Bioresources and Bioprocessing

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Exotic glycerol dehydrogenase expressing *Escherichia coli* increases yield of 2,3-butanediol

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Abstract

Background: The thriving of biodiesel industry has led to produce 10% (v/v) crude glycerol, thus creating an overflow problem. Biofuel production is restricted by *Escherichia coli* due to its toxicity to bacterial cells. Therefore, a platform chemical and fuel additive 2,3-butanediol (2,3-BD) with low toxicity to microbes could be a promising alternative for biofuel production by recombinant *E. coli* using glycerol as the sole substrate.

Results: A novel expression system of *E. coli* was developed to express the *dhaD* gene encoding glycerol dehydrogenase (GDH) to produce value-added metabolic products through aerobic biotransformation of glycerol. The *dhaD* gene obtained from *Klebsiella pneumoniae* SRP2 was expressed in *E. coli* BL21(DE3)pLysS using an *E. coli–K. pneumoniae* shuttle vector pJET1.2/blunt consisting of chloramphenicol-resistance gene under the control of the T7lac promotor. RT-PCR analysis and *dhaD* overexpression confirmed that the 2,3-BD synthesis pathway gene was expressed on RNA and protein levels. Therefore, the recombinant *E. coli* exhibited a 38.9-fold higher enzyme activity (312.57 units/mg protein), yielding 8.97 g/L 2,3-BD, a 2.4-fold increase with respect to the non-recombinant strain.

Conclusions: The engineered strain *E. coli* BL21(DE3)pLysS/pJET1.2/blunt-*dhaD*, carrying the 2,3-BD pathway gene *dhaD* from our newly isolated *Klebsiella pneumoniae* SRP2 strain, displayed the best ability to synthesize 2,3-BD from low-cost biomass glycerol. The value of expression of an important glycerol metabolism gene *dhaD* is the highest ever achieved with an engineered *E. coli* strain. From these results, the first reported *dhaD* expression system has paved the way for improvement of 2,3-BD production and is efficient for another heterologous gene expression in *E. coli*.

Background

With increasing fossil fuel price and environmental concern, alternative and renewable energy sources have become attractive. Biodiesel, a renewable and promising combustion fuel, is synthesized from vegetable oils and animal fats. However, the biodiesel synthesis process (transesterification) generates 10% crude glycerol as a core by-product which is the cheapest feedstock or negative-value biomass to produce a high-value green product, 2,3-BD (Rahman et al. 2015). 2,3-BD is an important platform chemical, and it is also known as an excellent building block in the synthesis of valuable chiral chemicals (Celinska and Grajek 2009; Zeng and Sabra 2011). Chemical biosynthesis processes for the production of optically pure 2,3-BD are difficult to control, complicated and expensive. Thus, the biotransformation process has been considered as the preferred method for the production of optically pure 2,3-BD (Celinska and Grajek 2009; Ji et al. 2011; Zeng and Sabra 2011). Several engineered strains of microorganisms including *Saccharomyces cerevisiae*, *Enterobacter cloacae*, *Bacillus licheniformis* and *E. coli* have been used for the production of optically pure 2,3-BD (Yan et al. 2009; Lian et al. 2014; Li et al. 2015; Wang et al. 2012).

In the past few years, several microorganisms including cyanobacteria, fungi and bacteria have been proved to produce biofuels and fuel additives using different biomasses (Domínguez de María 2011; Gross 2012; Yan et al. 2009]. *E. coli* is extensively used as a model organism

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for biofuel production using pentose and hexose sugars from lignocellulosic biomass (Bokinsky et al. 2011). Now, it has been proved that recombinant *E. coli* can produce numerous biofuels including ethanol, acetone, butanol, α-pinene, isoprenol, isobutanol and fatty alcohols through biosynthetic pathways (Bokinsky et al. 2011; May et al. 2013; Atsumi et al. 2008; Zhang et al. 2013; Yang et al. 2013). Nevertheless, these biofuels are highly toxic to *E. coli,* and the production of new end products which are less or non-toxic to microbial cells is needed to obtain high product yield (Baez et al. 2011) using low-cost or negative-cost biomass. Thus, less toxic metabolic products such as 2,3-BD, 1,3-propanediol (1,3-PDO) and acetoin can be produced through biotechnological routes (Ji et al. 2011). Moreover, an industrially important platform chemical 2,3-BD could be produced through the oxidative pathway of recombinant *E. coli* (Xu et al. 2007). A high heating value (27,200 J/g) bulk chemical 2,3-BD could be used as liquid fuel or fuel additive (Xiao et al. 2012); it has very low toxicity to bacterial cells (Oliver et al. 2013). Therefore, 2,3-BD could be a promising alternative for biofuel production through recombinant *E. coli* strains.

Several bacterial species including *Klebsiella pneumoniae*, *K. variicola*, *K. oxytoca*, *Serratia marcescens* and *Enterobacter cloacae* have been used to produce 2,3-BD with high yields through optimization of culture conditions or genetic engineering (Celinska and Grajek 2009; Kim et al. 2013), but these strains are pathogenic or opportunistic pathogens and have been categorized under risk group-2 microorganisms, unsuitable for industrial-scale biotransformation (Ji et al. 2011; Kim et al. 2013). The microorganisms that may cause disease in humans and animals but are unlikely to be a serious hazard to laboratory personnel, the community, animals or the environment are called risk group 2. However, the strain *E. coli* BL21(DE3)pLysS has been known to be non-pathogenic and does not carry any virulence factor or pathogenic mechanism causing infections (Chart et al. 2000). Consequently, *E. coli* BL21(DE3)pLysS strain could be the best candidate for the safe synthesis of bioproducts (Chart et al. 2000; Zhang et al. 2013). Therefore, in this research work, a preliminary attempt has been made to establish a method for 2,3-BD production efficiently using *E. coli* BL21(DE3)pLysS. Moreover, in the oxidative pathway of glycerol metabolisms, there are three key enzymes, viz., glycerol dehydrogenase (GDH), α-acetolactate synthase and acetoin reductase which are involved in 2,3-BD biosynthesis (Celinska and Grajek 2009; Zhang et al. 2013). Consequently, GDH is the first enzyme in the oxidative pathway for converting glycerol to dihydroxyacetone(DHA), and then 2,3-BD is produced through pyruvate (Rahman et al. 2015) (Fig. 1). Several works have been reported on the production of 2,3-BD by recombinant *E. coli* through metabolic engineering of the genes, budB and budC, responsible for α-acetolactate synthase and acetoin reductase enzymes, respectively (Li et al. 2012), but there is no report on the *dhaD* gene in the same pathway which is responsible for GDH enzyme production. Therefore, it is important to construct an efficient 2,3-BD biosynthesis pathway that includes the related gene cluster to improve 2,3-BD production. In this backdrop, our aim was to construct a novel *dhaD* expression system in *E. coli* and its application for 2,3-BD production under completely aerobic condition. In this work, a systematic approach has been taken to construct and optimize 2,3-BD production by an efficient engineered *E. coli* BL21(DE3)pLysS strain. This research work is the first step for systematic metabolic engineering, which we successfully made as the novel expression system of the *dhaD* gene, and a high enzyme activity (GDH) was achieved through batch biotransformation process using glycerol as the sole carbon source.

Methods

Enzymes and chemicals

2,3-Butanediol (99.0%) was purchased from Sigma Aldrich (Canada). The restriction enzymes, Fast Pfu DNA polymerase and T4 DNA ligase, were purchased from Thermo Fisher Scientific, Canada. Isopropyl-β-D-thiogalactoside (IPTG), ampicillin and chloramphenicol were purchased from BioShop, Canada. All other chemicals used in this research work were analytical-grade reagents and commercially available.

Bacterial strains and vector

Newly isolated *K. pneumoniae* SRP2 was used for isolation and amplification of the *dhaD* gene. *E. coli* JM109 and *E. coli* BL21(DE3)pLysS strains were used as the hosts for gene cloning and expression, respectively. *E. coli* BL21(DE3)pLysS was also used as a host strain for 2,3-BD production. Cloning vector pJET1.2/blunt (Thermo Fisher Scientific, Canada) and *E. coli* BL21(DE3)pLysS contained ampicillin- and chloramphenicol-resistant genes, respectively.

Media and growth conditions

Klebsiella pneumoniae SRP2 and *E. coli* JM109 were grown in a Luria–Bertani (LB) medium containing (g/L): peptone 10.0 g/L, yeast extract 5.0 g/L and sodium chloride 5.0 g/L. The LB broth medium was supplemented with 100 μg/mL ampicillin and 100 μg/mL ampicillin plus 34 μg/mL chloramphenicol for *E. coli* JM109 and *E. coli* BL21(DE3)pLysS/*dhaD*, respectively, when necessary to maintain the plasmids. For biotransformation or expression study of *E. coli* BL21(DE3)pLysS/*dhaD*, MS-2

medium (K_2HPO_4 0.1 g/L, $NaNO_3$ 0.1 g/L, $MgSO_4.7H_2O$ 0.05 g/L, KCl 0.1 g/L, yeast extract 2.5 g/L and peptone 5.0 g/L) supplemented with 25.0 g/L glycerol, 100 µg/mL ampicillin and 34 µg/mL chloramphenicol was used. The initial pH of the medium was adjusted to 7.0 by adding NaOH/HCl. The biotransformation was carried out in a 250-mL flask containing 50.0 mL medium with 5.0 mL seed culture using a rotary shaker incubator at 200 rpm and 37 °C under aerobic condition. All the seeds and culture media of the *E. coli* BL21(DE3)pLysS/*dhaD* were supplemented with ampicillin (100 µg/mL) and chloramphenicol (34 µg/mL) to maintain the plasmid.

Construction of plasmid with the *dhaD* gene

The *dhaD* gene encoding glycerol dehydrogenase was amplified through PCR using *K. pneumoniae* SRP2 genomic DNA as the template. The primers used in PCR were: forward primer—GGATCCATGCGCACTTATTTGAGGGTGA (with *Bam*H1 restriction site) and reverse primer—AAGCTTACGCGCCAGCCACTGGCCT (with *Hin*dIII restriction site). The PCR conditions were as follows: initial denaturation at 94 °C for 3 min; then 30 cycles of 30 s at 94 °C, 30 s at 58 °C and 1 min at 72 °C; and final extension at 72 °C 10 min. The amplified product was ligated into cloning vector pJET1.2/blunt at the PCR product site and transferred into *E. coli* JM109 using calcium chloride heat shock method (Ausubel et al. 1987), resulting in a recombinant plasmid designated as pJET1.2/blunt-*dhaD*. The ampicillin-resistant colonies were selected on the LB agar plate supplemented with 100 µg/mL ampicillin and purified. However, the plasmid containing the *dhaD* gene was extracted from *E. coli* JM109, purified and transferred to the competent cell of *E. coli* BL21(DE3)pLysS using calcium chloride heat shock method (Ausubel et al. 1987). The recombinant *E. coli* BL21(DE3)pLysS/pJET1.2/blunt-*dhaD* designated as *E. coli* BL21(DE3)pLysS/*dhaD* was obtained using the heat shock transformation method (Ausubel et al. 1987).

Expression and SDS-PAGE analysis of *dhaD*

The transformed *E. coli* BL21(DE3)pLysS containing plasmid pJET1.2/blunt-*dhaD* was grown at 37 °C in LB medium supplemented with 100 µg/mL ampicillin and 34 µg/mL chloramphenicol for 24 h with shaking at 200 rpm. This overnight culture was inoculated into fresh LB medium containing antibiotics to an OD_{600} of 0.05–0.1 (1:50 dilution of the overnight culture). One set of glycerol stock culture was stored at − 80 °C until the clone that best expressed the targeted protein was obtained. The cultures were grown until they reach mid-log phase (OD_{600} = 0.4–0.5, 2–3 h). Subsequent incubation, the cultures were induced by adding IPTG to a final concentration of 0.5 mM, and incubated for an additional 2–4 h, took time points to analyze for optimal expression

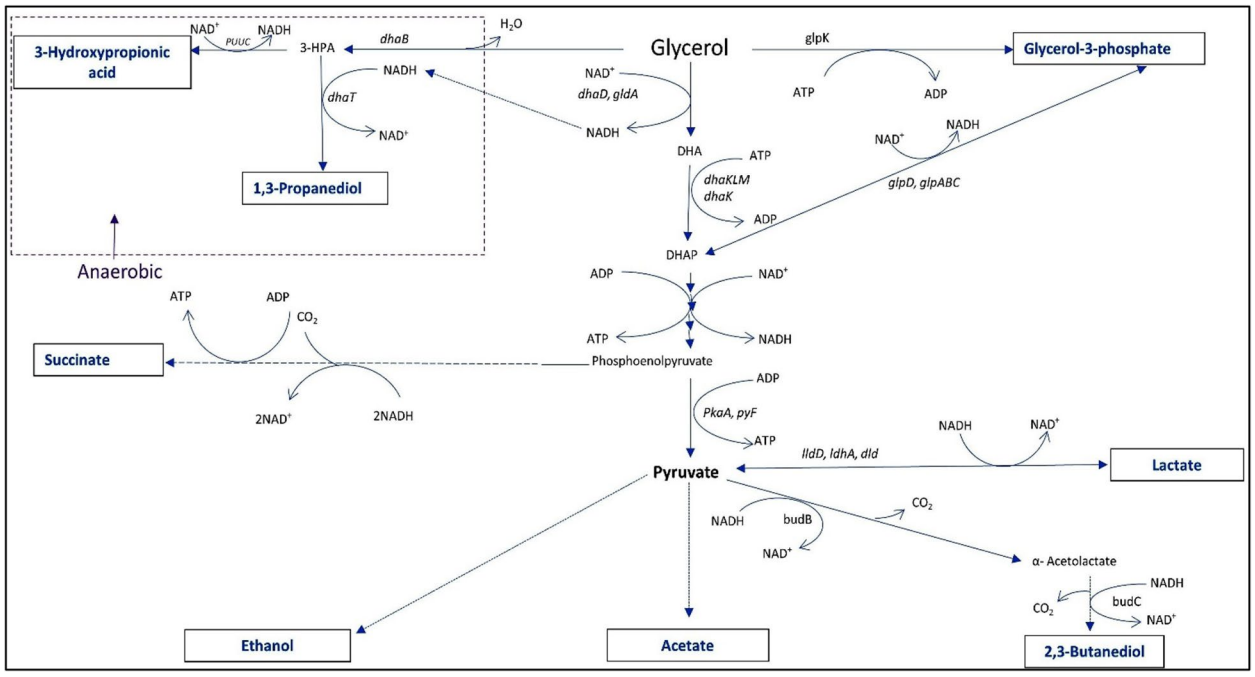

Fig. 1 Metabolism pathway for the conversion of glycerol to 2,3-BD. Pyruvate is reduced to various organic compounds, depending on the microorganism and on the biotransformation conditions

of targeted protein. Clones were then used for enzymatic assay to determine which clone best expressed the protein of interest. Cells were harvested after 4–7 h of incubation and resuspended in 100 mM potassium phosphate buffer containing 50 mM KCl, sonicated at 4 °C for 2 min (10 s at a time, and until 2 min) and centrifuged (3–5 min at 15,000×*g*). The supernatant was kept at low temperature (4 °C) and used for protein and GDH enzyme assays. The protein samples were separated by SDS-PAGE using 12% (w/v) SDS-polyacrylamide gel and identified by staining with Coomassie Brilliant Blue R-250 (Merck). SDS-PAGE analysis was carried out using the Laemmli method (Laemmli 1970). The SDS-PAGE analysis was performed on the Mini-PROTEAN Tetra System Electrophoresis (Bio-Rad, Canada). The Broad Range Protein Molecular Marker (Fermentas) was used to estimate the molecular weight of proteins.

Expression: RT-PCR analysis and enzyme assay

To study the expression of the *dhaD* gene responsible for glycerol utilization in IPTG-induced strain *E. coli* BL21(DE3)pLysS/*dhaD*, the experiment was carried out with quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was extracted using the PureLink™ RNA extraction kit (Ambion, Thermo Fisher Scientific, USA), following the manufacturer's instructions. The expression of the *dhaD* gene was evaluated via qRT-PCR. First-strand cDNA was prepared by reverse transcription using the cDNA synthesis kit (Tetro cDNA synthesis Kit, Bioline, UK) in which RNA was used as a template. Quantitative gene expression was carried out using SensiFAST™ SYBR No-ROX Kit (Bioline, UK) on C1000™ thermal cycler quantitative real-time PCR (qRT-PCR) detector system (Bio-Rad, USA). The 16S rRNA obtained based on the primers 5′-GCGGTTGTTACAGTCAGATG-3′ and 5′-GCCTCAGCGTCAGTATCG-3′ was used as an internal standard. The $2^{-\Delta\Delta CT}$method was used to analyze the fold change gene expression over the control (Lival and Schmittgen 2001).

However, the intracellular GDH activity was determined at room temperature by measuring the reduction of NAD^+ to the substrate-dependent absorbance change of NAD(H) at 340 nm ($\varepsilon 340 = 6.22\ mM^{-1}\ cm^{-1}$) using the method described by Ahrens et al. (1998) with slight modification (Rahman et al. 2015). Briefly, 1 mL of the reaction mixture contains 50 mM potassium phosphate buffer (pH 8.0), 30 mM ammonium sulfate, 0.2 M glycerol and 1.2 mM NAD. The assay was initiated by adding 50 µL of cell extract in 250 µL reaction mixture, and the absorbance increase (NADH) was observed from the spectrophotometer for 2–3 min. One unit of activity is the amount of enzyme required to reduce 1 µmol of substrate per minute. The specific activity of GDH is expressed as µmol of substrate reduced/min/mg of cell protein, and represents the averages for at least three cell preparations. The Bradford method was used for the determination of protein concentration (Bradford 1976) and bovine serum albumin served as the standard protein.

Analytical

The biomass concentration was measured from absorbance at 600 nm. The optical density at 600 nm (OD_{600}) was obtained from a microplate spectrophotometer (EPOCH, BioTek). The metabolic product was identified by GC–MS (Varian 1200 Quadrupole). The concentrations of major metabolic product 2,3-BD, as well as glycerol, were quantified using a GC–FID (Shimatzu GC 14A) under the following conditions: sample volume 1 µL; column temperature range from 45 °C (2 min) to 240 °C at the rate of 10 °C min^{-1}; the injector and detector temperature 250 °C; carrier gas, nitrogen; column, DB-WAXetr. The injecting sample was purified by centrifugation (Fisher Scientific, Germany, accu Spin Micro 17), and membrane filter (0.22 µm pore size), respectively.

Statistical analysis

All the experiments were performed in triplicate and the results expressed in terms of mean ± SD (standard deviation). The statistical analysis of data was performed to test the significant difference by one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) test ($p < 0.05$).

Results and discussion

Construction of plasmid with the *dhaD* gene

The vector pJET1.2/blunt was used for cloning the *dhaD* gene encoding glycerol dehydrogenase (GDH). After ligation of the amplified product (*dhaD*) into the cloning vector pJET1.2/blunt, the vector was transferred into *E. coli* JM109, resulting in recombinant *E. coli* JM109/pJET1.2/blunt-*dhaD* (Fig. 2). The ampicillin-resistant colonies were selected from LB agar plates supplemented with 100 µg/mL ampicillin and purified. For the confirmation of *dhaD*, the plasmid was extracted from transformed *E. coli* JM109/pJET1.2/blunt-*dhaD* strain. Transformation of the plasmid containing the *dhaD* gene into *E. coli* JM109 was confirmed by agarose gel electrophoresis analysis (Fig. 3). The size of 1125 bp of the *dhaD* gene, amplified by PCR from the vector (plasmid) using *dhaD* gene primers (forward and reverse primers), was also confirmed by sequencing.

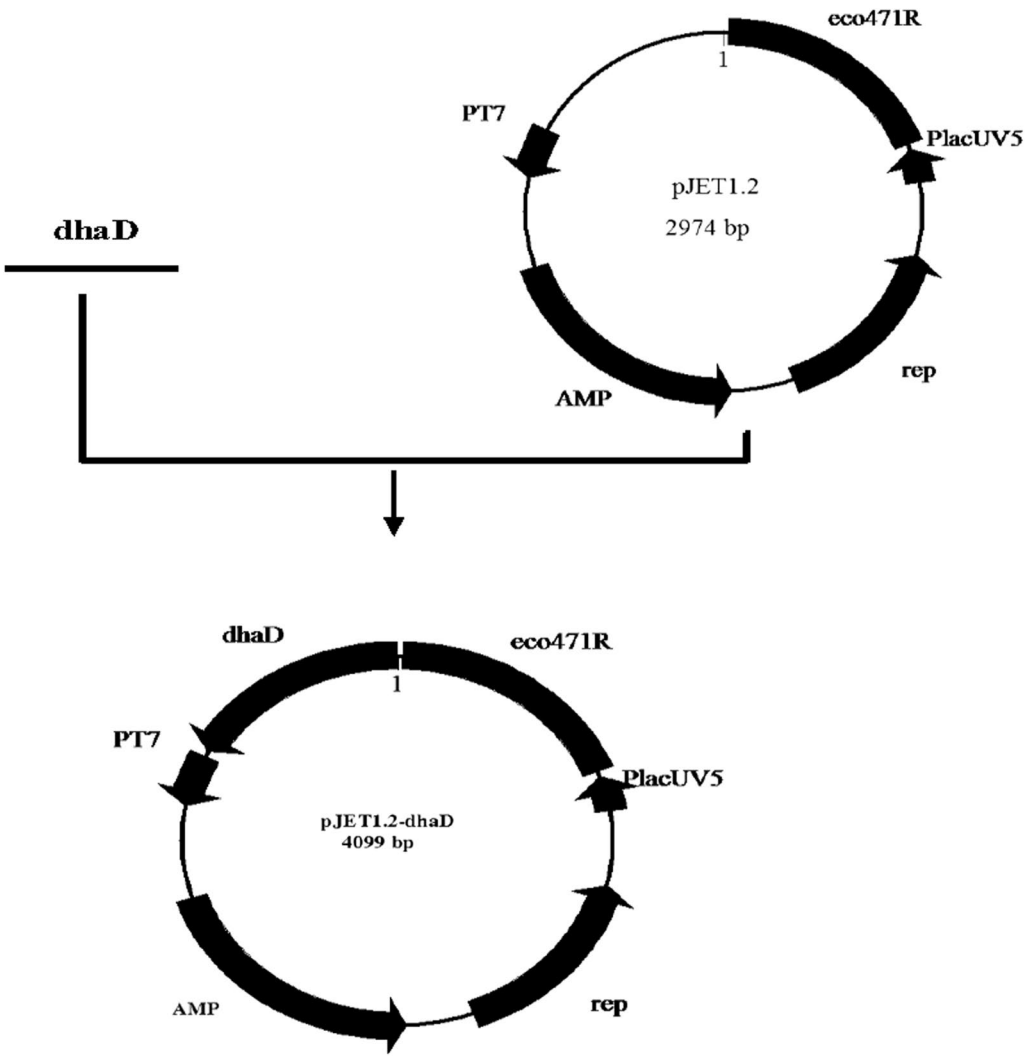

Fig. 2 Construction of recombinant vector for *dhaD* expression. *PlacUV5* modified P_{lac} promotor for expression of the eco47IR gene at a level sufficient to allow for positive selection, *eco47IR* lethal gene enables positive selection of recombinant plasmid, *dhaD* inserted gene glycerol dehydrogenase, *PT7* T7 RNA polymerase promotor for in vitro transcription of the cloned insert, *AMP* gene resistant to ampicillin, *rep* replicon from pMBI plasmid responsible for the replication of pJET1.2

Clone selection of engineered *E. coli* BL21(DE3)pLysS/*dhaD* strains

However, for selection of the best clone, totally six transformed *E. coli* BL21(DE3)pLysS/*dhaD* clones were tested for their GDH enzyme activity after 3 h of IPTG induction. All the six clones were almost identical in their enzyme activities, and one of the clones exhibited the highest activity of GDH which was 312.57 U/mg protein (Table 1). Thus, this clone was finally selected for the expression study, nominated as *E. coli* BL21(DE3)pLysS/*dhaD* strain. Therefore, this recombinant stain *E. coli* BL21(DE3) pLysS/*dhaD* also exhibited the highest biomass production ($OD_{600} = 1.27$) compared to that of non-recombinant *E. coli* BL21(DE3)pLysS and *K. pneumoniae* SRP2 (Table 1).

However, to confirm that the *dhaD* gene had inserted into *E. coli* BL21(DE3)pLysS, agarose gel electrophoresis analysis and gene sequencing were performed. The open reading frame (ORF) of *dhaD* gene was 1125 bp (Fig. 4). Also, the correct size of 1125 bp of the PCR product (*dhaD* gene), obtained from the recombinant vector containing the *dhaD* gene using forward and reverse primers, was confirmed by sequencing.

Enzyme activity

To compare the expression levels of non-recombinant *E. coli* BL21(DE3)pLysS, recombinant *E. coli* BL21(DE3) pLysS/*dhaD* and a wild-type *K. pneumoniae* SRP2 strains after induced IPTG (0.5 mM), GDH enzyme activities

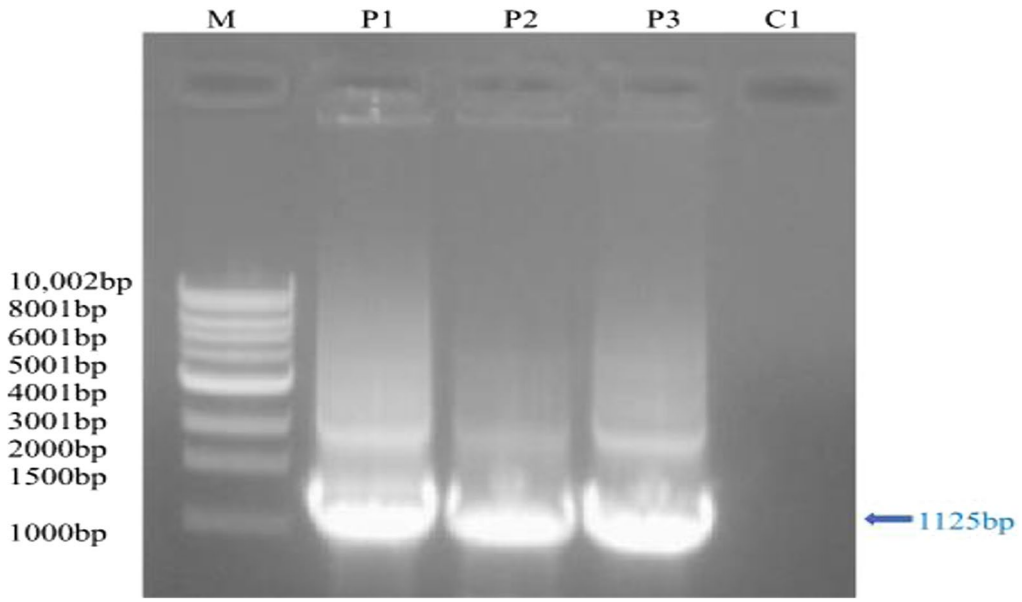

Fig. 3 Agarose gel electrophoresis analysis of *dhaD* gene amplified from the plasmid of *E. coli* JM109. Lane P1, P2 and P2—PCR products from the plasmid of *E. coli* JM109 containing *dhaD* genes; lane C1—negative control (no plasmid); lane M—molecular mass markers. Recombinant glycerol dehydrogenase (*dhaD*) from *K. pneumoniae* SRP2 is indicated by 1125 bp

Table 1 Glycerol dehydrogenase (GDH) activity of the clone constructed with *dhaD* containing plasmid

Strains	GDH activity (U/mg protein)	Biomass (OD_{600})
E. coli BL21(DE3) pLysS/*dhaD* (clone)	312.57 ± 14.81	1.27 ± 0.06
E. coli BL21(DE3)pLysS	7.82 ± 0.47	0.55 ± 0.03
K. pneumoniae SRP2	31.12 ± 0.11.5	0.53 ± 0.04

were determined in vitro. As shown in Fig. 5, when the GDH-expressing plasmid pJET1.2/blunt-*dhaD* was introduced into *E. coli* BL21(DE3)pLysS, the resultant strain *E. coli* BL21(DE3)pLysS/*dhaD* displayed a significant increase in GDH activity which was 312.57 U/mg protein, 38.9 times more than that of *E. coli* BL21(DE3)pLysS and 9.99 times more than wild-type strain *K. pneumoniae* SRP2 (Fig. 5a and Table 1). Similarly, the overexpression of the GDH gene in *E. coli* BL21(DE3)pLysS did not lead to a significantly enhanced GDH activity after induction with IPTG (Fig. 5a). Moreover, the recombinant GDH was not only highly induced by different concentrations of IPTG, but also markedly stimulated at 37 °C incubation temperature (Fig. 5c). Consequently, at an incubation temperature of 37 °C, the highest intracellular GDH activity was obtained using 0.5 mM IPTG (Fig. 5c). Similar effects had been observed previously in *Gluconobacter oxydans* MF1 when the gene coding for glucose dehydrogenase or gluconate-5-dehydrogenase was overexpressed (Merfort et al. 2006).

Moreover, the GDH enzyme activity was evaluated with the strain *E. coli* BL21(DE3)pLysS and its plasmid containing strain *E. coli* BL21(DE3)pLysS/*dhaD*, and was found to be on average 38.9.00-fold increased in *E. coli* BL21(DE3)pLysS/*dhaD*, which was directly correlated with an increase in the expression of GDH from plasmid pJET1.2/blunt. As shown in Fig. 5a, b, the GDH activity was increased during the late log and stationary phases of growth with the strain *E. coli* BL21(DE3)pLysS/*dhaD*, which was much higher than *E. coli* BL21(DE3)pLysS. Furthermore, the recombinant strain BL21(DE3) pLysS/*dhaD* exhibited the highest enzyme activity at a pH 7.0 (Fig. 5d).

Gene expression by RT-PCR analysis

However, for determining the expressions of the GDH gene in *E. coli* BL21(DE3)pLysS, *E. coli* BL21(DE3) pLysS/*dhaD* and *K. pneumoniae* SRP2 strains, qRT-PCR analysis was performed. qRT-PCR analysis was performed on bacteria having undergone glycerol transformation by the *dhaD* gene expression construct, which was obtained from *K. pneumoniae* SRP2. The use of two antibiotics (ampicillin, chloramphenicol) allowed the stable maintenance of plasmid in the recombinant bacterium. qRT-PCR analysis exhibited the expression of the *dhaD* gene from *K. pneumoniae* after the addition of IPTG as an inducer (Fig. 6). As gene expression often

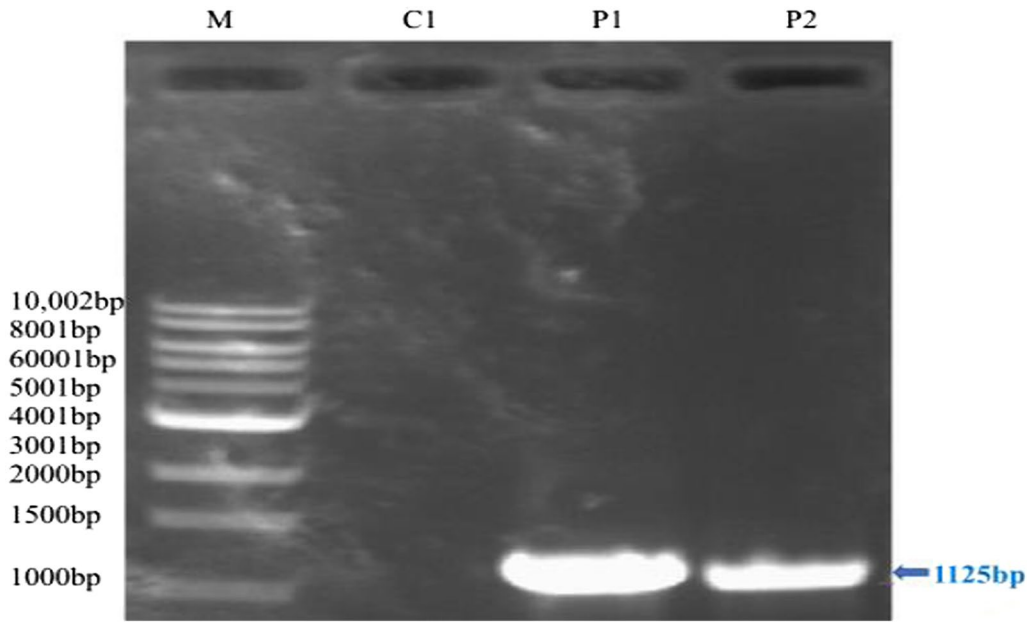

Fig. 4 Agarose gel electrophoresis analysis of *dhaD* gene amplified from the plasmid of *E. coli* BL21(DE3)pLysS/*dhaD*. Lane P1 and P2—PCR products from the plasmid of *E. coli* BL21(DE3)pLysS containing the *dhaD* gene; lane C2—negative control (no plasmid); lane M—molecular mass markers. Recombinant glycerol dehydrogenase (*dhaD*) (1125 bp) (lane P1 or P2) from *K. pneumoniae* SRP2 is indicated by a line

varied significantly in different growth phases (Quintero et al. 2009), all bacterial cells used for RNA isolation were cultured for a constant period to minimize its effect on RT-PCR analysis. It was undoubtedly observed that the transcription level of the GDH gene in *E. coli* BL21(DE3) pLysS/*dhaD* was much higher than that of the control strain *E. coli* BL21(DE3)pLysS (Fig. 6). In *E. coli* BL21(DE3)pLysS/*dhaD*, the expression level of the GDH gene was about 84 times more abundant than that in *E. coli* BL21(DE3)pLysS after 3 h of IPTG induction, due to GDH overexpression. Therefore, the results confirmed the expression of *dhaD* in the *E. coli* BL21(DE3) pLysS/*dhaD* expression system.

Overexpression of the pJET1.2/blunt-*dhaD* construct

The *E. coli* BL21(DE3)pLysS strain contained a fragment of the DE3 phage genome in the genome system has a T7 RNA polymerase gene under the control of the lacUV5 promoter. Logically, the bacterium contains the lacI gene in the chromosome and expression plasmid, which encodes a repressor that binds to the T7lac promoter on the expression plasmid and the lacUV5 operator–promoter, thereby blocking the expression of the gene encoding RNA polymerase. This repression is induced by IPTG which allows induced transcription from the T7lac promoter (ThermoFisher Scientific 2010). The gene *dhaD* was transcribed in a similar way and controlled by the T7lac promoter. The results indicate efficient overexpression of the *dhaD* gene from *K. pneumoniae* SRP2 in *E. coli* BL21(DE3)pLysS (Fig. 6). SDS-PAGE analysis of the expression products from genes inserted into the pJET1.2/blunt-*dhaD* construct indicates a higher level of the expressed protein in *E. coli* BL21(DE3)pLysS strain (Fig. 7). As shown in Fig. 7, the sample showed a protein brand of about 41 kDa, which is the GDH enzyme.

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

The transformed *E. coli* BL21(DE3)pLysS/*dhaD* strain was used for biotransformation of glycerol to 2,3-BD. The time course data for the metabolic product, glycerol consumed and cell growth of batch cultivation at 37 °C by *E. coli* BL21(DE3)pLysS, *E. coli* BL21(DE3) pLysS/*dhaD* and *K. pneumoniae* SRP2 is presented in Fig. 8. The purpose of the culture was both to assess the production of 2,3-BD, glycerol consumption, the efficiency and selectivity of the process. The concentrations of the desired metabolite (2,3-BD) as well as of unused glycerol in the culture medium were analyzed using GC–FID. IPTG was added to the culture medium (MS-2 medium supplemented with 25.0 g/L glycerol) after 6 h. As the GDH activity was increased when the GDH gene was overexpressed, a study of the time course of glycerol metabolism by recombinant *E. coli* BL21(DE3) pLysS/*dhaD*, non-recombinant *E. coli* BL21(DE3)pLysS

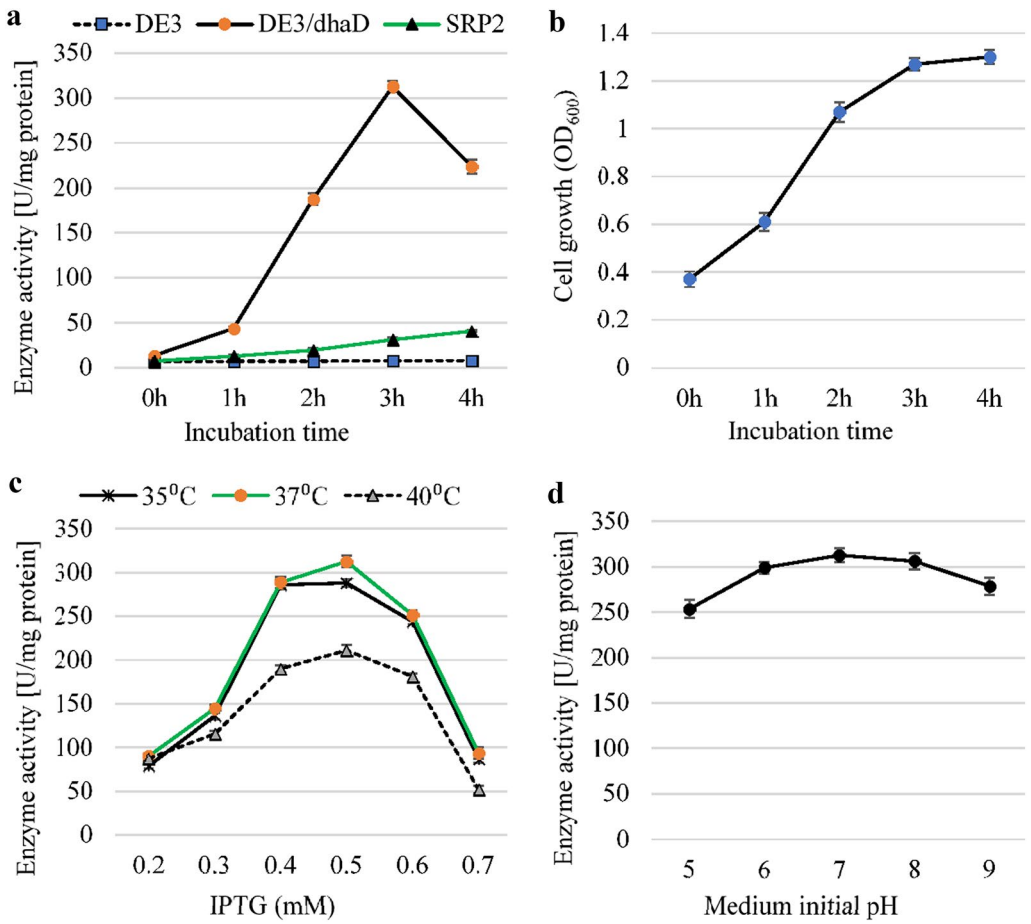

Fig. 5 Comparison of GDH enzyme activities of the strains *E. coli* BL21(DE3)pLysS(DE3), *E. coli* BL21(DE3)pLysS/*dhaD* (DE3/*dhaD*) and *K. pneumoniae* SRP2 (SRP2): **a** effect of incubation time on GDH activity after addition of 0.5 mM IPTG; **b** effect of incubation time on biomass production after addition of 0.5 mM IPTG; **c** effect of incubation temperature and IPTG concentrations on GDH activity after 3 h of IPTG induction; **d** effect of medium initial pH on GDH activity after 3 h of 0.5 mM IPTG induction

as well as the wild-type strain *K. pneumoniae* SRP2 was carried out in shake flasks. The recombinant bacterial strain *E. coli* BL21(DE3)pLysS/*dhaD* exhibited 8.97 g/L of 2,3-BD using 24.67 g/L of glycerol after 48 h incubation. This product yield of 2,3-BD is not as high as that of strain *K. pneumoniae* SRP2 reported in our earlier study (Rahman et al. 2015). The strain *E. coli* BL21(DE3) pLysS/*dhaD* might produce metabolic products including 1,3-PDO, ethanol, acetate, succinate or lactate with 2,3-BD (Xu et al. 2014). Therefore, the other two important genes, budB and budC, responsible for 2,3-BD production should be inserted into the glycerol metabolism pathway of *E. coli* BL21(DE3)pLysS/*dhaD* stain to get a high yield of 2,3-BD. Although there is no report on the pathway of 2,3-BD production in *E. coli*, several works have been done on systematic metabolic engineering of *E. coli* for high product yield of 2,3-BD by introducing budB and budC genes (Xu et al. 2014; Chu et al. 2015).

We have demonstrated that overexpression of GDH in a *E. coli* strain led to a significant improvement of GDH activity and 2,3-BD production. The increased GDH activity led to a 2.4-fold increase in 2,3-BD product yield compared with *E. coli* BL21(DE3)pLysS in a batch biotransformation when 25.0 g/L glycerol was supplied and the reaction time was shortened (Figs. 5a, 8). It is also possible that higher levels of GDH could increase the tolerance of the strain against product(s) inhibition, as suggested by Gätgens et al. (2007). Since in utilized a sufficient amount of glycerol, a relatively low yield of 2,3-BD was attained (Fig. 8a, b), possibly the recombinant strain produced bioproducts other than 2,3-BD due to lack of sufficient activities of other genes in the metabolic pathway of 2,3-BD. Therefore, further improvements in yield will have to construct recombinant strain by introducing budB and budC genes from highly efficient strain, and involve changes in fermentation procedures such as

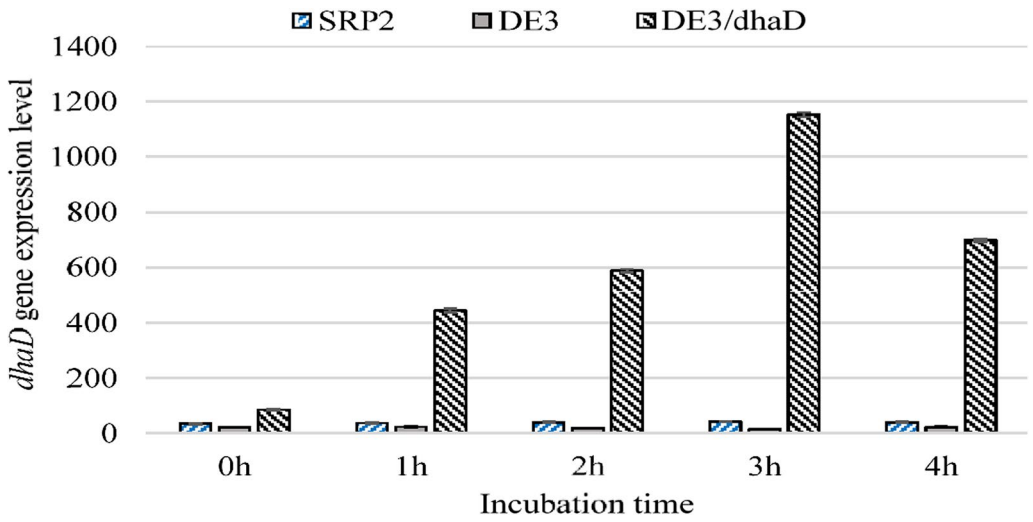

Fig. 6 Comparison of expression levels of *dhaD* of the strains *E. coli* BL21(DE3)pLysS (DE3), *E. coli* BL21(DE3)pLysS/*dhaD* (DE3/*dhaD*) and K. pneumoniae SRP2 (SRP2). Incubation temperature and IPTG concentration were 37 °C and 0.5 mM, respectively

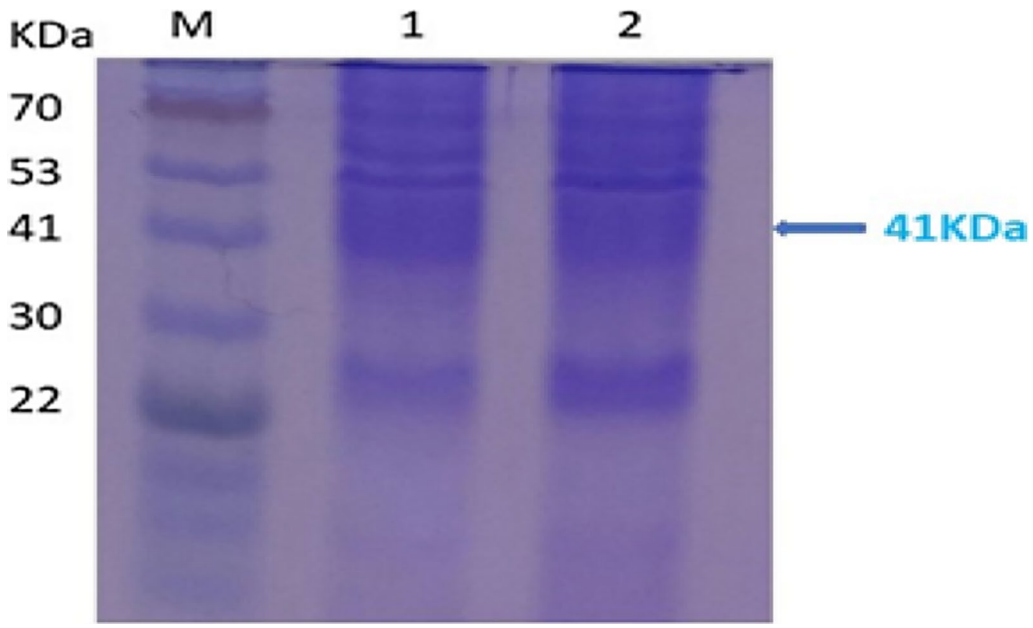

Fig. 7 SDS-PAGE analysis of the location and existing state of glycerol dehydrogenase (GDH) expression protein. Lane M—protein marker; lanes 1 and 2—cell lysates from the IPTG-induced *E. coli* BL21(DE3)pLysS/*dhaD* strain. Glycerol dehydrogenase (*dhaD*) protein is indicated by 41 kDa

those described by Hekmat et al. (2003) who designed a reactor system consisting of a shaking tank and a permeable column harboring immobilized cells.

The current experiments were carried out with pure glycerol. In a commercial setting, most likely biodiesel-derived glycerol would have to be used. This type of glycerol typically contains various impurities that could cause severe inhibition at high concentrations, or even be toxic to the cells (Sabourin-Provost and Hallenbeck 2009). It remains to be determined if our recombinant strain will be able to provide high yields under those conditions.

Conclusion

A promising strain for industrial production of 2,3-BD was developed by overexpressing the GDH gene in the *E. coli* BL21(DE3)pLysS strain. Taking advantage of

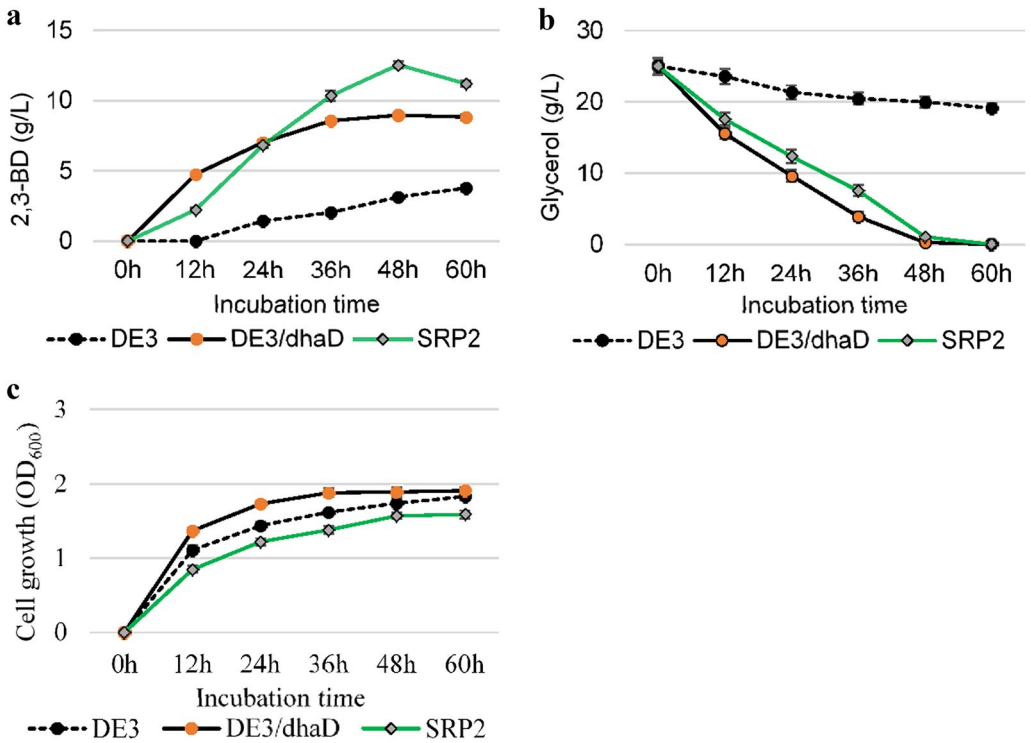

Fig. 8 Comparison of 2,3-BD product yield, biomass production (cell growth) and glycerol consumed with the strains *E. coli* BL21(DE3)pLysS (DE3), *E. coli* BL21(DE3)pLysS/*dhaD* (DE3/*dhaD*) and *K. pneumoniae* SRP2 (SRP2). The incubation temperature and IPTG concentration were 37 °C and 0.5 mM, respectively

the elevated activity of GDH, the recombinant *E. coli* BL21(DE3)pLysS/*dhaD* strain can produce 2,3-BD at an acceptable concentration and display a substantially increased GDH activity when the IPTG inducer is used. The recombinant strain thus has potential for industrial production of 2,3-BD. From these results, the first reported *dhaD* expression system has paved the way for improvement of 2,3-BD production and is efficient for another heterologous gene expression in *E. coli*. For further improvement in yield, the recombinant strain could be constructed by introducing budB and budC genes in addition to the *dhaD* gene from a highly efficient strain.

Authors' contributions

Provided the research idea and designed the experiments: MSR, CX and WQ. Performed the experiments and analyzed the data: MSR. Wrote the paper: MSR. Edited: QW. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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All data generated or analyzed during this study are included in this article.

Consent for publication

All authors provided consent for publishing the manuscript to Bioresources and Bioprocessing.

Ethics approval and consent to participate

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