

A new NAD(H)-dependent *meso*-2,3-butanediol dehydrogenase from an industrially potential strain *Serratia marcescens* H30

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Abstract The *budC* gene coding for a new *meso*-2,3-butanediol dehydrogenase (BDH) from *Serratia marcescens* H30 was cloned and expressed in *Escherichia coli* BL21(DE3), purified, and characterized for its properties. The recombinant BDH with a molecular weight of 27.4 kDa exhibited a reversible transformation between acetoin and 2,3-butanediol. In the presence of NADH, BDH could catalyze the reduction of diacetyl and (3*R*)-acetoin to (3*S*)-acetoin and *meso*-2,3-butanediol, respectively, while (3*S*)-acetoin as a substrate could be further transformed into (2*S*, 3*S*)-2,3-butanediol at pH 9.0. For diol oxidation reactions, (3*R*)-acetoin and (3*S*)-acetoin were obtained when *meso*-2,3-butanediol and (2*S*,3*S*)-2,3-butanediol were used as the substrates with BDH and NAD⁺. (2*R*,3*R*)-2,3-butanediol was not a substrate for the BDH at all. The low K_m value (4.1 mM) in *meso*-2,3-butanediol oxidation reaction and no activity for diacetyl, acetoin, and 2,3-butanediol as the substrates with NADP⁺/NADPH suggested that the *budC* gene

product belongs to a NAD(H)-dependent *meso*-2,3-BDH. Maximum activities for diacetyl and (3*S*/3*R*)-acetoin reduction were observed at pH 8.0 and pH 5.0 while for *meso*-2,3-butanediol oxidation it was pH 8.0. However, the optimum temperature for oxidation and reduction reactions was about 40 °C. In addition, the BDH activity for *meso*-2,3-butanediol oxidation was enhanced in the presence of Fe²⁺ and for diacetyl and (3*S*/3*R*)-acetoin reduction in the presence of Mg²⁺ and Mn²⁺, while several metal ions inhibited its activity, particularly Fe³⁺ for reduction of diacetyl and acetoin. Sequence analysis showed that the BDH from *S. marcescens* H30 possessed two conserved sequences including the coenzyme binding motif (GxxxGxG) and the active-site motif (YxxxK), which are present in the short-chain dehydrogenase/reductase superfamily.

Keywords *Serratia marcescens* · *Meso*-2,3-butanediol dehydrogenase · Cloning · Expression · Enzymatic properties

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Introduction

2,3-Butanediol (2,3-BD) and its dehydrogenation product, acetoin (AC), are important platform compounds due to its potential industrial applications (Ji et al. 2009; Liu et al. 2011). 2,3-BD contains two stereo centers and has three stereo isomers including (2*R*,3*R*)-, *meso*- and (2*S*,3*S*)-forms, and AC exists in two stereoisomeric forms: (3*R*)-acetoin and (3*S*)-acetoin, which are important potential pharmaceutical intermediates (Yan et al. 2009; Ji et al. 2011). All of the isomers of 2,3-BD and AC could be produced by the homologous pathways involving pyruvate in a mixed acid fermentation process in several native strains such as *Klebsiella pneumoniae* (Borim et al. 2012), *Klebsiella oxytoca*

(Yu and Saddler 1983; Han et al. 2013), *Enterobacter aerogenes* (Zeng et al. 1990), *Enterobacter cloacae* (Li et al. 2011), *Bacillus polymyxa* (De Mas et al. 1987), and *Serratia marcescens* (Zhang et al. 2010; Sun et al. 2012; Rao et al. 2012). α -Acetolactate, diacetyl, and acetoin are the main three intermediate compounds of 2,3-BD fermentation in bacteria (Celinska and Grajek 2009; Zhang et al. 2012). In general, the stereoisomeric composition of 2,3-BD and AC formed by bacteria differs among strains (Table 1), a phenomenon that is related to the existence of various 2,3-butanediol dehydrogenases (BDHs) differing in their stereospecificities (Ui et al. 1998).

Several BDHs from different strains have been characterized in previous studies (Table 2). In summary, BDHs can be divided into three classes: *meso*-BDH, (2*S*,3*S*)-BDH and (2*R*,3*R*)-BDH. According to the configuration of the AC product from 2,3-BD as a substrate, BDHs also were classified into two types: *D*-(-)-Acetoin forming and *L*-(+)-Acetoin forming. *meso*-BDHs in *K. pneumoniae* CICC10011 and *Enterobacter* sp. 638 could catalyze the conversion of (3*R*)-AC to *meso*-2,3-BD and (3*S*)-AC to (2*S*,3*S*)-2,3-BD in the presence of NADH. Meanwhile, *meso*- and (2*S*,3*S*)-2,3-BD as the substrates could also be transformed into (3*R*)- and (3*S*)-AC, respectively, with the BDHs and NAD⁺ (Liu et al. 2011; Li et al. 2011). While (2*S*,3*S*)-BDH from *Brevibacterium saccharolyticum* C-1012

was found to possess substrate specificity in the interconversion between (3*S*)-AC and (2*S*,3*S*)-2,3-BD (Ui et al. 1998; Takusagawa et al. 2001), which has not yet been reported in other strains. Recently, three (2*R*,3*R*)-BDHs from *Paenibacillus polymyxa* ATCC12321, *Bacillus subtilis* 168, and *Saccharomyces cerevisiae* were identified, and analysis of their sequences revealed that the three enzymes belonged to the medium-chain dehydrogenase/reductase superfamily. The three BDHs showed the abilities in the interconversion of (3*S*)-AC/*meso*-2,3-BD and (3*R*)-AC/(2*R*,3*R*)-2,3-BD (Gonzalez et al. 2000; Xiao et al. 2010; Yu et al. 2011; Liu et al. 2011). During the 2,3-BD fermentation process, (3*R*)-AC is the decarboxylation product of α -acetolactate catalyzed by α -acetolactate decarboxylase (Xiao and Xu 2007). The precursor of (3*S*)-AC is diacetyl (DA), a byproduct of 2,3-BD fermentation produced by the non-enzymatic oxidation of α -acetolactate (Nicholson 2008). Due to the low concentration of DA in microbial fermentation, (3*S*)-AC can only be limited at low concentrations (Liu et al. 2011).

In our previous study, we reported a *S. marcescens* strain H30 possessing high productivity and yield for AC and 2,3-BD production (Zhang et al. 2010; Sun et al. 2012). During the fermentation process, the strain H30 exhibited broad substrate spectrum, cultural adaptability, and better resistance to bacteria contamination due to prodigiosin production. Therefore, it is considered as a promising producer

Table 1 AC and 2,3-BD stereoisomers produced by different species

Organism	Isomer (AC)	Isomer (2,3-BD)	References
<i>K. pneumoniae</i> IAM 1063	(3 <i>S</i>)-AC	<i>meso</i> -2,3-BD	Ui et al. (1983)
	(3 <i>R</i>)-AC	(2 <i>S</i> ,3 <i>S</i>)-2,3-BD (2 <i>R</i> ,3 <i>R</i>)-2,3-BD	
<i>K. pneumoniae</i> CICC10011	(3 <i>S</i>)-AC	<i>meso</i> -2,3-BD	Liu et al. (2011)
	(3 <i>R</i>)-AC	(2 <i>S</i> ,3 <i>S</i>)-2,3-BD	
<i>K. oxytoca</i>	(3 <i>S</i>)-AC	<i>meso</i> -2,3-BD	Celinska and Grajek (2009)
	(3 <i>R</i>)-AC	(2 <i>S</i> ,3 <i>S</i>)-2,3-BD	
<i>E. aerogenes</i>	(3 <i>S</i>)-AC	<i>meso</i> -2,3-BD	Celinska and Grajek (2009)
	(3 <i>R</i>)-AC	(2 <i>S</i> ,3 <i>S</i>)-2,3-BD	
<i>Enterobacter</i> sp. 638	(3 <i>S</i>)-AC	<i>meso</i> -2,3-BD	Li et al. (2011)
	(3 <i>R</i>)-AC	(2 <i>S</i> ,3 <i>S</i>)-2,3-BD	
<i>S. marcescens</i> H30	(3 <i>S</i>)-AC	<i>meso</i> -2,3-BD	Zhang et al. (2010)
	(3 <i>R</i>)-AC	(2 <i>S</i> ,3 <i>S</i>)-2,3-BD	
<i>B. saccharolyticum</i> C-1012	(3 <i>S</i>)-AC	(2 <i>S</i> ,3 <i>S</i>)-2,3-BD	Ui et al. (1998)
	(3 <i>R</i>)-AC		
<i>A. hydrophila</i>	(3 <i>S</i>)-AC	(2 <i>R</i> ,3 <i>R</i>)-2,3-BD	Celinska and Grajek (2009)
	(3 <i>R</i>)-AC		
<i>P. polymyxa</i> ATCC12321	(3 <i>S</i>)-AC	<i>meso</i> -2,3-BD	Yu et al. (2011)
	(3 <i>R</i>)-AC	(2 <i>R</i> ,3 <i>R</i>)-2,3-BD	
<i>B. subtilis</i>	(3 <i>S</i>)-AC	<i>meso</i> -2,3-BD	Celinska and Grajek (2009)
	(3 <i>R</i>)-AC	(2 <i>R</i> ,3 <i>R</i>)-2,3-BD	
<i>B. amyloliquefaciens</i>	(3 <i>S</i>)-AC	<i>meso</i> -2,3-BD	Celinska and Grajek (2009)
	(3 <i>R</i>)-AC	(2 <i>R</i> ,3 <i>R</i>)-2,3-BD	
<i>S. cerevisiae</i>	(3 <i>S</i>)-AC	<i>meso</i> -2,3-BD	Gonzalez et al. (2000)
	(3 <i>R</i>)-AC	(2 <i>R</i> ,3 <i>R</i>)-2,3-BD	

Table 2 Stereospecificity of 2,3-butanediol dehydrogenases (2,3-BDH) by different species using varying substrates

Organism	Enzyme	product	substrate	References
<i>K. pneumoniae</i> CICC10011	<i>meso</i> -2,3-BDH	(3 <i>S</i>)-AC	DA	Liu et al. (2011)
		(2 <i>S</i> ,3 <i>S</i>)-2,3-BD	(3 <i>S</i>)-AC	
		<i>meso</i> -2,3-BD	(3 <i>R</i>)-AC	
		(3 <i>S</i>)-AC	(2 <i>S</i> ,3 <i>S</i>)-2,3-BD	
		(3 <i>R</i>)-AC	<i>meso</i> -2,3-BD	
<i>Enterobacter</i> sp. 638	<i>meso</i> -2,3-BDH	–	(2 <i>R</i> ,3 <i>R</i>)-2,3-BD	Li et al. (2011)
		(3 <i>S</i>)-AC	DA	
		(2 <i>S</i> ,3 <i>S</i>)-2,3-BD	(3 <i>S</i>)-AC	
		<i>meso</i> -2,3-BD	(3 <i>R</i>)-AC	
		(3 <i>S</i>)-AC	(2 <i>S</i> ,3 <i>S</i>)-2,3-BD	
<i>S. marcescens</i> H30	<i>meso</i> -2,3-BDH	(3 <i>R</i>)-AC	<i>meso</i> -2,3-BD	This study
		–	(2 <i>R</i> ,3 <i>R</i>)-2,3-BD	
		(3 <i>S</i>)-AC	DA	
		(2 <i>S</i> ,3 <i>S</i>)-2,3-BD	(3 <i>S</i>)-AC	
		(3 <i>R</i>)-AC	(3 <i>R</i>)-AC	
<i>B. saccharolyticum</i> C-1012	(2 <i>S</i> ,3 <i>S</i>)-2,3-BDH	–	(2 <i>S</i> ,3 <i>S</i>)-2,3-BD	Takusagawa et al. (2001)
		ND	DA	
		(2 <i>S</i> ,3 <i>S</i>)-2,3-BD	(3 <i>S</i>)-AC	
		–	(3 <i>R</i>)-AC	
		(3 <i>S</i>)-AC	(2 <i>S</i> ,3 <i>S</i>)-2,3-BD	
<i>P. polymyxa</i> ATCC12321	(2 <i>R</i> ,3 <i>R</i>)-2,3-BDH	–	<i>meso</i> -2,3-BD	Yu et al. (2011)
		(3 <i>R</i>)-AC	DA	
		<i>meso</i> -2,3-BD	(3 <i>S</i>)-AC	
		(2 <i>R</i> ,3 <i>R</i>)-2,3-BD	(3 <i>R</i>)-AC	
		–	(2 <i>S</i> ,3 <i>S</i>)-2,3-BD	
<i>S. cerevisiae</i>	(2 <i>R</i> ,3 <i>R</i>)-2,3-BDH	(3 <i>S</i>)-AC	<i>meso</i> -2,3-BD	Gonzalez et al. (2000)
		(3 <i>R</i>)-AC	(2 <i>R</i> ,3 <i>R</i>)-2,3-BD	
		ND	DA	
		<i>meso</i> -2,3-BD	(3 <i>S</i>)-AC	
		(2 <i>R</i> ,3 <i>R</i>)-2,3-BD	(3 <i>R</i>)-AC	
–	(2 <i>S</i> ,3 <i>S</i>)-2,3-BD			
		(3 <i>S</i>)-AC	<i>meso</i> -2,3-BD	
		(3 <i>R</i>)-AC	(2 <i>R</i> ,3 <i>R</i>)-2,3-BD	

– no activity, *ND* not determined

with high industrial potential (Zhang et al. 2010; Ji et al. 2011). Interestingly, unlike other 2,3-BD producing Gram-negative strains, this strain mainly produced *meso*-2,3-BD (a weight fraction of over 98 %) with a little (2*S*,3*S*)-2,3-BD (Zhang et al. 2010). To understand the characteristics of BDH from *S. marcescens* H30 and conduct a study on fermentation, the BDH gene (*budC*) was cloned, and the encoded amino acid sequence was compared with those of other BDHs reported previously. The enzyme was characterized according to the optimal pH, temperature, and substrate stereospecificity after the expression and purification.

Materials and methods

Strains and bacterial growth condition

S. marcescens H30 (deposited in the China Center for Industrial Culture Collection, accession number: CICC 20066) used as the source of *budC* gene was grown at 30 °C. *Escherichia coli* DH5 α and BL21(DE3) as the cloning and expression hosts were grown at 37 °C. Plasmid pET-28a (+) (Novagen) was used for the BDH expression. All strains were cultured in Luria–Bertani (LB) medium.

Antibiotics were added in the following amounts (per milliliter) if necessary: 50 µg kanamycin.

Reagents, primers, and genomic isolation

Restriction enzyme, T4 DNA ligase, and Taq DNA polymerase high fidelity were from TaKaRa Biotech (Dalian, China). DNA and protein marker were purchased from Tiangen Biotech (Shanghai, China). Oligonucleotided primers were synthesized in SBSbio (Shanghai, China). Genomic DNA from *S. marcescens* H30, which were used as template for PCR amplification, was prepared with the Bacterial Genomic DNA Mini-prep Kit (BIODEV Corp., Beijing, China). (3*S*/3*R*)-AC, (2*S*,3*S*)-2,3-BD (97.0 %), (2*R*,3*R*)-2,3-BD (97.0 %), *meso*-2,3-BD (99.0 %), and other chemicals, unless otherwise indicated, were obtained from Sigma-Aldrich (Shanghai, China).

Development of the *budC* recombinant strain

The encoding sequence of *budC* gene was amplified by PCR with the genomic DNA of *S. marcescens* H30 as template using the following primers: *budC*-F (5'-TCC GAA TTC ATG CGT TTT GAC AAT AAA G-3') and *budC*-R (5'-GAC AAG CTT TTA GAC GAT CTT CGG TTG G-3'). Amplification was carried out in a TaKaRa PCR thermal cycler with the parameter settings as follows: 5 min at 94 °C for pre-denaturation, 30 s at 94 °C for denaturation, 30 s at 55 °C for annealing and 50 s at 72 °C for elongation. After 30 cycles of amplification, an additional elongation period for 10 min at 72 °C was used to ensure the completeness of the products. The amplified product was ligated into the vector pET-28a (+) at *Eco*RI and *Hind*III sites, resulting in the recombinant plasmid designated as pET28a-*budC*. The recombinant *E. coli* BL21(DE3)/pET28a-*budC* was obtained using heat shock transformation.

Preparation and purification of recombinant BDH

The recombinant strain was cultured at 37 °C in a 250-ml flask containing 50 ml LB medium (pH 7.0) with kanamycin (50 µg/ml). The cells were induced at about 0.6 OD₆₀₀ with 0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) and harvested by centrifugation after 6 h. The precipitate was resuspended in binding buffer (20 mM phosphate, 500 mM NaCl, and 20 mM imidazole, pH 7.4) and disrupted by sonication in an ice bath. The homogenate was centrifuged at 13,000×g for 10 min to remove the debris. The soluble fraction was subjected to purification under nondenaturing conditions with Ni-affinity chromatography using a HisTrap HP column according the purification protocol (GE Healthcare, USA). The eluate from the column was pooled and desalted by a Hitrap desalting column (GE Healthcare, USA). To avoid the effect of His₆-tag on the properties of BDH enzyme, the

His₆-tag of the purified enzyme was cut off using the THROMBIN Kit (Jianglaibio, Shanghai). The obtained enzyme was analyzed via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Enzyme assays

Enzyme activity was determined spectrophotometrically by measuring the changes in absorbance at 340 nm and 40 °C corresponding to the oxidation of NADH or the reduction of NAD⁺. The reaction mixtures containing 50 mM potassium phosphate buffer (pH 8.0), 4 mM NAD⁺ for the oxidation reactions or 50 mM sodium acetate buffer (pH 5.0), 0.2 mM NADH for the reduction reactions were incubated at 40 °C for 5 min. After adding 10 µl of enzyme solution, the reaction was started by the addition of the substrates. One unit of BDH activity was defined as the amount of enzyme required to reduce 1 µmol of NAD(H) in 1 min. All enzyme activities were determined in triplicate.

Stereospecificity of BDH

Stereospecificity of the purified BDH enzyme was investigated in the oxidation–reduction processes of the 2,3-BD/AC/DA interconversion. For the oxidation processes, a mixture containing 100 mM *meso*-2,3-BD/(2*S*,3*S*)-2,3-BD, 4 mM NAD⁺, 50 mM of potassium phosphate buffer (pH 8.0) and 10 µl of purified BDH enzyme in a final volume of 1 ml was incubated at 40 °C for 2 h. The reduction processes was carried out in 1-ml reaction system containing 100 mM DA/(3*S*/3*R*)-AC, 0.2 mM NADH, 50 mM of sodium acetate buffer (pH 5.0) and 10 µl of purified BDH enzyme at 40 °C for 2 h. The products in these reaction systems were extracted by ethyl acetate and then used to check the enzyme stereospecificity using a GC system (Agilent GC9860) equipped with a chiral column (Supelco β-DEXTM 120, 30 m length, 0.25 mm inner diameter). The operation conditions were as follows: N₂ was used as the carrier gas at flow rate of 1.2 ml/min; the injector temperature and the detector temperature were 215 and 245 °C, respectively; and the column temperature was maintained at 50 °C for 1.5 min, then raised to 180 °C at a rate of 15 °C/min.

Results

Cloning and sequence analysis of the *budC* gene

The *budC* gene was obtained by PCR method using the primers (*budC*-F/*budC*-R) designed according to our previous submitted sequence (Genbank accession number AFH00999). The amino acid sequence as deduced from

the nucleotide sequence of 756 bp was compared with other reported BDHs by multiple alignment (Table 3). The BDH showed low identity with other known functional BDHs except that from *S. marcescens* MG1 with 98 % identity. Using InterProScan web server (<http://www.ebi.ac.uk/Tools/pfa/iprscan/>) to search the conserved domain of the BDH enzyme, two conserved sequences for the coenzyme binding motif (GxxxGxG) in the N-terminal and the active site motif (YxxxK) in the C-terminal were found (Fig. 1), which are present in the short-chain dehydrogenase/reductase (SDR) superfamily (Jornvall et al. 1995; Kallberg et al. 2002). So far, almost all the *meso*-BDHs and (2*S*,3*S*)-BDH characterized belong to the SDR superfamily (Yu et al. 2011). Such observations indicated that the *budC* gene product from *S. marcescens* H30 may be *meso*-BDH or (2*S*,3*S*)-BDH belonging to the SDR superfamily.

Expression and purification of BDH enzyme

The recombinant *E. coli* BL21(DE3)/pET28a-*budC* was induced with 0.5 mM IPTG at 30 °C when OD₆₀₀ reached 0.6. SDS-PAGE analysis on the soluble fractions from the cell lysate revealed the presence of over-expressed protein (Fig. 2), implying that the target protein was successfully expressed.

The purified His₆-tag BDH and His₆-tag-free BDH were given in Fig. 2. The BDH activity was determined using two buffer systems for the oxidation-reduction reactions. For the oxidation reaction, the reaction mixtures contained 50 mM potassium phosphate buffer (pH 8.0), 4 mM NAD⁺ and 100 mM *meso*-2,3-BD, while in the reduction reactions 50 mM sodium acetate buffer (pH 5.0), 0.2 mM NADH and 100 mM (3*S*/3*R*)-AC were employed. The results showed that the highest activities were determined as 215 U/mg for reduction of (3*S*/3*R*)-AC and 67 U/mg for oxidation of *meso*-2,3-BD. After purification, the BDH was purified 1.9-fold during the purification process.

Specificity of BDH for substrates and coenzymes

The substrate specificity of BDH enzyme in the oxidation reactions was studied using 100 mM diols with 2 different coenzymes, NAD⁺ and NADP⁺. Reduction activities were measured with 100 mM ketones in the presence of NADH and NADPH as coenzymes. As shown in Table 4, the recombinant BDH could oxidize *meso*-2,3-BD and (2*S*,3*S*)-2,3-BD, while (2*R*,3*R*)-2,3-BD was not a substrate for the recombinant BDH at all. In addition, several primary alcohols (such as glycerol, 1,2-pentanediol and 1,2-propanediol) could also be oxidized, though showing less activity than *meso*-2,3-BD. (3*S*/3*R*)-AC was the best substrate in the ketone reduction reaction, followed by diacetyl which had 75 % of the specific activity of (3*S*/3*R*)-AC. No activity (data not shown) was detected for DA, AC and 2,3-BD as the substrates with NADPH/NADP⁺, indicating that the *budC* gene product belongs to a NAD(H)-dependent 2,3-BDH.

The comparative data of apparent K_m and K_{cat} values for BDH from *S. marcescens* H30 were given in Table 4. The K_m and K_{cat} values of BDH were 4.1 mM and 6.2 s⁻¹ for *meso*-2,3-BD, 31.2 mM and 1.02 s⁻¹ for (2*S*,3*S*)-2,3-BD, 0.97 mM, and 19.7 s⁻¹ for (3*S*/3*R*)-AC, and 3.3 mM and 11.5 s⁻¹ for diacetyl, respectively. The K_{cat}/K_m ratio for *meso*-2,3-BD is much higher than that for (2*S*,3*S*)-2,3-BD, and combined with the results from substrate specificity test, BDH from *S. marcescens* H30, therefore, could be categorized as a NAD(H)-dependent *meso*-2,3-BDH.

Effects of temperature and pH on BDH activity

The effects of temperature and pH on BDH activity were determined using 100 mM DA, 100 mM (3*S*/3*R*)-AC, and 100 mM *meso*-2,3-BD as substrates and NAD(H) as coenzymes. The pH effects were determined in the range of

Table 3 Alignment of the sequence from the *budC* gene of *S. marcescens* H30

Organism	Gene ^a		Protein ^a	
	Length (bp)	Identity (%)	Length (aa)	Amino acid identity (%)
<i>S. marcescens</i> H30	756	100	251	100
<i>S. marcescens</i> MG1	756	94	251	98
<i>K. pneumoniae</i> XJ-Li	771	52	256	31
<i>K. oxytoca</i> E718	771	51	256	32
<i>B. saccharolyticum</i> C-1012	777	51	258	31
<i>Enterobacter</i> sp. 638	771	49	256	30

^a The gene sequences used for the *budC* gene homologous analysis are from GenBank data bank, and their deduced acids sequences were used to perform the protein identities. Accession numbers of the gene sequence are JF519738 (*meso*-2,3-BDH, *S. marcescens* MG1), JN865245 (*meso*-2,3-BDH, *K. pneumoniae* XJ-Li), NC018106 (*meso*-2,3-BDH, *K. oxytoca* E718), AB009078 ((2*S*,3*S*)-2,3-BDH, *B. saccharolyticum* C-1012), NC009436 (*meso*-2,3-BDH, *Enterobacter* sp. 638)

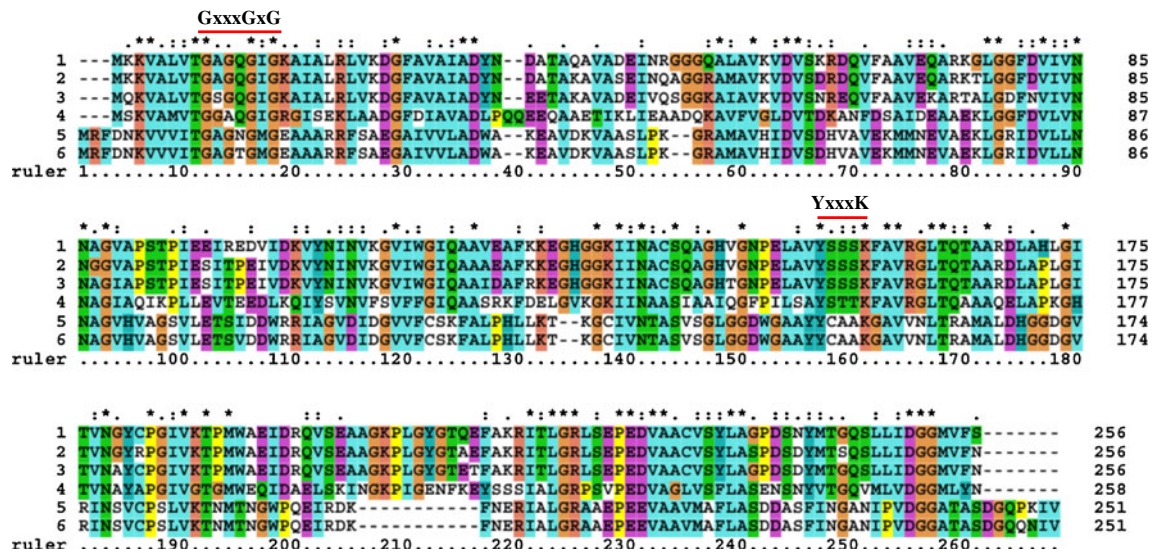


Fig. 1 Amino acid sequence multiple alignment of BDH from *S. marcescens* H30 with other reported (2S,3S)-BDH or *meso*-BDH. 1 *K. oxytoca* E718, 2 *K. pneumoniae* CICC10011, 3 *Enterobacter* sp.

638, 4 *B. saccharolyticum* C-1012, 5 *S. marcescens* H30, and 6 *S. marcescens* MG1

pH 4–10 at 40 °C using 50 mM sodium acetate (pH 4–5), potassium phosphate (pH 6–8), and glycine–NaOH buffers (pH 9–10). As shown in Fig. 3a, maximum activity for (3S/3R)-AC and DA reduction was observed at pH 5.0 and pH 8.0, while it was pH 8.0 for the *meso*-2,3-BD oxidation reaction.

Temperature effects were studied in the range of 20–70 °C for *meso*-2,3-BD oxidation and DA reduction at pH 8.0, and for (3S/3R)-AC reduction at pH 5.0 (Fig. 3b). The effects of

temperature on the BDH activity appeared to be similar in both reduction and oxidation reactions, and the optimal temperature

Table 4 Substrate specificity of *meso*-2,3-butanediol dehydrogenase from *S. marcescens* H30^a

Substrates	Activity	K_m^b (mM)	K_{cat} (s ⁻¹)
Oxidation ^a	%		
<i>meso</i> -2,3-Butanediol	100	4.1±0.3	6.2±0.2
(2R,3R)-2,3-Butanediol	ND		
(2S,3S)-2,3-Butanediol	11±3	31.2±0.4	1.02±0.06
Glycerol	9±2		
1,2-Pentanediol	13±3		
1,4-Butanediol	ND		
1,3-Propanediol	ND		
1,2-Propanediol	24±3		
1,5-Pentanediol	ND		
Reduction ^a	%		
(3S/3R)-Acetoin	100	0.97±0.08	19.7±0.8
Diacetyl	75±3	3.3±0.1	11.5±0.6

ND not detected

^a Assay conditions: 50 mM potassium phosphate buffer (pH 8.0), 100 mM diols, 4 mM NAD⁺ for the oxidation reactions; 50 mM sodium acetate buffer (pH 5.0), 100 mM ketones, 0.2 mM NADH for the reduction reactions

^b To determine the kinetic parameters, the oxidation reactions were prepared with potassium phosphate buffer (50 mM, pH 8.0) and 4 mM NAD⁺, the reduction reactions were prepared with sodium acetate buffer (50 mM, pH 5.0) and 0.2 mM NADH. Various concentration of *meso*-2,3-BD, (2S,3S)-2,3-BD, (3S/3R)-AC and DA ranging from 10 μM to 20 mM at room temperature. The K_m and K_{cat} values were obtained by nonlinear fitting with the Michaelis–Menten equation

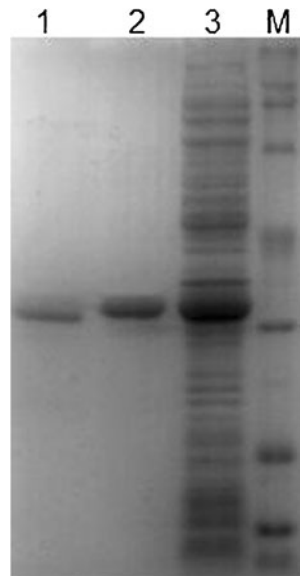


Fig. 2 Expression and purification of the BDH protein: M protein marker (200, 116, 97.2, 66.4, 44.3, 29, 20.1, 14.3, 6.5 kDa); 1 purified His₆-tag-free BDH protein; 2 purified His₆-tag BDH protein; 3 soluble protein from the cell lysate, were electrophoresed on a 12 % polyacrylamide gel under denaturing conditions

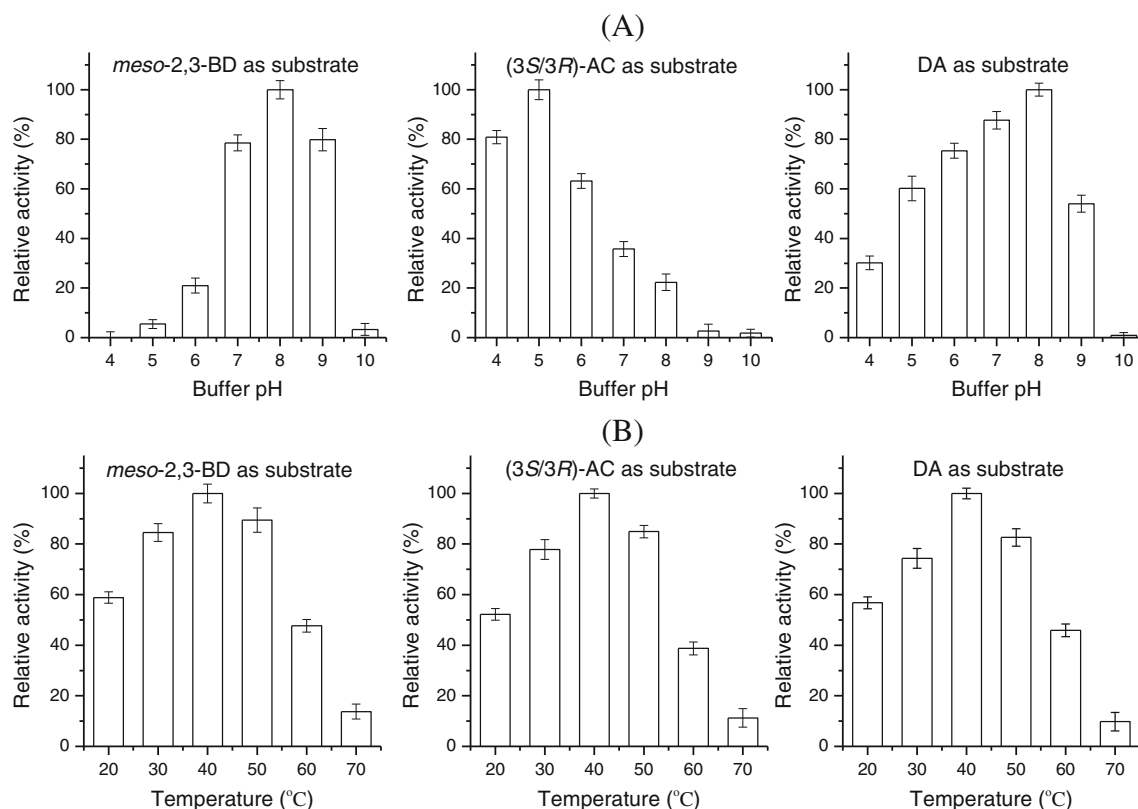


Fig. 3 Effects of pH and temperature on BDH activity. **a** Relative activity at different pH conditions and **b** relative activity at different temperatures

of 40 °C for these three substrates was determined. More than 50 % of maximum activity was retained between 20 and 50 °C, but the recombinant BDH became unstable at 60 and 70 °C, most likely due to thermal denaturation.

Effects of metal ions on BDH activity

The effects of different metal ions (NH_4^+ , Na^+ , Mn^{2+} , Mg^{2+} , Zn^{2+} , Ca^{2+} , Fe^{2+} , and Fe^{3+}), all in the form of chloride/sulfate salts, on the BDH activity were evaluated at 1 mM. As shown in Table 5, the BDH activity in the oxidation and reduction processes could be inhibited by most of metal ions such as NH_4^+ , Na^+ , Zn^{2+} , Ca^{2+} , and Fe^{3+} . Especially Fe^{3+} exhibited strong inhibition with less than 20 % of maximum activity for the DA and (3*S*/3*R*)-AC reduction. In contrast, Mg^{2+} and Mn^{2+} could slightly activate and increase its activity (<10 %). For *meso*-2,3-BD oxidation, the activity of BDH could obviously be enhanced by 27 % in the presence of Fe^{2+} , which resulted in the loss of 64 and 52 % with DA and (3*S*/3*R*)-AC as substrates and NADH as coenzyme.

Stereospecificity of the BDH

Figure 4 demonstrated the BDH stereospecificity in the oxidation-reduction processes of the 2,3-BD/AC/DA interconversion. With respect to 2,3-BD oxidation reactions,

when *meso*-2,3-BD was used as the substrate with NAD^+ and BDH, (3*R*)-AC was the only product detected (Fig. 4b).

Table 5 Effect of various metal ions on BDH activity in the oxidation and reduction reactions

Metals	Relative activity (%)		
	DA as substrate ^a	(3 <i>S</i> /3 <i>R</i>)-AC as substrate ^b	<i>meso</i> -2,3-BD as substrate ^c
Control	100.00	100.00	100
NH_4Cl	71±3	86±2	84±3
NaCl	90±3	85±4	84±2
MnSO_4	105±3	107±2	65±4
FeSO_4	36±3	48±3	127±2
FeCl_3	19±4	6±4	67±4
ZnSO_4	95±3	85±2.8	50±3
CaCl_2	91±2	85±4	66±3
MgCl_2	109±2	105±3	48±3

^a Assay conditions: 50 mM potassium phosphate buffer (pH 8.0) with 100 mM diacetyl as substrate and 0.2 mM NADH as coenzyme

^b Assay conditions: 50 mM sodium acetate buffer (pH 5.0) with 100 mM (3*S*/3*R*)-acetoin as substrate and 0.2 mM NADH as coenzyme

^c Assay conditions: 50 mM potassium phosphate buffer (pH 8.0) with 100 mM *meso*-2,3-butanediol as substrate and 4 mM NAD^+ as coenzyme

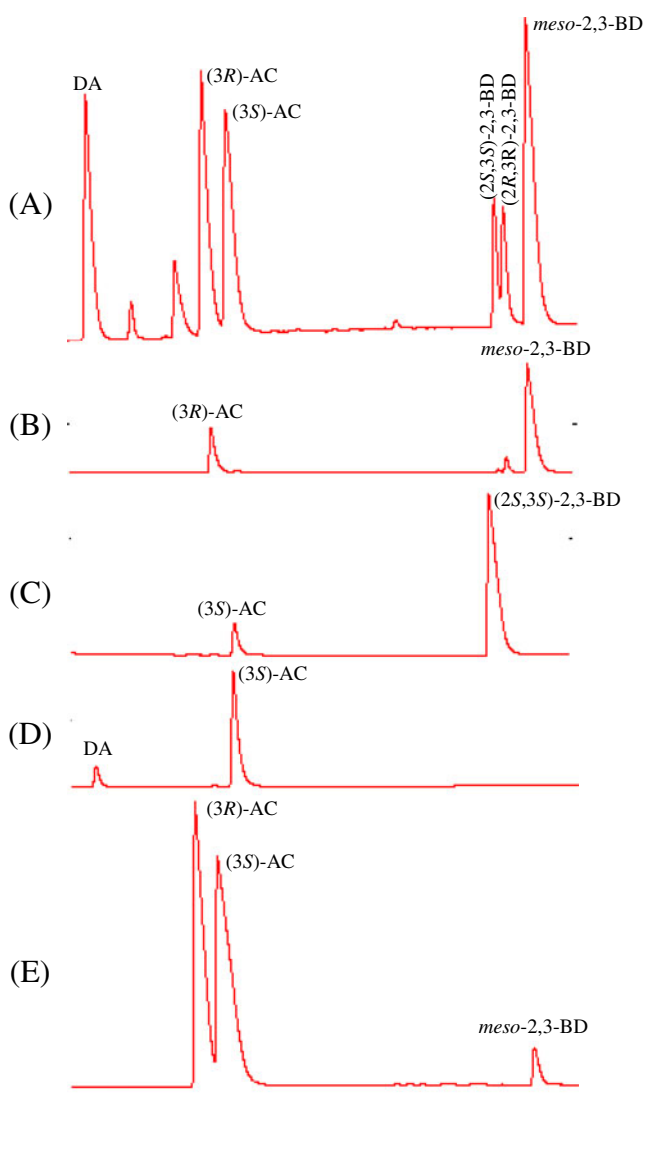


Fig. 4 Chiral-column GC analysis of the substrates and products in the oxidation and reduction reaction catalyzed by *meso*-2,3-butanediol dehydrogenase from *S. marcescens* H30. **a** Profile of mixture of standard chemicals. **b** The product from *meso*-2,3-butanediol. **c** The product from (2*S*,3*S*)-2,3-butanediol. **d** The product from diacetyl. **e** The product from (3*S*/3*R*)-acetoin (the peak of diacetyl was in front of solvent peak and not shown in the picture)

Accordingly, (3*S*)-AC was, as expected, the only product obtained from (2*S*,3*S*)-2,3-BD (Fig. 4c). And more, (3*S*)-AC could be obtained from DA by BDH (Fig. 4d). Unexpectedly, (3*S*)-AC could not further be transformed into any form of 2,3-BD at pH 5.0 (Fig. 4d), which is different from other *meso*-2,3-BDHs reported previously. To investigate the reason, the DA reduction reactions were carried out with different buffer systems from pH 5.0 to pH 9.0. The results (Fig. 5) showed that only (3*S*)-AC was obtained from DA when the pH value was below 8.0, while the (3*S*)-AC product from DA could be further transformed into (2*S*,3*S*)-2,3-BD at pH 9.0.

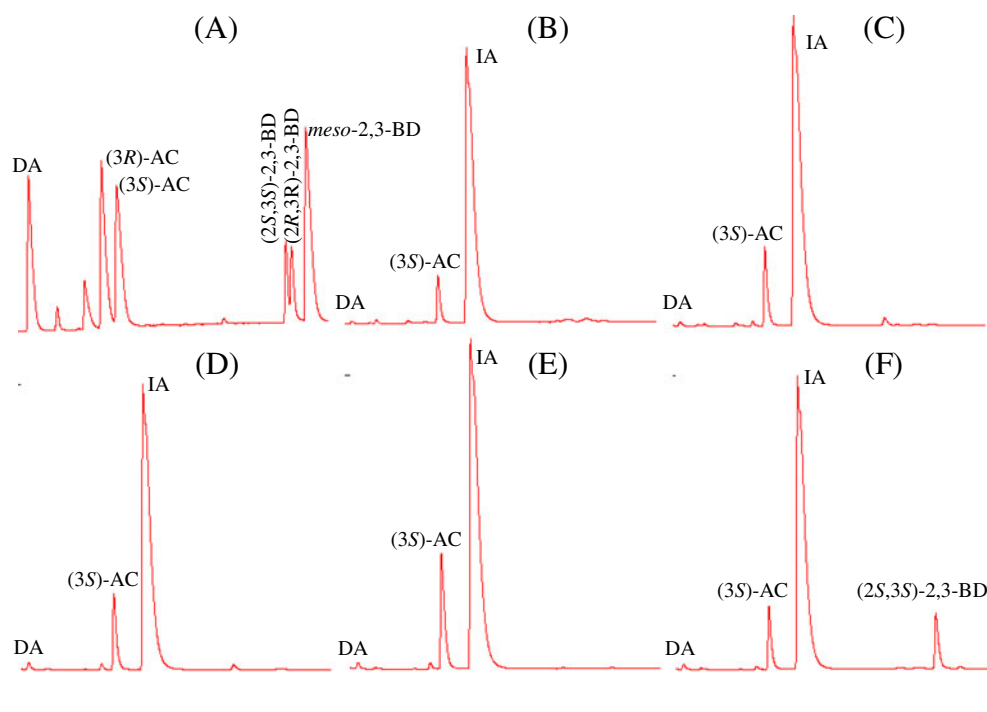
When a racemic mixture of (3*S*/3*R*)-AC was incubated with BDH and NADH, only *meso*-2,3-BD was formed (Fig. 4e). Considering that (2*S*,3*S*)-2,3-BD is the only product from (3*S*)-AC, therefore, *meso*-2,3-BD was produced from (3*R*)-AC as a substrate.

Discussion

Single configuration production of AC and 2,3-BD was recently paid increasing attention due to their potential industrial and pharmaceutical applications (Ji et al. 2011). However, natural microorganism usually produced a mixture of (2*S*,3*S*)-2,3-BD/*meso*-2,3-BD or (2*R*,3*R*)-2,3-BD/*meso*-2,3-BD with (3*S*/3*R*)-AC, such as (2*S*,3*S*)-2,3-BD/*meso*-2,3-BD with (3*S*/3*R*)-AC were formed by *K. pneumoniae* (Liu et al. 2011), and *P. polymyxa* could produce (2*R*,3*R*)-2,3-BD and (3*S*/3*R*)-AC with a little *meso*-2,3-BD (Ji et al. 2011; Yu et al. 2011). Hence, it is very difficult that single configuration of AC and 2,3-BD in the direct fermentative process was achieved by natural strains. There are several possible explanations for the mixed formation of AC and 2,3-BD isomers, including aeration conditions (redox balance), non-stereospecific dehydrogenases, multiple pathways, and multiple stereospecific dehydrogenases (Syu 2011; Yan et al. 2009). In previous studies, several 2,3-butanediol dehydrogenases from different strains were characterized, exhibiting the variation of their stereospecificities and potential applications. Ui et al. introduced gene fragments containing genes encoding acetolactate synthase (ALS), acetolactate decarboxylase (ALDC), and a single *meso*-2,3-BDH from *K. pneumoniae* IAM 1063 into *E. coli*, and obtained production of pure *meso*-2,3-BD (Ui et al. 1997). While whole cells of engineered *E. coli* with overexpression of (2*S*,3*S*)-2,3-BDH from *B. saccharolyticum* C-1012 were developed for biocatalytic production of pure (3*S*)-AC and (3*S*,3*S*)-2,3-BD from DA (Ui et al. 2004). Pure (2*R*,3*R*)-2,3-BD has been achieved by introducing three genes encoding ALS, ALDC, and a (2*R*,3*R*)-2,3-BDH from *B. subtilis* into *E. coli* JCL16 and JCL 260 (Yan et al. 2009). These studies indicated that mixed formation of AC and 2,3-BD isomers is mainly due to the existence of multiple pathways or dehydrogenases.

In this study, the *budC* gene encoding the BDH from *S. marcescens* H30 was cloned and expressed in *E. coli* BL21(DE3), purified and characterized for its properties. Amino acid sequence alignment showed the BDH shared low identity (<35 %) with other reported BDHs except that with a 98 % identity from *S. marcescens* MG1, implying that the enzyme from *S. marcescens* is a new BDH. However, the BDH similar to that from *K. pneumoniae* and *B. saccharolyticum* possessed two conserved residues:

Fig. 5 Chiral-column GC analysis of the substrate and products in the DA reduction reaction catalyzed by *meso*-2,3-butanediol dehydrogenase from *S. marcescens* H30 with different pH system. **a** Profile of mixture of standard chemicals. **b** pH 5.0, **c** pH 6.0, **d** pH 7.0, **e** pH 8.0, **f** pH 9.0. *IA* was the abbreviation of isoamyl alcohol, which was used as internal standard for GC analysis



coenzyme binding motif (GxxxGxG) in the N-terminal part and substrate binding region (YxxxK) in the C-terminal. So the BDH from this study should also belong to the SDR superfamily. In the oxidation and reduction reactions, no activity could be detected with NADP(H) as coenzyme, NAD(H) was the only coenzyme for the BDH, indicating that the *budC* gene product is a NAD(H)-specific BDH. The maximum activity and low K_m value for *meso*-2,3-BD in the oxidation process were determined, and combined with the results from substrate specificity test, therefore, the BDH from *S. marcescens* H30 could be categorized as a NAD(H)-dependent *meso*-2,3-BDH.

The BDH optimal values of pH 5 and pH 8 determined with (3*S*/3*R*)-AC and *meso*-2,3-BD as substrates demonstrated that pH value played an important role between oxidation and reduction reactions. The BDH activity could also be influenced by several metal ions. Fe^{2+} could increase its activity by 27 % with *meso*-2,3-BD as a substrate. In contrast, Fe^{2+} exhibited inhibition activity in DA and (3*S*/3*R*)-AC reduction process and resulted in the loss of 64 and 52 %, respectively. In addition, Mg^{2+} and Mn^{2+} could slightly improve the 2,3-BDH activity in the DA and (3*S*/3*R*)-AC reduction, while its activity could be strongly inhibited (>90 %) by Fe^{3+} with (3*S*/3*R*)-AC as a substrate. Hence, the ratio between AC and 2,3-BD by *S. marcescens* H30 during the fermentation process could be controlled by the fermentative pH value adjusted and metal ions added. These results would provide further useful hints for fermentation strategy development of AC or 2,3-BD production.

The isomers formation mechanism of AC and 2,3-BD by native strains during the fermentation process was still elucidated unclearly as far. But stereospecificity of the BDH have been accepted as a key factor. Similar to the genus of *Klebsiella* and *Enterobacter*, the BDH from *S. marcescens* H30 exhibited the abilities in the inter-conversion of (3*R*)-AC/*meso*-2,3-BD and (3*S*)-AC/(2*S*,3*S*)-2,3-BD, and (3*S*)-AC was obtained from DA as a substrate by the BDH. The major difference was that (2*S*,3*S*)-2,3-BD produced from (3*S*)-AC was limited at the pH value of 9.0. When the pH value was below 8.0, (3*S*)-AC with the BDH enzyme and NADH as a coenzyme could not be further oxidized and transformed into (2*S*,3*S*)-2,3-BD. From another perspective, this characteristics of the BDH from *S. marcescens* H30 is very helpful for chiral (3*S*)-AC production from DA by whole cells catalysis with over-expression, since the pH adjustment could effectively prevent (3*S*)-AC from forming (2*S*,3*S*)-2,3-BD.

In conclusion, we reported a new NAD(H)-dependent *meso*-2,3-BDH from *S. marcescens* H30. And the *meso*-2,3-BDH was purified and characterized, exhibiting some different characteristics compared with others reported *meso*-2,3-BDH. These properties should lead to its potential application for chiral (3*S*)-AC production, and more suitable fermentation strategy for AC or 2,3-BD production by *S. marcescens* H30 in industry.

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