the seed and fermentation medium of the recombinant *E. coli* were supplemented with CM to maintain the plasmids. At OD_{600} of approximate 0.4 - 0.6, the IPTG was added to the final concentration of 1 mM for fermentation.

Determination of 1, 3-propanediol by gas chromatograph

The determination of 1, 3-propanediol was carried out with a gas chromatograph (SHIMAZU GC-14B, FID-detector, 2 m × Ø 5 mm stainless steel column) packed with Chromosorb101 and operated with N_2 as carrier gas at flow rate of 40 ml min^{-1}, detector temperature 220℃ and column temperature 210℃.

RESULTS

Construction of the recombinant plasmid

It was reported that the genes *dha*B and *dha*T are transcribed in opposite direction from a common region in the native genome DNA of *K. pneumoniae* (Tong et al., 1991). In order to consolidate the functions of both genes *dha*B and *dha*T in *E. coli*, we designed an expression construct that allows their sequential expression using the plasmid pACYCDuet-1 with two multiple cloning sites preceded by a phage T_7 RNA polymerase promoter and ribosome binding site, So the two genes would transcribe more efficiently in the same direction. The expression plasmids pACT, pACB and pACTB were constructed as described in the “Materials and methods” section. The plasmid pACT harboring the *dha*T was confirmed by digestion with *Bgl* II and *Xho* I. As shown in Figure 2, one *Bgl* II site and one *Xho* I site that can divided the plasmid into two major fragments with the sizes of 4000 and 1200 bp, respectively, which are consistent with the map of pACT (Figure 1). The plasmid pACB harboring the *dha*B was confirmed by digestion with *Eco*RI and *Hin*dIII. As shown in Figure 2, *Eco*R I and *Hin*dIII that can divided the plasmid into two major fragments with the sizes of 4000 and 2700 bp, respectively, which are consistent with the map of pACB (Figure 1). The plasmid pACTB was analyzed by double digestion with *Eco*R I/*Hin*dIII and the results showed that two DNA fragments (5200 and 2700 bp) were observed (Figure 1). Three recombinant expression plasmids pACT, pACB and pACTB were transformed into the competent *E. coli* BL21 (DE3) cells. Then the positive clones *E. coli*/pACT, *E. coli*/pACB and *E. coli*/p ACTB were achieved after verified by DNA sequence determination.

Co-expression of *dha*T and *dha*B in the recombinant *E. coli* BL21

The culture and inducing conditions were carried out as described in materials and methods. The proteins expression was analyzed by SDS-PAGE analysis. The results showed that the protein DHAT was expressed highly in *E. coli*/pACT and DHAB was produced in *E. coli*/pACB (Figure 3). The proteins DHAT and DHAB were both predominantly produced in *E. coli*/pACTB.

Enzyme activity assays

In order to investigate the biological functions of DHAT and DHAB in the recombinant cells, the specific activity were detected in the cell-free extracts of *E. coli*/pACT,

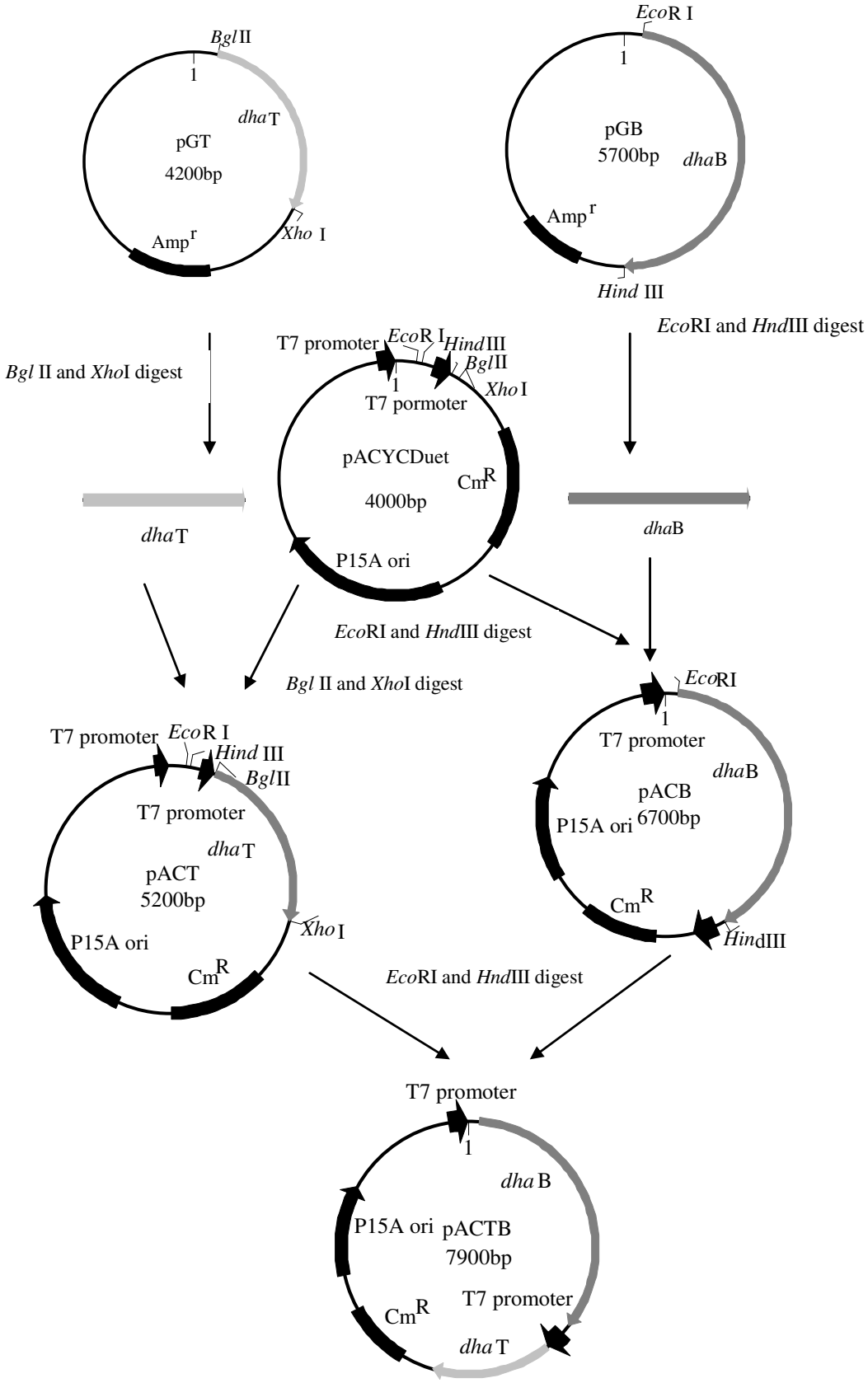

Figure 1. The sketch map of recombinant pACTB construction. The arrows indicate the flowing direction during construction of the key plasmid pACTB.

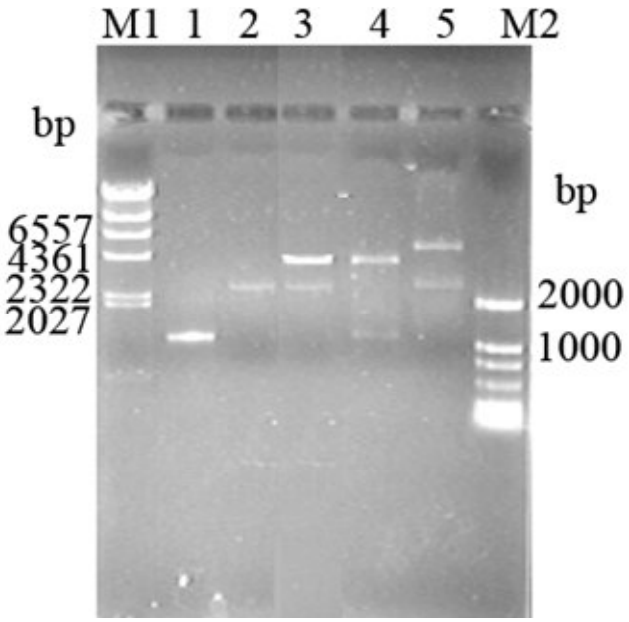

Figure 2. Identification of the recombinant plasmids by digestion with restriction enzymes. Lane 1, the gene *dha*T digested with *Bgl*II and *Xho*I; Lane 2, the gene *dha*B digested with *Eco*RI and *Hin*dIII; Lane 3, the plasmid pACB digested with *Eco*RI and *Hin*dIII; Lane 4, plasmid pACT digested with *Bgl*II and *Xho*I; Lane 5, plasmid pACTB digested with *Eco*RI and *Hin*dIII.

E. coli/pACTB and *E. coli*/pACTB. As showed in Table 2, the specific activity of DHAT was 6.5 U/mg in *E. coli*/pACT and DHAB was 8.5 U/mg in *E. coli*/pACB. In the co-expression system *E. coli*/pACTB, the enzyme activities of DHAT and DHAB were 6.2 and 8.3 U/mg in *E. coli*/pACTB, respectively. These results indicated that the enzymes DHAT and DHAB both functionally expressed and their specific activities were not affected by the co-expression of both enzymes in *E. coli*.

Fermentation of recombinant *E. coli*/pACTB

To test whether the engineered *E. coli*/pACTB could convert glycerol to 1,3-propanediol, the fermentation experiment was carried out with the medium containing glycerol under the aerobic conditions. By fermentation of glycerol, the recombinant *E. coli*/pACTB produced 1,3-propanediol of 11.3 g/L with the consumption of 40 g/L glycerol. But the same product was not detected in the fermentation media of the wild type *E. coli* BL21 or *E. coli*/pACT by gas chromatography. These results suggested that both enzymes DHAT and DHAB were functionally expressed and their functions were well consolidated in *E. coli* as well. More interestingly, a small quantity of 1,3-propanediol could be detected in the fermentation media of the recombinant *E. coli*/pACB (Table 2), which maybe due to the NADPH-linked alcohol dehydrogenase encoded by *yqh*D from *E. coli* can non-specifically catalyze 3-HPA to 1, 3-propanediol (Emptage et al., 2003).

DISCUSSION

In the metabolic pathway of glycerol to 1,3-PD, the enzyme DHAB dehydrates glycerol to form intermediate (3-HPA) and then the enzyme DHAT reduces 3-HPA to 1,3-PD. In this study, in order to realize the possibility of producing 1,3-PD from glycerol, the novel co-expression system *E. coli*/pACTB harboring their coding genes *dha*B and *dha*T was constructed. The results showed that both enzymes DHAB and DHAT were functionally co-expressed in the recombinant *E. coli* and their specific enzyme activity were 6.2 and 8.3 U/mg. Compared to those of the recombinant *E. coli*/pACT and *E. coli*/pACB, no change was detectable in the specific activity of DHAT or DHAB. By gas chromatography the product 1,3-PD of 11.3 g/L was detected in the fermentation media with glycerol as the substrate, further indicating the genes *dha* B and *dha*T were well consolidated in *E. coli*. Although several groups have reported 1,3-propaediol production in the fermentation of glycerol by the other micro-organisms (Menzel et al., 1997; Zeng et al., 1993), this is the first report to realize the pathway by an co-expression construct that allows the sequential expression of key enzymes in *E. coli*. This work is our first step, the improved expression systems of the target enzymes for the synthesis of 1,3-PD in organisms other than *E. coli* will be further developed to increase the production of 1,3-PD. For example, the *dha* operon was expressed in natural producers of 1,3-PD or in organisms for high tolerance to glycerol or 1,3-PD. The 1,3-PD pathway construction of *Klebsiella* in *E. coli* will supply the opportunity to investigate the interactions between its metabolic processes in the foreign biochemical background. The work will also provide the basis to improve the production of 1,3-PD from glycerol and extend the substrate range of other abundant renewable resources, such as sugars and starch. An exciting example was reported that the over-expressed DHAT and DHAB in *Candida glycerinogenes* strain WL2002-5 could produce 120 g/L glycerol in the media containing 25% D-glucose (Zhuge et al., 2001). Likewise, the engineered *C. glycerinogenes* capable of producing 1,3-PD from glucose will be constructed in our later works.

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Table 2. The enzyme activities of glycerol dehydratase (DHAB) and 1,3-propanediol oxidoreductase (DHAT) and production of 1,3-propanediol by the recombinant *E. coli*.

Strain	DHAB (U/mg protein)	DHAT (U/mg protein)	1,3-propanediol (g/L)
E. coli BL21	0	0	0
E. coli /pAC	0	0	0
E. coli /pACB	8.5 ± 0.1	0	0.81 ± 0.01
E. coli /pACT	0	6.5 ± 0.2	0
E. coli /pACTB	8.3 ± 0.2	6.2 ± 0.3	11.3 ± 0.3

The different strains were cultivated at 37°C for 30 h in the fermentation medium containing 40 g/L glycerol. The values showed were averaged from two independent triplicate experiments with means less than 5%.

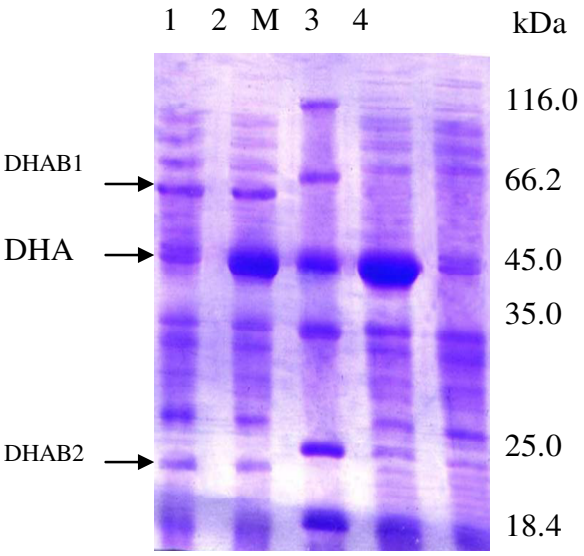

Figure 3. SDS-PAGE analysis of protein expression in *E. coli* BL21 (DE3). M, protein molecular weight marker; Lane 1, expression of DHAB in *E. coli*/pACB; Lane 2, Expression of DHAT and DHAB in *E. coli*/pACTB; Lane 3, expression of DHAT in *E. coli*/pACT; Lane 4, crude extract before IPTG induction in *E. coli* BL21 (DE3). The target proteins were marked with arrows. Proteins were visualized with Coomassie Brilliant Blue R-250 and destained in a 50% (v/v) methanol/10% (v/v) acetic acid solution.

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