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Cloning, Expression, and Characterization of *budC* Gene Encoding *meso*-2,3-Butanediol Dehydrogenase from *Bacillus licheniformis*

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Abstract The *budC* gene encoding a *meso*-2,3-butanediol dehydrogenase (*Bl*BDH) from *Bacillus licheniformis* was cloned and overexpressed in *Escherichia coli* BL21(DE3). Sequence analysis reveals that this *Bl*BDH belongs to short-chain dehydrogenase/reductase (SDR) superfamily. In the presence of NADH, *Bl*BDH catalyzes the reduction of diacetyl to (3*S*)-acetoin (97.3 % *ee*), and further to (2*S*,3*S*)-2,3-butanediol (97.3 % *ee* and 96.5 % *de*). Similar to other *meso*-2,3-BDHs, it shows oxidative activity to racemic 2,3-butanediol whereas no activity toward racemic acetoin in the presence of NAD^+. For diacetyl reduction and 2,3-butanediol oxidation, the pH optimum of *Bl*BDH is 5.0 and 10.0, respectively. Unusually, it shows relatively high activity over a wide pH range from 5.0 to 8.0 for racemic acetoin reduction. *Bl*BDH shows lower K_m and higher catalytic efficiency toward racemic acetoin (K_m=0.47 mM, k_{cat}/K_m=432 $s^{-1}\cdot mM^{-1}$) when compared with 2,3-butanediol (K_m=7.25 mM, k_{cat}/K_m=81.5 $s^{-1}\cdot mM^{-1}$), indicating its physiological role in favor of reducing racemic acetoin into 2,3-butanediol. The enzymatic characterization of *Bl*BDH provides evidence for the directed engineering of *B. licheniformis* for producing enantiopure 2,3-butanediol.

Keywords *Bacillus licheniformis* · *meso*-2,3-Butanediol dehydrogenase · Expression · Characterization

Introduction

2,3-Butanediol (2,3-BD) is a crucial vicinal diol which can be used for the synthesis of bulk chemicals such as 2-butanone and 1,3-butadiene. It has also been regarded as a promising

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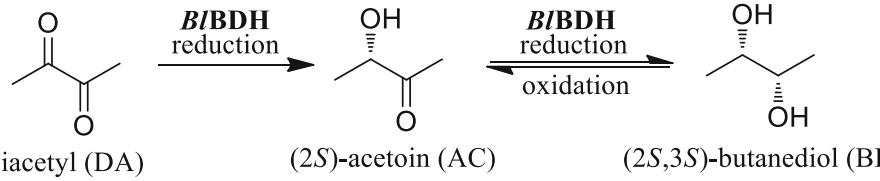
biofuel with relatively high fuel value and could be extensively applied in the production of fine chemicals including printing inks, perfumes, fumigants, explosives, foods, pharmaceuticals, polymers, etc. [1, 2]. Due to the existence of two chiral centers, 2,3-BD has three stereoisomers including *meso*-2,3-BD, (2*R*,3*R*)-2,3-BD, and (2*S*,3*S*)-2,3-BD. Optically pure 2,3-BDs are important building blocks for the synthesis of chiral compounds that contain two vicinal stereogenic centers [3]. Most importantly, different application has been verified among three chiral 2,3-BDs. For example, (2*R*,3*R*)-2,3-BD can be used as an antifreeze agent because of its low freezing point (−60 °C), while (2*S*,3*S*)-2,3-BD is a valuable pharmaceutical intermediate [4]. Hence, the production of chiral 2,3-BD is of great interests.

Various chemical and biotechnological routes have been developed for the production of 2,3-BD. In view of environmental friendliness, warm conditions, easy operation, high yield, and optical purities, biotechnology strategies have emerged as the preferred approach for the preparation of chiral 2,3-BD [5]. In the microbial metabolism, all isomers of 2,3-BD could be produced from α-acetolacetate pathway. A number of native microorganisms capable of producing 2,3-BD have been identified, including pathogenic strains *Klebsiella pneumonia* [6], *Serratia marcescens* [7], *Klebsiella oxytoca* [8], *Enterobacter cloacae* [9] band"" non-pathogenic strains *Bacillus licheniformis* [10], *Paenibacillus polymyxa* [11], *Bacillus subtilis* [12], etc. However, 2,3-BDs are usually produced in racemic form [2]. There are several possible explanations for the simultaneous formation of three 2,3-BD isomers, including aeration conditions (redox balance), nonstereospecific dehydrogenases, multiple pathways, and multiple stereospecific dehydrogenases [13]. Xu and coworkers found the existence of multiple stereospecific dehydrogenases to be a key factor for the production of 2,3-BD in *K. pneumonia* [6], *S. marcescens* [7], *E. cloacae* [9], and *B. licheniformis* [10]. A complete *alsSD* operon responsible for the synthesis of 2,3-BD was identified in above strains. In the operon, α-acetolactate synthase encoded by *als*S could catalyze the conversion of pyruvate to α-acetolactate, while α-acetolactate decarboxylase encoded by *als*D catalyzes the transformation of α-acetolactate to acetoin (AC). Then, acetoin could be reduced into 2,3-BDs by 2,3-butanediol dehydeogenase (2,3-BDH). Due to the stereosepecificity of α-acetolactate decarboxylase and 2,3-BDH, 2,3-BD could be produced in optically pure form [1]. Consequently, the characterization of 2,3-BDHs is of vital importance for the preparation, especially for the biocatalytic synthesis of optically pure 2,3-BD.

Several 2,3-BDHs from different microbial strains have been purified and characterized previously. (2*R*,3*R*)-2,3-BDHs from *Saccharomyces cerevisiae* [14], *P. polymyxa* [15], and *Bacillus subtilis* [16], catalyzing the conversion of (3*R*)-acetoin to (2*R*,3*R*)-2,3-BD and (3*S*)-acetoin to *meso*-2,3-BD, were analyzed belonging to medium-chain dehydrogenase/reductase (MDR) superfamily. Whereas *meso*-2,3-BDHs from *S. marcescens* [17], *Bacillus stearothermophilus* [18], and *Rhodococcus erythropolis* [19] were reported to catalyze the conversion of (3*R*)-acetoin and (3*S*)-acetoin into *meso*-2,3-BD and (2*S*,3*S*)-2,3-BD, respectively, and were classified into short-chain dehydrogenase/reductase (SDR) superfamily. Additionally, some glycerol dehydrogenases (GDHs) belonging to zinc-dependent MDR were also proved to exhibit similar activity as (2*R*,3*R*)-2,3-BDH [20, 21].

As a thermophilic and generally recognized as safe (GRAS) strain, *B. licheniformis* has gained much attentions due to its great potential in the production of 2,3-BD [1, 10, 22]. However, 2,3-BD is always produced by *B. licheniformis* as a mixture of (2*R*,3*R*)-2,3-BD and *meso*-2,3-BD due to its complicated BDH system. A *meso*-2,3-BDH (encoded by *budC*) was reported to be responsible for the formation of *meso*-2,3-BD by sequential reduction of diacetyl into acetoin and 2,3-butanediol, as shown in Scheme 1 [1]. Furthermore, Qi and



Scheme 1 Asymmetric reduction of diacetyl to (2*S*, 3*S*)-butanediol catalyzed by *Bl*BDH

coworkers constructed a *budC* knockout strain which exhibited enhanced (2*R*,3*R*)-2,3-BD production [4]. To better understand the potential of *B. licheniformis* in chiral 2,3-BDH production, the key *meso*-2,3-BDH of *B. licheniformis* was cloned and heterogeneously expressed in *E. coli*. Herein, the classification and enzymatic characteristics of purified *meso*-2,3-BDH are the main focus of this work.

Materials and Methods

Chemicals

Diacetyl (DA), racemic AC, and racemic 2,3-BD were purchased from TCI (Shanghai, China) Ltd. PCR polymerase, T4 ligase, and restriction endonucleases were obtained from Takara (Dalian, China). All the other reagents used in this study were of analytical grade and commercially available.

Bacterial Strains, Plasmids, and Culture Conditions

B. licheniformis was used as the template for *budC* gene. *E. coli* JM109 and *E. coli* BL21(DE3) stored in our laboratory were used as the cloning and expression hosts. Plasmid pET-28a(+) (Novagen) was used for expression of *budC*.

Cloning, Expression, and Purification of Recombinant *meso*-2,3-BDH

The *budC* gene encoding *meso*-2,3-BDH was amplified by PCR using *B. licheniformis* genome as the template. Primers used were as follows: forward primer 5′-CGCGGATCCATGAGTAAAGTATCTGGAAAAAT-3′ and reverse primer 5′-CCGGAATTCTTAATTAAATACCATTCCGCC-3′ (undelined restriction endonucleases sites). The PCR conditions were set as 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C after denaturation for 5 min at 94 °C. The amplified products were double digested and ligated into the linerized pET-28a(+) between *Bam*HI and *Eco*RI sites to form a recombinant plasmid designated as pET28a-*bud*C. The resultant pET28a-*bud*C were transformed into expressing host cells by heat shock transformation to obtain *E. coli* BL21(DE3)/pET28-*bud*C.

The recombinant strain harboring *bud*C gene was cultivated in LB medium supplemented with 50 μg mL^{-1} kanamycin. When OD_{600} reached to 0.6–0.8, 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added into and further induced under 25 °C for 6 h. Then, the cell pellets were harvested by centrifugation at 8000×*g* and 4 °C. For purification, the cells were resuspended in binding solution buffer (20 mM

sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4) and disrupted by ultrasonication (400 W, work 3 s, stop 2 s) for 15 min in an ice bath. Afterward, the lysed cells were centrifuged at 10,000×*g* for 30 min to remove the cell debris. The soluble fraction was filtrated through 0.22-μm filter and directly loaded to an equilibrated Histrap column (GE Healthcare, USA), and then eluted with a linear gradient of imidazole concentration (20–500 mM). The eluents containing protein peaks were collected, pooled, and desalted against a Hitrap desalting column (GE Healthcare, USA). The purified *Bl*BDH was verified via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stored at 4 °C for further characterization.

Gel Filtration Chromatography

The molecular mass of the recombinant *Bl*BDH was determined by gel filtration chromatography with a Superdex™ 200 column (GE Healthcare, USA). In total, 0.5 mL purified enzyme (0.1 mg/mL) was applied onto the column, and then equilibrated with 100 mM Tris–HCl buffer (pH 7.0). Standard proteins used for calibration were aprotinin (6.5 kDa), ribonuclease A (13.7 kDa), *β*-lactoglobulin (35 kDa), bovine serum albumin (67 kDa), and ferritin (440 kDa).

Enzyme Activity Assays

Reduction and oxidation activities were measured spectrophotometrically by monitoring the increase or decrease in absorbance of NADH at 340 nm. Unless otherwise stated, all reactions were performed at 30 °C under standard reaction conditions as described below. Each oxidation reaction was performed in a reaction mixture containing 100 mM glycine-NaOH buffer (pH 10.0), 5 mM alcohol (2,3-BD), and 1 mM NAD^+. Each reduction reaction was performed in a reaction mixture containing 100 mM potassium phosphate buffer (pH 6.0), 5 mM ketone, and 1 mM NADH. The enzyme activity assay was started by adding an appropriate amount of purified enzyme. One unit of BDH activity was apparently defined as the amount of enzyme required to reduce 1 μmol NADH for one carbonyl group (2 μmol NADH for diacetyl group) or oxidize 1 μmol NAD^+ for one hydroxyl group (2 μmol NAD^+ for dihydroxyl group) in 1 min at the activity assay condition [23]. Protein concentrations of all samples were determined using standard Bradford method with BSA as standard protein. All the activities were assayed in triplicate.

Enzymatic Properties of *meso*-2,3-BDH

The pH-profiles of *Bl*BDH toward different substrates were determined at room temperature in the range of pH 4.0–12.0 using 100 mM sodium citrate (pH 4.0–6.0), sodium phosphate (pH 6.0–8.0), and glycine-NaOH buffers (pH 8.0–12.0). The optimal temperature was determined at 4–46 °C using the abovementioned protocol. And, the thermostability of the purified *Bl*BDH was determined under 50 °C by measuring the residual activity using diacetyl as substrate. Different metal ions in the form of chloride/sulfate salts were added to the purified enzyme solution at a final concentration of 1 mM, incubated at 30 °C for 30 min, and afterward measured the residual activity in a mixture of 100 mM Tris–HCl buffer (pH 7.0), 5 mM diacetyl, and 1 mM NADH or 100 mM Tris–HCl buffer (pH 8.9), 5 mM 2,3-BD, and 1 mM

NAD^+. The residual activity of enzyme without metal ions was regarded as control. All the activities were measured in triplicate.

The substrate specificity was studied by detecting the activities with mixture of 100 mM sodium citrate (pH 5.0), 5 mM ketones, and 1 mM NADH for reduction reactions and mixture of 100 mM glycine-NaOH (pH 10.0), 10 mM alcohols, and 1 mM NAD^+ for oxidation reactions, respectively. Kinetic parameters for diacetyl, racemic acetoin, racemic 2,3-BD, NADH, and NAD^+ were determined as previously described [24]. The K_m and k_{cat} were calculated by nonlinear fitting with Michealis-Menten equation.

Stereospecificity of *meso*-2,3-BDH

Stereospecificity of *Bl*BDH was analyzed by carrying out the asymmetric reduction of DA. Reaction mixture (5 mL) consisted of 100 U purified *Bl*BDH, 100 U GDH for cofactor regeneration, 1.25 mmol glucose, 0.5 mmol DA, and 2.5 μmol NAD^+ in appropriate of PBS buffer (pH 6.5, 100 mM) was kept static at 30 °C for 12 h. Then, the reaction mixture was extracted with equal volume of ethyl acetate supplemented with dodecane as internal standard. The upper organic phase was separated and dried over anhydrous Na_2SO_4 for 12 h. The sample was analyzed through gas chromatography (Varian CP3900) equipped with FID detector and CP7502-Chirasil-DEX CB column as previously reported [13]. Temperature program was set as incubating at 50 °C for 2 min, and increasing to 80 °C at 10 °C/min, further increasing to 180 °C at 10 °C/min and holding for 10 min. The split ratio was 1:50. The retention times were as follows: (*R*)-AC, 8.07 min; (*S*)-AC, 8.89 min; (2*S*,3*S*)-2,3-BD, 15.53 min; (2*R*,3*R*)-2,3-BD, 15.18 min; and *meso*-2,3-BD, 15.00 min.

Results and Discussion

Sequence Analysis of *meso*-2,3-BDH from *B. licheniformis*

Based on the genomic sequence (NC_006322.1) of *B. licheniformis*, *bud*C gene encoding *meso*-2,3-butanediol dehydrogenase (*meso*-2,3-BDH) was selected in our study. The *bud*C gene (GenBank no. 3100198) is 783 bp in length and is under the control of α-acetolactate synthease (ALS) pathway. The protein encoded by *bud*C comprises 260 amino acids and was designated as *Bl*BDH. Phylogenetic analysis reveals that there are three main groups of enzymes exhibiting 2,3-butanediol dehydrogenase activity, including *meso*-2,3-BDH (or acetoin dehydrogenase), GDH, and (2*R*,3*R*)-2,3-BDH (Fig. 1). The dehydrogenase coded by *bud*C falls into the branch of *meso*-2,3-BDH and displays distinct difference to the members of GDH and (2*R*,3*R*)-2,3-BDH.

*Bl*BDH shares high sequence identity with other *meso*-2,3-BDH from *K. pneumonia* [6], *E. cloacae* [9], *P. polymyxa* [15], *S. marcescens* [19], and *R. erythropolis* [21]. As illustrated in Fig. 2, all of them share similar backbone in secondary structure and belong to SDR superfamily. Further BLAST analysis of *Bl*BDH in SDR database reveals that it is classified into SDR69C subfamily, namely acetoin dehydrongease (http://www.sdr-enzymes.org). As shown Table 1, key motifs of SDR proteins, including coenzyme binding region, catalytic triad, structure-stabilizing residues, and reaction-directed residues, are conserved in *Bl*BDH [25]. The classic Rossaman fold for NADH-binding motif TGGGQGIGEA was identifed

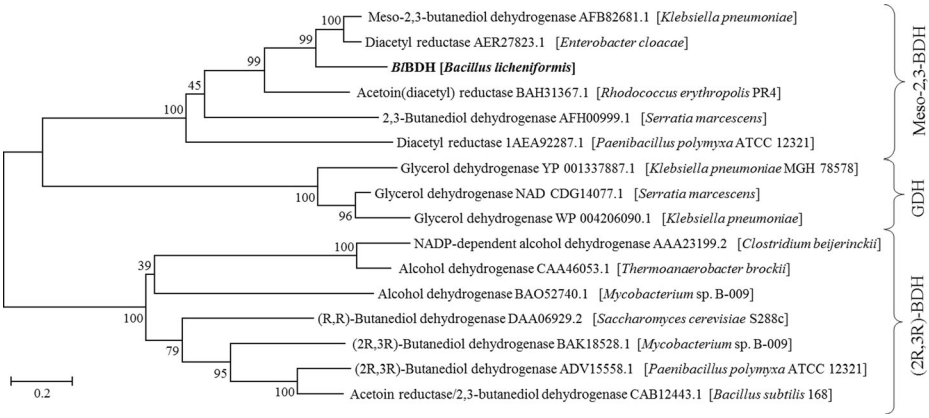


Fig. 1 Phylogenetic tree of *meso*-2,3-BDHs, glycerol dehydrogenases, and (2*R*,3*R*)-2,3-BDHs

within the *N*-terminal of *Bl*BDH, indicating it belongs to classic SDR (Table 1). The catalytic triad S-Y-K was also conserved in *Bl*BDH, at residues 143, 156, and 160.

Cloning and Expression of *Bl*BDH

The recombinant *Bl*BDH was soluble expressed in *E. coli* BL21(DE3) as shown in Fig. 3. The apparent size of recombinant *Bl*BDH as migrated on the SDS-PAGE is in good agreement with the calculated molecular weight (around 30 kDa). Crude extract of *Bl*BDH could catalyze the cascade reduction of DA to AC, and further to BD, showing an apparent activity of 60.7 U/mg crude extract toward DA.

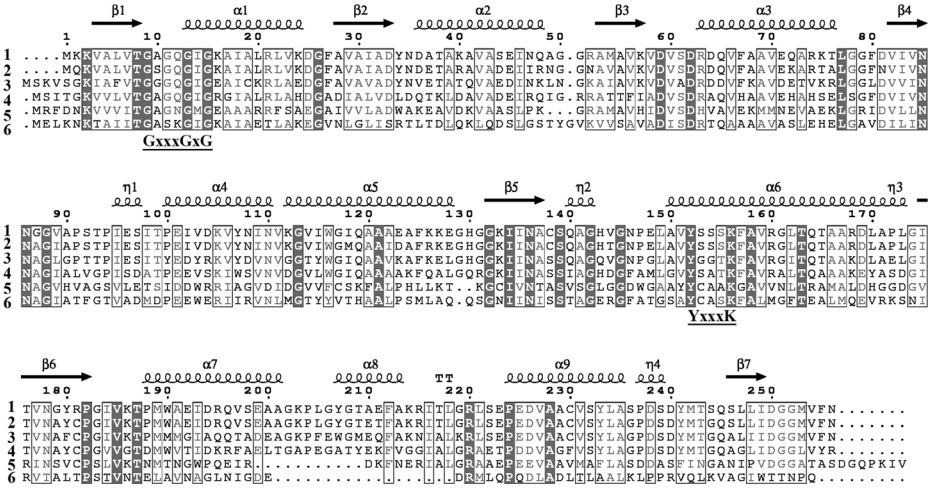


Fig. 2 Amino acid sequence alignment of *meso*-2,3-BDH from *Bacillus licheniformis* with other reported *meso*-2,3-BDHs. *1, K. pneumonia*; *2, E. cloacae*; *3, B. licheniformis*; *4, R. erythropolis*; *5, S. marcescens*; *6, P. polymyxa*

Table 1 Typical sequence motifs of SDR identified in *Bl*BDH and homologous proteins

Sequence motif of SDR	Position in SDR	Function	Position in *Bl*BDH
(T)Gxx(x)Gx(G)Xa	12–19	Coenzyme binding region, maintenance of central β-sheet	12–20[a]
D	60	Stabilization of adenine ring pocket, weak binding to cofactor	63
N	111	Active site	114
S	140	Active site	143
YxxxK	151–154	Active site	156–160[b]
N	179	Connection of substrate binding loop and active site	182
PG	183–184	Reaction direction	186–187
T	188	H bonding to carboxamide of nicotinamide ring	191

[a] TGGGQGIGEA

[b] YGGTK

Purification of the *Bl*BDH

The crude extract of *N*-terminal His-tagged recombinant *Bl*BDH (218 mg) was successfully purified to homogeneity through affinity chromatography as confirmed by a single band of 30 kDa on SDS-PAGE (Fig. 3). After desalting, about 89.2 mg of *Bl*BDH was yielded. The apparent purity was about 90 % based on the area of elution peak. The molecular mass of the native enzyme was estimated to be 125 kDa, and this *Bl*BDH is a homotetramer consisted of subunits of about 31.3 kDa (data not shown).

Full activity was detected with NADH as cofactor whereas no activity with NADPH, indicating *Bl*BDH is NADH-dependent. The specific activity of purified *Bl*BDH (DA as substrate) is 120 U/mg, representing a purification fold of 1.98. As shown in Scheme 1, the

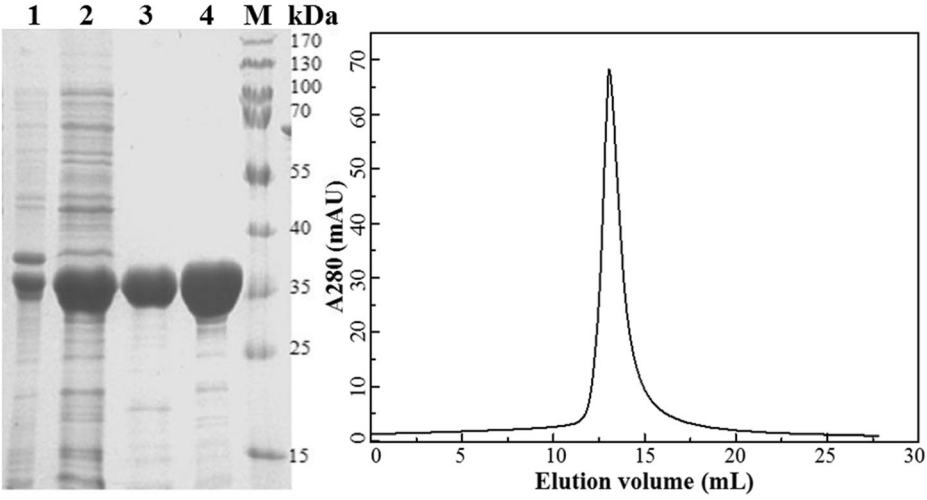


Fig. 3 Determination of molecule mass of *Bl*BDH. *Lanes 1 and 2*, precipitant and supernatant of recombinant *E. coli* BL21/pET28-*bud*C; *lane 3*, purified *Bl*BDH from Histrap column; *lane 4*, purified *Bl*BDH from gel filtration chromatography; *lane M*, protein molecular weight marker

*Bl*BDH could catalyze the asymmetric reduction of DA mainly into (*S*)-AC, and (*S*)-AC into (2*S*,3*S*)-BD, as well as the oxidation of (2*S*,3*S*)-BD into (*S*)-AC.

Effect of pH and Temperature on *Bl*BDH

Due to the mutifunction of *Bl*BDH, the optimum pH was investigated using different substrates (DA, AC, and BD). The pH profiles toward DA, AC, and BD are shown in Fig. 4a–c. In the reduction of DA, the highest enzymatic activity was determined at pH 5.0 (Fig. 4a). The activity was sharply decreased at pHs higher or lower than 5.0, and almost no reducing activity toward DA was detected at pH >8 (glycine-NaOH buffer). As shown in Fig. 4b, the highest activity toward AC was reached at pHs from 5.0 to 8.0. And, more than 60 % of activity was retained between pH 4.5 and 10.0, indicating that *Bl*BDH could remain active over a wide pH range in the asymmetric reduction of AC. With regard to the oxidation of BD (Fig. 4c), the pH optimum was around pH 10.0. It is commonly known that the oxidation reaction is usually catalyzed by dehydrogenases under basic conditions [23]. At pHs over or less than 10.0, the activity of *meso*-2,3-BDH was quickly decreased. And, only <20 % activity was detected at pH ≤8.0. Above results demonstrated that different electric status might influence the substrate binding and thus the catalytic efficiency. The different pH profiles of *Bl*BDH toward various substrates provides evidence for its complicated catalytic mechanism and the utilization of *Bl*BDH for different purposes, which were in coincidence with other *meso*-2,3-BDHs [17–19]. Remarkable difference in pH profiles for reduction and oxidation confers *Bl*BDH with high versatility under different circumstance [23].

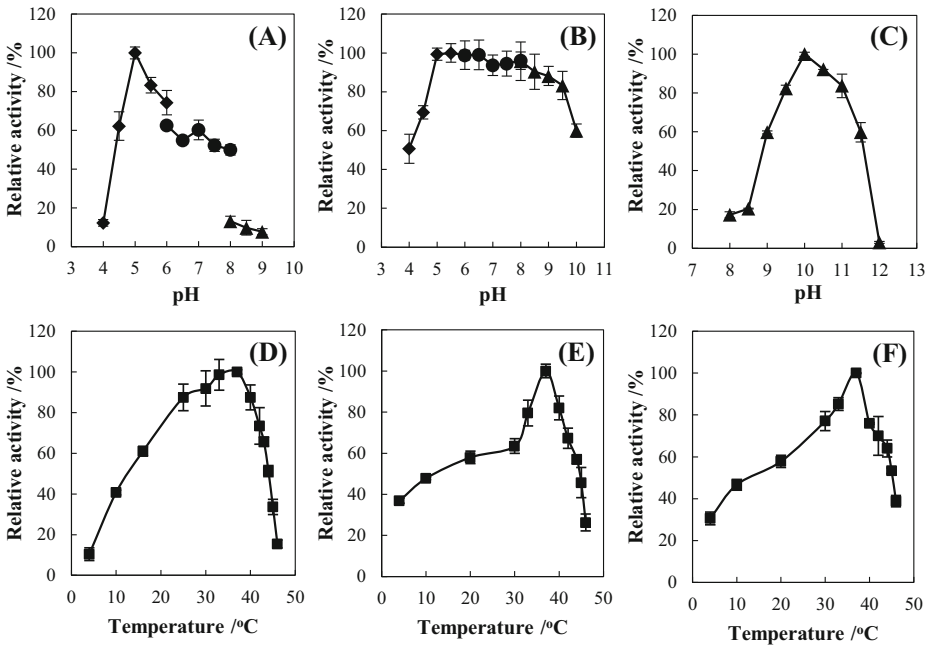


Fig. 4 pH and temperature profiles of purified *Bl*BDH toward different substrates. **a**–**c** pH profiles to DA, AC, and BD; **d**–**f** temperature profiles to DA, AC, and BD. *Diamonds*, citrate buffer; *circles*, PBS buffer; *triangles*, glycine-NaOH buffer

The effect of temperature on enzyme activity was also performed using three different substrates (DA, AC, and BD) over a temperature range of 4–46 °C. No obvious difference was observed among these substrates (Fig. 4d–f). The highest activity was observed at 37 °C, above which the activity sharply decreased, indicating *Bl*BDH is a mesophilic enzyme and has a moderate deactivation energy. Since thermophilic *B. licheniformis* could efficiently produce 2,3-BD at higher temperature (e.g., 50 °C), the thermostability of *Bl*BDH was examined at 50 °C using DA as substrate. The enzymatic activity dropped rapidly to half of its initial activity within 40 min, then further decreased to 20 % after 330 min, suggesting that it was liable at higher temperatures. Consequently, the thermostablility of *Bl*BDH could be affected by the relatively lower incubation temperature (25 °C) during its recombinant expression. Compared with other *meso*-2,3-BDHs, this *Bl*BDH only displayed moderate thermostability and relative low high-temperature dependence.

Effect of Metal Ions on *Bl*BDH

This *Bl*BDH belongs to SDR superfamily and might be divalent metal ion dependent as other SDR members [26]. Several cations (including Zn^{2+}, Fe^{2+}, and Mg^{2+}) have been reported to influence the activity of enzymes involved in the interconversion between DA and 2,3-BD by stabilizing enzyme conformation and participating substrate binding. Hence, the effects of different cations on *Bl*BDH activity were studied using DA and BD as substrates (Table 2). For reducing activity, the addition of metal ions such as Mn^{2+} and Co^{2+} could slightly activate the enzyme, while Al^{3+}, Cu^{2+}, Ag^{+}, and Fe^{3+} showed strong inhibition on the activity. The addition of other metal ions also has certain inhibitory effect on the activity, which was reduced by 5–25 %. No obvious inhibitory effect was found with metal chelating agent. Similar phenomena were observed with the oxidizing activity of *Bl*BDH. It is speculated that

Table 2 Effect of various metal ions on *Bl*BDH

Metal ions	Relative activity (%)	
	Diacetyl	2,3-Butanediol
Control	100	100
$MgSO_4$	95.6±4.8	96.8±3.2
$MnSO_4$	110.5±5.5	105.8±4.5
$ZnSO_4$	75.0±5.3	80.3±4.2
$Al_2(SO_4)_3$	3.9±1.0	5.7±0.9
$NiSO_4$	96.2±5.8	95.9±4.8
$BaCl_2$	96.8±11.9	96.2±5.8
$CoCl_2$	104.5±2.5	102.5±3.2
$CuSO_4$	0.2±0.1	1.1±0.2
$LiC_2H_3O_2$	100.8±6.4	101.5±5.5
$AgNO_3$	0.5±0.2	0.5±0.2
$CaCl_2$	97.9±7.1	98.8±4.8
$FeCl_3$	1.9±2.2	3.5±0.6
NaCl	90.9±5.2	92.2±3.8
KCl	84.9±5.4	85.7±4.0
EDTA	91.2±1.2	89.8±4.5

this enzyme is not metal ion dependent. The decrease of enzyme activity by some metal ions could be caused by the disturbed local conformation of protein stereostructure. Most of the reported *meso*-2,3-BDHs were metal ion independent, except for some from medium-chain dehdyrogenases/reductases superfamily [23].

Substrate Specificity of *meso*-2,3-BDH

The *Bl*BDH could catalyze the reduction of ketone and the oxidation of alcohol. The substrate specificity toward a set of ketones, alcohols, and diols was investigated (Table 3). In the reduction reaction, the enzyme displayed the highest reducing activity toward DA (120 U/mg) and racemic AC (116 U/mg), while no activity was detected for 2-butanone which has only one carbonyl group. The enzyme also showed activity toward 2,3-hexanedione, 2,3-pentanedione, and 3,4-hexanedione, which possess two vicinal carbonyl groups. Additionally, activities decreased alongside the increase of chain length. No activity was detected for 2,5-hexanedione and 2,4-pentanedione, which possess two distinct carbonyl groups. Therefore, it is speculated that the two vicinal carbonyl groups was essential for the reducing activity of *Bl*BDH.

For the oxidation reaction, various alcohols including vicinal and distinct diols were examined (Table 3). *Bl*BDH showed the highest activity toward racemic 2,3-BD. Compared with 2,3-BD, only 0.57 % activity was detected for 1,2-propanediol, which has one less carbon than BD. No activity was found with 1,4-butanediol (1,4-BD), an isomers of 2,3-BD. With regard to alcohols, including methanol, mannitol, glycerol, and acetoin, no activity was detected, which was the same as the acetoin reductase/2,3-butanediol dehydrogenase from *Clostridium beijerinckii* NCIMB 8052 [23]. Consequently, the oxidation activity of *Bl*BDH depends specifically on 2,3-BD.

Table 3 Substrate specificity of *Bl*BDH

Reaction	Substrate	Relative activity (%)
Reduction	Diacetyl	100±3
	Acetoin	97±2
	2,3-Hexanedione	66±2
	2,3-Pentanedione	69±4
	3,4-Hexanedione	10±1
	2,5-Hexanedione	*n.d.*
	2,4-Pentanedione	*n.d.*
	2-Butanone	*n.d.*
Oxidation	2,3-Butanediol	100±2
	1,2-Propanediol	0.57±0.15
	1,4-Butanediol	*n.d.*
	Methanol	*n.d.*
	Mannitol	*n.d.*
	Glycerol	*n.d.*
	Acetoin	*n.d.*

[a] *n.d.* no activity was detected

Table 4 Kinetic parameters of *Bl*BDH toward diacetyl, acetoin, 2,3-butanediol, NADH, and NAD^+

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)
Diacetyl	72.4±0.4	1222±5	16.9±1.1
Acetoin	0.47±0.03	202±2	432±5
2,3-Butanediol	7.25±0.05	591±3	81.5±3.5
NADH	0.25±0.02	1274±5	5072±110
NAD^+	0.34±0.04	748±3	2192±44

Kinetic Parameters of *Bl*BDH

The kinetic parameters of *Bl*BDH were determined (Table 4). The K_m and k_{cat} values are 72.4 mM and 1222 s^{-1} for DA, 0.47 mM and 202 s^{-1} for AC, 7.25 mM and 591 s^{-1} for BD, 0.25 mM and 1274 s^{-1} for NADH, 0.34 mM and 748 s^{-1} for NAD^+, respectively. The enzyme showed lower K_m and k_{cat}/K_m values to AC in comparison with that for DA, suggesting its physiological role in favor of 2,3-BD formation in *B. licheniformis*. This *Bl*BDH is a robust dehydrogenase due to its high k_{cat} values toward DA (1222 s^{-1}) and NADH (1274 s^{-1}), which was much higher than *meso*-2,3-BDHs from *P. polymyxa*, *R. erythropolis*, and *C. beijerinckii* [15, 19, 23]. The naturally evolved function of *Bl*BDH favors the efficient conversion of DA to 2,3-BD using NADH as cofactor.

Stereospecificity of *Bl*BDH

To understand the stereospecificity of *Bl*BDH in the oxido-reduction processes of DA/AC/2,3-BD interconversion, the biocatalytic reactions were performed using purified enzyme.

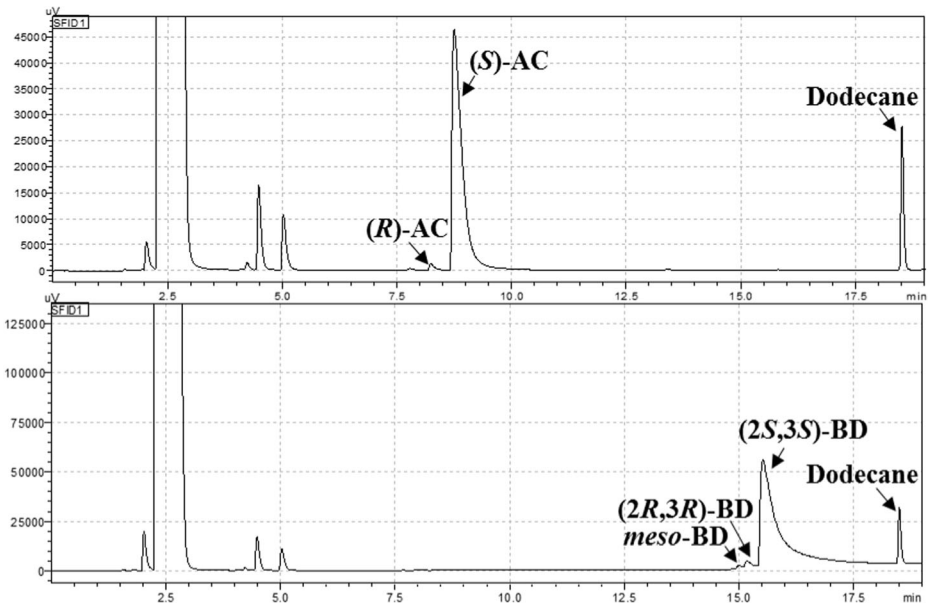


Fig. 5 Stereospecificity of *Bl*BDH in the asymmetric reduction of DA

According to Fig. 5, DA was converted into (*S*)-AC with 97.3 % *ee*, and further converted into mainly (2*S*,3*S*)-2,3-BD, and a little *meso*-2,3-BD and (2*R*,3*R*)-2,3-BD with 97.3 % *ee* and 96.5 % *de*. The stereospecificity of *Bl*BDH is not highly strict. *B. licheniformis* strain harboring *Bl*BDH coding gene *budC* could hardly produce enantiopure 2,3-BD [10]. In addition to the complicated dehydrogenase systems in *B. licheniformis*, the low stereospecificity of *Bl*BDH is also a critical factor affecting the configuration of the fermentation product 2,3-BD. To engineering *B. licheniformis* strain for the production of 2,3-BD with high optical purity, the introduction of highly stereospecific *Bl*BDH by protein engineering is a promising solution.

Conclusion

A *meso*-2,3-BDH (*Bl*BDH) encoding gene *bud*C was cloned from *B. licheniformis* and heterogeneously overexpressed in *E. coli*. The *Bl*BDH is responsible for the production of 2,3-BD by interconversion of DA/AC/2,3-BD. Enzymatic properties of this *Bl*BDH is important for the engineering and application of *B. licheniformis* in enantiopure 2,3-BD production. *Bl*BDH is a potential member of classic SDR superfamily and SDR69C subfamily. Most key motifs of SDR are conserved in the *Bl*BDH. The *Bl*BDH is an NADH-dependent enzyme with a specific activity of 120 U/mg purified enzyme (DA as substrate). The optimum pHs for DA reduction and 2,3-BDH oxidation are around pH 5.0 and 10.0, while a wide pH range is found for AC reduction. The optimum temperature is 37 °C. Its high k_{cat}/K_m value toward AC demonstrates that the naturally evolved function of *Bl*BDH favors DA reduction. The enzyme preferred vicinal diketone and diols as substrate, especially diacetyl and butanediol. The stereosepecificity of *Bl*BDH is 97.3 % in the reduction of DA into (*S*)-AC. This study provides evidence for the properties and application of *Bl*BDH and contributes to the engineering of *B. licheniformis* for the production of chiral 2,3-BD.

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Compliance with Ethical Standards

Conflict of Interest All the authors certify that this manuscript is original and has not been published and will not be submitted elsewhere for publication while being considered by Applied Biochemistry and Biotechnology. Moreover, the study is not split up into several parts to increase the quantity of submissions and submitted to various journals or to one journal over time. No data have been fabricated or manipulated (including images) to support your conclusions. No data, text, or theories by others are presented as if they were our own. The submission has been received explicitly from all coauthors. Furthermore, authors whose names appear on the submission have contributed sufficiently to the scientific work and therefore share collective responsibility and accountability for the results. The authors declare that they have no conflict of interest.

Ethics Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Informed consent was obtained from all individual participants included in the study.

References

1. Li, L. X., Zhang, L. J., Li, K., Wang, Y., Gao, C., Han, B. B., Ma, C. Q., & Xu, P. (2013). A newly isolated *Bacillus licheniformis* strain thermophilically produces 2,3-butanediol, a platform and fuel biochemical. *Biotechnology for Biofuels, 6*, 123.
2. Ji, X. J., Huang, H., & Ouyang, P. K. (2011). Microbial 2,3-butanediol production: a state-of-the-art review. *Biotechnology Advances, 29*, 351–364.
3. Li, L. X., Wang, Y., Zhang, L. J., Ma, C. Q., Wang, A. L., Tao, F., & Xu, P. (2012). Biocatalytic production of (2*S*,3*S*)-2,3-butanediol from diacetyl using whole cells of engineered *Escherichia coli. Bioresource Technology, 115*, 111–116.
4. Qi, G. F., Kang, Y. F., Li, L., Xiao, A. F., Zhang, S. M., Wen, Z. Y., Xu, D. H., & Chen, S. W. (2014). Deletion of meso-2,3-butanediol dehydrogenase gene budC for enhanced D-2,3-butanediol production in *Bacillus licheniformis. Biotechnology for Biofuels, 7*, 16.
5. Xu, G. C., & Ni, Y. (2015). Bioreductive preparation of ACE inhibitors precursor (*R*)-2-hydroxy-4-phenylbutanoate esters: recent advances and future perspectives. *Bioresources and Bioprocessing, 2*, 15.
6. Ma, C. Q., Wang, A. L., Qin, J. Y., Li, L. X., Ai, X. L., Jiang, T. Y., Tang, H. Z., & Xu, P. (2009). Enhanced 2,3-butanediol production by *Klebsiella pneumoniae* SDM. *Applied Microbiology and Biotechnology, 82*, 49–57.
7. Zhang, L. Y., Yang, Y. L., Sun, J. A., Shen, Y. L., Wei, D. Z., Zhu, J. W., & Chu, J. (2010). Microbial production of 2,3-butanediol by a mutagenized strain of *Serratia marcescens* H30. *Bioresource Technology, 101*, 1961–1967.
8. Ji, X. J., Huang, H., Du, J., Zhu, J. G., Ren, L. J., Hu, N., & Li, S. (2009). Enhanced 2,3-butanediol production by *Klebsiella oxytoca* using a two-stage agitation speed control strategy. *Bioresource Technology, 100*, 3410–3414.
9. Wang, A., Xu, Y. Q., Ma, C., Gao, C., Li, L. X., Wang, Y., Tao, F., & Xu, P. (2012). Efficient 2,3-butanediol production from cassava powder by a crop-biomass-utilizer, *Enterobacter cloacae* subsp. dissolvens SDM. *PLoS One, 7*, e40442.
10. Jurchescu, I. M., Hamann, J., Zhou, X., Ortmann, T., Kuenz, A., Prüβe, U., & Lang, S. (2013). Enhanced 2, 3-butanediol production in fed-batch cultures of free and immobilized *Bacillus licheniformis* DSM 8785. *Applied Microbiology and Biotechnology, 97*, 6715–6723.
11. Hassler, T., Schieder, D., Pfaller, R., Faulstich, M., & Sieber, V. (2012). Enhanced fed-batch fermentation of 2,3-butanediol by *Paenibacillus polymyxa* DSM 365. *Bioresource Technology, 124*, 237–244.
12. Biswas, R., Yamaoka, M., Nakayama, H., Kondo, T., Yoshida, K., Bisaria, V. S., & Kondo, A. (2012). Enhanced production of 2,3-butanediol by engineered *Bacillus subtilis. Applied Microbiology and Biotechnology, 94*, 651–658.
13. Yan, Y. J., Lee, C. C., & Liao, J. C. (2009). Enantioselective synthesis of pure (R, R)-2,3-butanediol in *Escherichia coli* with stereospecific secondary alcohol dehydrogen. *Organic and Biomolecular Chemistry, 7*, 3914–3917.
14. González, E., Fernández, M. R., Larroy, C., Solà, L., Pericàs, M. A., Parés, X., & Biosca, J. A. (2000). Characterization of a (2*R*,3*R*)-2,3-butanediol dehydrogenase as the *Saccharomyces cerevisiae* YAL060W gene product. Disruption and induction of the gene. *Journal of Biological Chemistry, 275*, 35876–35885.
15. Yu, B., Sun, J., Bommareddy, R. R., Song, L., & Zeng, A. P. (2011). Novel (2*R*,3*R*)-2,3-butanediol dehydrogenase from potential industrial strain *Paenibacillus polymyxa* ATCC 12321. *Applied and Environmental Microbiology, 77*, 4230–4233.
16. Nicholson, W. L. (2008). The *Bacillus subtilis* ydjL (bdhA) gene encodes acetoin reductase/2,3-butanediol dehydrogenase. *Applied and Environmental Microbiology, 74*, 6832–6838.
17. Zhang, L. Y., Xu, Q. M., Zhan, S. R., Li, Y. Y., Lin, H., Sun, S. J., Sha, L., Hu, K. H., Guan, X., & Shen, Y. L. (2014). A new NAD(H)-dependent *meso*-2,3-butanediol dehydrogenase from an industrially potential strain *Serratia marcescens* H30. *Applied Microbiology and Biotechnology, 98*, 1175–1184.
18. Giovannini, P. P., Medici, A., Bergamini, C. M., & Rippa, M. (1996). Properties of diacetyl (acetoin) reductase from *Bacillus stearothermophilus. Bioorganic and Medicinal Chemistry, 4*, 1197–1201.
19. Wang, Z., Song, Q. Q., Yu, M. L., Wang, Y. F., Xiong, B., Zhang, Y. J., Zheng, J. Y., & Ying, X. X. (2014). Characterization of a stereospecific acetoin (diacetyl) reductase from *Rhodococcus erythropolis* WZ010 and its application for the synthesis of (2*S*,3*S*)-2,3-butanediol. *Applied Microbiology and Biotechnology, 98*, 641–650.
20. Zhang, L. Y., Xu, Q. M., Peng, X. Q., Xu, B. H., Wu, Y. H., Yang, Y. L., Sun, S. J., Hu, K. H., & Shen, Y. L. (2014). Cloning, expression and characterization of glycerol dehydrogenase involved in 2,3-butanediol formation in *Serratia marcesce. Journal of Industrial Microbiology and Biotechnology, 41*, 1319–1327.
21. Wang, Y., Tao, F., & Xu, P. (2014). Glycerol dehydrogenase plays a dual role in glycerol metabolism and 2,3-butanediol formation in *Klebsiella pneumoniae. Journal of Biological Chemistry, 289*, 6080–6090.

22. Thanh, T. N., Jürgen, B., Bauch, M., Liebeke, M., Lalk, M., Ehrenreich, A., Evers, S., Maurer, K. H., Antelmann, H., Ernst, F., Homuth, G., Hecker, M., & Schweder, T. (2010). Regulation of acetoin and 2,3-butanediol utilization in *Bacillus licheniformis. Applied Microbiology and Biotechnology, 87*, 2227–2235.
23. Raedts, J., Siemerink, M. A. J., Levisson, M., van der Oost, J., & Kengen, S. W. M. (2014). Molecular characterization of an NADPH-dependent acetoin reductase 2,3-butanediol dehydrogenase from *Clostridium beijerinckii* NCIMB8052. *Applied and Environmental Microbiology, 80*, 2011–2020.
24. Xu, G. C., Yu, H. L., Zhang, X. Y., & Xu, J. H. (2012). Access to optically active aryl halohyddrins using a substrate-tolerant carbonyl reductase discovered from *Kluyveromyces thermotolerans. ACS Catalysis, 2*, 2566–2571.
25. Filling, C., Berndt, K. D., Benach, J., Knapp, S., Prozorovski, T., Nordling, E., Ladenstein, R., Jörnvall, H., & Oppermann, U. (2002). Critical residues for structure and catalysis in short-chain dehydrogenases/reductases. *Journal of Biological Chemistry, 277*, 25677–25684.
26. Jörnvall, H., Hedlund, J., Bergman, T., Oppermann, U., & Persson, B. (2010). Superfamilies SDR and MDR: from early ancestry to present forms. Emergence of three lines, a Zn-metalloenzyme, and distinct variabilities. *Biochemical and Biophysical Research Communications, 396*, 125–130.