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Identification and characterization of a novel 2R,3R-*Butanediol dehydrogenase* from *Bacillus* sp. DL01

Mostafa Elmahmoudy [a], Nora Elfeky [a,c], Pu Zhongji [a], Yue Zhang [a], Yongming Bao [a,b,*]

[a] School of Bioengineering, Dalian University of Technology, Dalian 116024, Liaoning, China
[b] School of Ocean Science and Technology, Dalian University of Technology, Panjin 124221, Liaoning, China
[c] Botany Department, Faculty of Science, Menoufia University, Shebin El-Kom 32511, Egypt

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ABSTRACT

Background: 2R,3R-butanediol dehydrogenase (R-BDH) and other BDHs contribute to metabolism of 3R/3S-Acetoin (3R/3S-AC) and 2,3-butanediol (2,3-BD), which are important bulk chemicals used in different industries. R-BDH is responsible for oxidizing the hydroxyl group at their (R) configuration. *Bacillus* species is a promising producer of 3R/3S-AC and 2,3-BD. In this study, R-*bdh* gene encoding R-BDH from *Bacillus* sp. DL01 was isolated, expressed and identified.

Results: R-BDH exerted reducing activities towards Diacetyl (DA) and 3R/3S-AC using NADH, and oxidizing activities towards 2R,3R-BD and Meso-BD using NAD^+, while no activity was detected with 2S,3S-BD. The R-BDH showed its activity at a wide range of temperature (25°C to 65°C) and pH (5.0–8.0). The R-BDH activity was increased significantly by Cd^{2+} when DA, 3R/3S-AC, and Meso-BD were used as substrates, while Fe^{2+} enhanced the activity remarkably at 2R,3R-BD oxidation. Kinetic parameters of the R-BDH from *Bacillus* sp. DL01 showed the lowest K_m, the highest V_{max}, and the highest K_{cat} towards the racemic 3R/3S-AC substrate, also displayed low K_m towards 2R,3R-BD and Meso-BD when compared with other reported R-BDHs.

Conclusions: The R-BDH from *B*acillus sp. DL01 was characterized as a novel R-BDH with high enantioselectivity for R-configuration. It considered NAD^+ and Zn^{2+} dependant enzyme, with a significant affinity towards 3R/3S-AC, 2R,3R-BD, and Meso-BD substrates. Thus, R-BDH is providing an approach to regulate the production of 3R/3S-AC or 2,3-BD from *Bacillus* sp. DL01.

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1. Introduction

3R/3S-Acetoin (3R/3S-AC) is an organic compound widely used as a preservative in food industry [1], and is one of the 30 most important platforms bulk chemicals [2]. 3R/3S-AC is a neutral compound produced by microorganisms as a defence mechanism against acidity inside the cell [3]. Furthermore, it alsoacts as an energy source when other cellular carbon sources were depleted [1]. 3R/3S-AC exists in two stereoisomeric forms (3R-AC and 3S-AC) and each of them is needed for specific applications [4]. However, commercial 3R/3S-AC products, mostly produced with chemical methods, are usually racemic mixtures due to the difficulties to obtain optically pure AC [5].

2,3-BUTANEDIOL (2,3-BD) is another bulk chemical, and is widely used in manufacturing of inks, softening agent, explosivesn, and medicine [6]. 2,3-BD competes with 3R/3S-AC in pyruvate metabolic pathway, and has three stereoisomers 2R,3R-BD, Meso-BD, and 2S,3S-BD [6]. Several microorganisms, such as *Bacillus* species [7,8,9], *Klebsiella* species [10], *E. coli* [11] and *Serratia* species [12], produce 3R/3S-AC and 2,3-BD. The isomers of 3R/3S-AC and 2,3-BD, as well as their production levels vary according to microbial strains and fermentation conditions.

Butanediol dehydrogenase complex enzymes (BDHs), including 2S,3S-BDH, 2R,3R-BDH (R-BDH), Meso-BDH and glycerol dehydrogenase (GDH), play a critical role in the metabolism of 3R/3S-AC and 2,3-BD production [13,14]. R-BDH is responsible for the reduction of diacetyl (DA) to 2R,3R-BD via 3R-AC, and 3S-AC to Meso-BD [12]. Furthermore, R-BDH can catalyze the oxidation of 2R,3R-BD and Meso-BD to 3R-AC and 3S-AC, respectively [12]. Previous

* Corresponding author.
E-mail address: biosci@dlut.edu.cn (Y. Bao).

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studies had confirmed that R-BDH catalyzes DA, which is transformed from α-acetolactate under the effect of non-enzymatic oxidative decarboxylation (NOD), to form 3R-AC in *Serratia* sp. T241 [12], *Bacillus licheniformis* [14], and *Paenibacillus polymyxa* ATCC 12321 [15]. On the other hand, neither the reductive activity of R-BDH to convert 3S-AC to 2S,3S-BD nor its oxidative activity to convert 2S,3S-BD to 3S-AC was detected in *Serratia* sp. [12], *Paenibacillus polymyxa* [15], *Saccharomyces Cerevisiae* [16], and *Bacillus subtilis* [8]. It implies that R-BDH from these microorganisms are potentially enantioselectivitive. The activity of R-BDH was confirmed by knockout of R-BDH in *Bacillus* species, the 3R/3S-AC yield increased remarkably while 2R,3R-BD diminished [17].

Bacillus sp. DL01, has been characterized as a strain with high 3R/3S-AC production, capable of achieving around 76 g/L in a 5-L fermenter with glucose as carbon source under low oxygen conditions, and most of the product was 3R-AC [18]. Overexpression of *als*S (involved in catalyzing pyruvate to α-acetolactate) and knockout of *als*D (involved in converting α-acetolactate to 3R-AC) in an engineered strain *Bacillus.* sp. DL01-*alsS*Δ*alsD* lead to increased production of DA [19]. Increase in DA production achieves a higher degree when the gene encoding phosphotransacetylase (*pta*) is furthere deleted in a new engineered strain *Bacillus* sp. DL01-*alsS*-Δ*alsD*Δ*pta* to block the acetate pathway [20]. These findings indicates that *Bacillus* sp. DL01 has active DA and 3R/3S-AC pathways.

Accordingly, this study focused on investigating the enantioselectivities in oxidoreduction of 3R/3S-AC and 2,3-BD by R-BDH from *Bacillus* sp. DL01, and elucidating its mechanism of catalyzing and regulating DA, 3R/3S-AC and 2,3-BD production, by isolating, identifying and characterizing the R-BDH.

2. Materials and methods

2.1. Strains, plasmids, primers and culture conditions

Bacillus sp. DL01, isolated from sea sediment, was grown with Luria–Bertani (LB) medium at 37°C in shaker with a speed of 200 rpm. *E. coli* DH5-α and *E. coli* BL21(DE3) were used as the cloning and expression hosts, respectively. All the strains were cultured with LB medium. Plasmid pMD 18T (TAKARA) was used for gene cloning and sequencing. Plasmid pET28a(+) was used for protein expression. Primers synthesis and DNA sequencing were carried out by BGI company (Beijing, China). Restriction enzymes, PCR reagents and related reagents for DNA manipulation were purchased from Takara Biotech (Dalian, China). The plasmids and genomic DNA were prepared with Mini-prep Kit Tiangen Biotech (Beijing, China) and Sangon Biotech (Shanghai, China). All other chemicals, unless otherwise indicated, were obtained from Sigma-Aldrich (USA).

2.2. Cloning of R-bdh

The encoding fragment of R-*bdh* gene was amplified by polymerase chain reaction (PCR) after genomic extraction from *Bacillus* sp. DL01 using a pair of specific primers (forward primer R-*bdh*-f: 5′ATGAAAGCGGCAAGATGGCAC3′, reverse primer R-*bdh*-r: 5′TTAGTTCGTTTGA CTAAG3′). PCR fragment was ligated into T-vector (pMD18. Takara), after amplification by the use LA Taq enzyme, and the recombinant plasmid was transformed to *E. coli* DH5-α. Positive colonies were picked, cultured and collected, and then the complete sequence of R-BDH was obtained by sequencing. After examination to ensure that cutting sites of *Bam*HI or *Xho*I did not exist within R-*bdh*, a new pair of primers (*Bam*HI-R-*bdh*-f: 5′CGCGGATCCATGAAA GCGGCAAGATGGCAC3′ and *Xho*I-R-*bdh*-r: 5′CCGCTCGAGTTAGTTCGGTTTGACTAA G3′) were synthesized. The PCR conditions were as following: a preliminary denaturation step at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 90 s, and then a final extension 72°C for 10 min. After double digestion with *Bam*HI and *Xho*I, the amplified R-*bdh* fragment was ligated into the pET28a(+) vector to construct pET28a-R-*bdh*. The recombinant plasmid was transformed into *E. coli* DH5-α by heat shock method [21], the positive colonies were selected from plates containing kanamycin, and the recombinant plasmid was extracted and confirmed by the sequencing. The correct recombinant pET28a-R-*bdh* was transformed into expression host *E. coli* BL21(DE3) with heat shock method.

2.3. Expression and purification of R-BDH protein

The recombinant strain was cultivated into 5 mL LB containing kanamycin and incubated overnight at 37°C in a rotatory shaker at 200 rpm. Then 2 mL of recombinant cells were inoculated into 500 mL flask containing 200 mL LB culture medium with kanamycin and incubated at 37°C in a rotatory shaker at 200 rpm till 0.5–0.6 OD. The cultures were induced by 0.5 M IPTG (isopropyl-beta-D-thiogalactopyranoside) and incubated at 25°C in a rotatory shaker at 180 rpm for 12–14 h. The cells were harvested and centrifuged at 10,000 *g* (Sorval RC-5C plus, USA) for 20 min at 4°C, the supernatant was discarded, and the pellets were suspended into binding buffer A (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 20 mM imidazole) and disrupted by sonication in the icebox for 15 min (5 s on and 5 s off strategy). The cell lysate was centrifuged at 10,000 × *g* for 30 min at 4°C, the supernatant was subjected to protein purification system (GE Health care, USA) using binding buffer A for column equilibration and Buffer B (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 500 mM imidazole) for protein elution at a speed of 2 mL/min. The eluted protein of R-BDH was combined and concentrated by the use of ultrafiltration membrane (Millipore, Milford, MA) with centrifugation at 4000 × *g* for 30 min at 4°C. The purified protein was examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using separating gel 12% at 200 voltages. The purified R-BDH was used directly or stored at −80°C for further experiments.

2.4. Activity assay of R-BDH

The activity of the enzyme was investigated with a spectrophotometer by recording the change in the absorbance of NADH at 340 nm corresponding to the oxidation of NADH or reduction of NAD^+ (extinction coefficient, ε340 = 6.22 mM^{-1} cm^{-1}). Reaction volume was 1 mL (100 mM phosphate buffer pH 7.4) contained 0.2 mM NADH for the reduction reaction with 12.5 mM substrate as a final concentration of DA, 3R/3S-AC, respectively; while, 0.2 mM NAD^+ for the oxidation reaction with 12.5 mM substrates of 2R, 3R-BD, *meso*-BD and 2S, 3S-BD, respectively; and an appropriate quantity (determined with Bradford assay) of the purified R-BDH catalyzing reactions.

2.5. R-BDH properties assay

The effect of temperature on the activity of R-BDH was detected from 25°C to 65°C. For determining the optimum pH for R-BDH, the reaction was carried out by incubating the enzyme at different pH degrees from 4 to 10.5. Acetic acid, phosphate buffer and glycine buffer were used to prepare buffers with pH of 4–5, 6–7.4, and 8–10.5, respectively.

The effect of metal ions (Zn^{2+}, Mn^{2+}, Ni^{2+}, Ca^{2+}, Fe^{2+}, Fe^{3+}, Cd^{2+}, Mg^{2+}, Ba^{2+}, and Cu^{2+}) in sulfate or chloride salts on the activity of R-BDH were studied. The effect of pH and metals were evaluated at room temperature. The kinetic parameters of R-BDH were

determined at room temperature with different concentrations of substrates (3R/3S-AC, DA, Meso-BD, and 2R,3R-BD), the calculation was performed using Lineweaver-Burke plot or double-reciprocal plot, The slope of the line was K_m/V_{max} and the y-intercept was $1/V_{max}$, then followed by calculation of K_{cat} and (K_{cat}/K_m). All the experiments were carried out at three separate times, and the mean and standard error were calculated.

2.6. Bioinformatics analysis

National centre of biotechnology information (NCBI) https://www.ncbi.nlm.nih.gov/ was used to blast the nucleotide and protein sequences, restriction mapper http://www.restrictionmapper.org/ was used to confirming that no cutting through the opening reading frame of the gene. The website http://reverse-complement.com/translate-protein/ROOT used for getting the reverse complementary sequences and translation from nucleotide sequence to the protein sequence. Snap gene software https://www.snapgene.com utilized for getting the ligation picture of R-BDH into the vectors. modeller software https://salilab.org/modeller used for designing the 3D structure of R-BDH, and pymol software https://pymol.org/2/ utilized for showing the 3D structure.

3. Results and discussion

3.1. Cloning and multiple alignments of R-bdh

The full gene R-*bdh* from *B*acillus sp. DL01 was successfully isolated using primers R-*bdh*-f and R-*bdh*-r, which were designed according to the genome sequence of *Bacillus velezensis* strain SRCM101413 (accession No. **CP021890** in Genbank). The isolated R-*bdh* gene sequence from *Bacillus* sp. DL01 was 1041 bp, with a GC content 48%, a calculated molecular weight of 37.44 KDa, and predicted isoelectric point of pH 5.05. The result of alignment implied that R-BDH belongs to zinc-containing alcohol dehydrogenase.

As shown in Table 1 and Fig. 1, the amino acid sequence of R-BDH from *Bacillus* sp. DL01 showed 93.64% identity with 2,3-*butanediol dehydrogenase* (ydjL, *bdh*A gene encoded) from *Bacillus subtilis* 168 [8], 70.57% with *Paenibacillus polymyxa* [15] and 62.15% with *Bacillus thuringiensis* [22]. Although, high similarity (99.7%) of the R-BDH from *Bacillus* sp. DL01 with R-BDH form *B. velezensis* and *B. amyloliquefaciens*, these R-BDHs were not studied before. The 3D structure of R-BDH from *Bacillus* sp. DL01, constructed based on the 3D structure of R-BDH from *B. subtilis* 168, showed that the α-helices and β-sheets were combined to Zn^{2+} domain and NAD^+ domain, separately (Fig. 2), indicating that R-BDH belongs to NAD^+ and Zn^{2+} dependent enzyme, as supported by the result of multiple alignments with R-BDH of *B. velezensis* SRCM101413. However, R-BDH from *Bacillus* sp. DL01 differed from R-BDH (*bdh*A) from *B. subtilis* 168, which contained Zn^{2+} domain, but not NAD^+ domain [23]. The relationship between structure and function need further investigation.

3.2. Expression and purification of R-BDH

Results of double digestion by *Bam*H1 and *Xho*I indicated successful recombination of the target DNA sequence into pET28a+, as shown in Fig. 3a. The sequenced recombinant plasmid (pET28a-R-*bdh*) was transformed into *E. coli* BL21(DE3). Temperature, IPTG concentration, and induction time were optimized, and the optimal conditions for protein expression were incubation at 25°C for 12–14 h in the presence of 0.5 M (IPTG concentration). Expression of the target protein was enhanced when the concentration of nutrients in the culture medium was duplicated, which agreed with Gomes and Mergulh [24].

SDS-PAGE analysis indicated that recombinant R-BDH was successfully expressed in the soluble form, as demonstrated by the band corresponding to a molecular weight around 37.44 KDa in lane 2 (Fig. 3b). After purified by His tag affinity chromatography (lane 5, Fig. 3b), the concentrated and desalted R-BDH (lane 6, Fig. 3b) was obtained for enzyme acvity assay in further.

The R-BDH showed no activity when $NADP^+$(H) was used as a coenzyme, whereas its enzymatic activity was observed when NAD^+(H) was used, indicating that this enzyme was strictly NAD^+(-H) dependant. R-BDH exhibited a reduction activity converting DA to 3R-AC, 3R-AC to 2R, 3R-BD and 3S-AC to *meso*-BD in the presence of NADH, as well as an oxidation activity converting 2R, 3R-BD to 3R-AC and meso-BD to 3S-AC in the presence of NAD^+. However, enzymatic activity was not detected when 2R, 3R-BD was replaced with 2S,3S-BD (data not shown).

3.3. R-BDH properties

3.3.1. Effect of temperature on R-BDH activity

The optimal temperatures of R-BDH catalyzing the reduction reaction of 3R/3S-AC and DA was 35 and 40°C, respectively, as shown in Fig. 4a. Whereas an optimal temperature of 40°C was observed for catalyzing the oxidation reactions both of 2R, 3R-BD and *meso*-BD. These results showed that the maximal activity was in the range of 35–40°C, consistent with the fermentation process of *Bacillus* sp. DL01 [18]. The relative activity of the R-BDH at 40°C was 90%, 120%, 110% and 120% for catalyzing 3R/3S-AC to 2,3-BD, DA to 3R-AC, 2R,3R-BD to 3R-AC, and *meso*-BD to 3S-AC, respectively. The optimal temperature of the R-BDH was similar to R-BDH from *Paenibacillus polymyxa* ATCC 12321 [15], while the R-BDH from mesophilic *Rhodococcus erythhropolis* WZ010 exhibited a wide range from 10 to 75°C [21].

3.3.2. Effect of pH on R-BDH activity

The optimal pH of the R-BDH catalyzing the reduction reaction of 3R/3S-AC and DA was 5.5 and 7.4, respectively, as shown in

Table 1
Multiple Alignments of R-BDH from *Bacillus* sp. DL01 and other sequences from different microorganisms.

Name of organisms	Name of an enzyme from NCBI	Gene		Protein		Reference
		Length (bp)	Identity (%)	Length (Aa)	Identity (%)	
Bacillus sp. DL01	2R,3R-BDH	1041	100	346	100	This study
B. subtilis 168	2R,3R-BDH	1041	NS	346	93.64	[8]
Paenibacillus polymyxa	2R,3R-BDH	1053	NS	350	70.57	[15]
B. thuringiensis	2R,3R-BDH	1053	NS	350	62.15	[22]
B. amyloliquefaciens	2R,3R-BDH	1041	99.62	346	99.71	Not studied
B. velezensis	2R,3R-BDH	1041	99.71	346	99.71	Not studied
Klebsiella pneumoniae HS11286	putative *oxidoreductase*	1044	NS	347	30.45	Not studied

NS: no similarity.

Fig. 1. Multiple amino acid sequence alignment. R-BDH from *Bacillus* sp. DL01 and R-BDHs from *Bacillus amyloliqufaciens* (Accession: **wp_061581404**), *Bacillus velezensis* (Accession: **wp_095284669.1**), *Bacillus subtilis* 168 (Accession: **NP_388505.1**), *Bacillus thuringiensis* (Accession: **WP_030023136.1**) and *Paenibacillus polymyxa* ATCC 12321 (Accession: **ADV15558.1**).

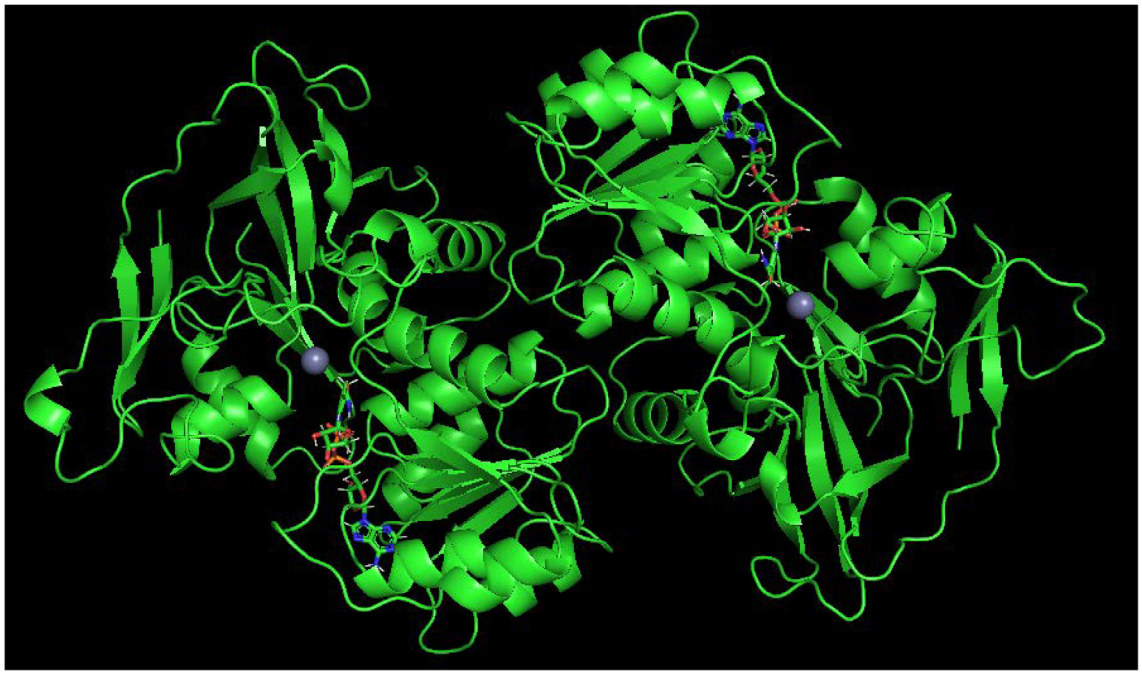

Fig. 2. 3D modeling structure of R-BDH from *Bacillus* sp. DL01. α helices and β sheets (light grey) of the enzyme incorporating zinc domain (black sphere) and NAD^+ domain (dark grey sticks).

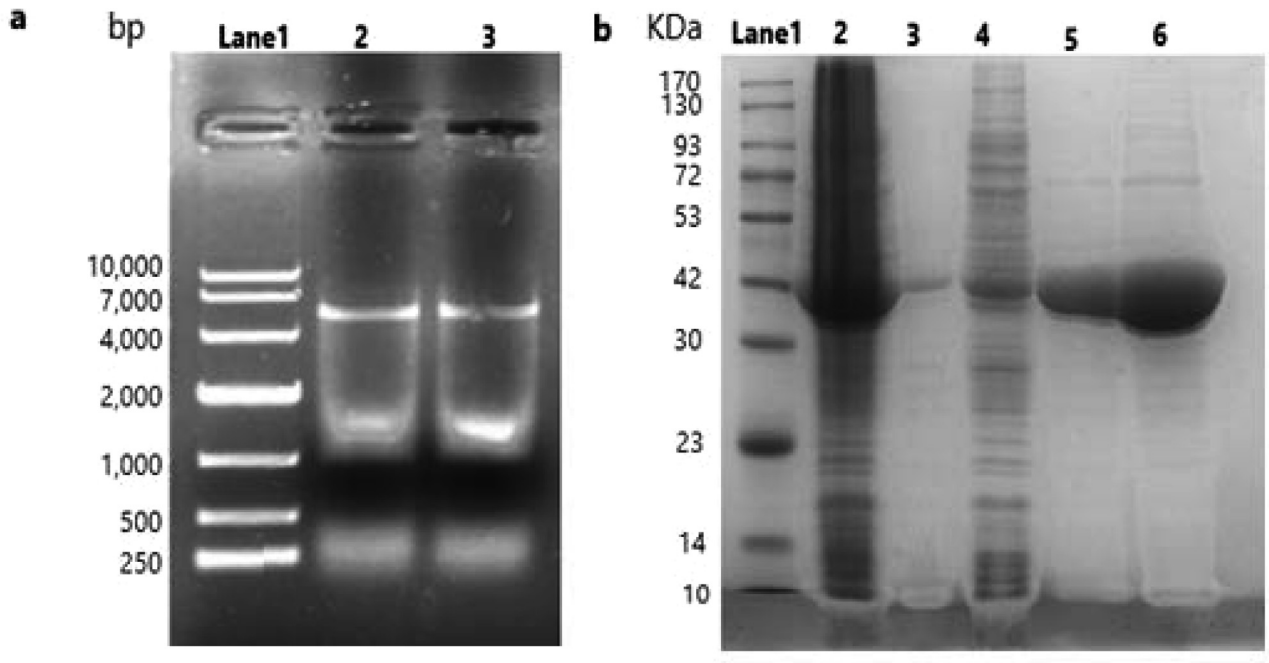

Fig. 3. Electrophoresis of recombinant pET-28a-R-*bdh* and SDS-PAGE of R-BDH. (a) Agarose electrophotogram of recombinant plasmid pET-28a-R-bdh. Lane1, DNA marker (DL10,000 TAKARA). Lane 2 and lane 3, showed recombinant plasmid of pET-28a-R-*bdh* double digestion. (b) SDS–PAGE analysis for expression and purification of R-bdh. Lane 1, protein marker. Lane 2, supernatant of cell lysate. Lane 3, flow-through sample of His-tag column chromatography. Lane 4, washing by low concentration imidazole buffer. lane 5, eluting sample by high concentration imidazole buffer. Lane 6, concentrated sample by membrane ultrafiltration.

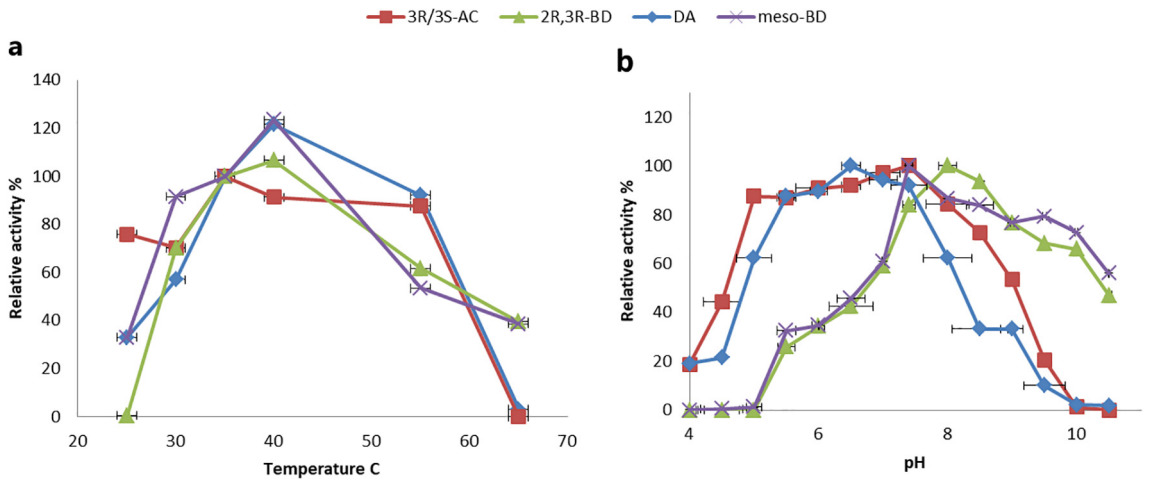

Fig. 4. Effects of temperature and pH on R-BDH activity. (a) Effect of temperature on the activity of R-BDH towards substrates DA, 3R/3S-AC, 2R,3R-BD, and Meso-BD. Assay conditions: 100 mM phosphate buffer (pH 7.4), 0.2 mM NADH for 12.5 mM 3R/3S-AC and DA or 0.2 mM NAD^+ for 12.5 mM 2R,3R-BD and Meso-BD at different temperature. Activity was determined as relative activity, the activity at 35°C used as control (100%). (b) Effect of pH on the activity of R-BDH with substrates DA, 3R/3S-AC, 2R,3R-BD and Meso-BD. Assay conditions: 100 mM buffers with different pH, 0.2 mM NADH for 12.5 mM 3R/3S-AC and DA or 0.2 mM NAD^+ for 12.5 mM 2R,3R-BD and Meso-BD at room temperature. Activity was determined as relative activity, the activity at pH 7.4 used as control (100%).

Fig. 4b. While, the optimal pH was 7.4 to 8 with 2R, 3R-BD and *meso*-BD oxidation, respectively. The results were similar to R-BDH from *Serratia* sp [25], typically similar to R-BDH from *Saccharomyces cerevisiae* [16], and different to R-BDH from *Paenibacillus polymyxa* which exhibited at pH 11 [15].

3.3.3. Effect of metal ions on R-BDH activity

The activity of the R-BDH was enhanced to 124.9% using 1 mM Cd^{2+} when catalyzing DA to 3R-AC (as shown in Fig. 5a and Table 2). The enzymatic activity was increased to 120% when 1 mM Mn^{+2} used, consistent with the result of a previous study where the culture medium was supplemented with Mn^{+2} to enhance the production of 3R/3S-AC (majority was 3R-AC) by *Bacillus* sp. DL01 [18], as well as the observation of an increase in the activity of R-BDH from *Saccharomyces cerevisiae* when Mn^{2+} was added [16]. Furthermore, the R-BDH activity was slightly enhanced by Zn^{2+} but declined by Fe^{2+}.

When the R-BDH catalyzed racemic 3R/3S-AC to mixture of 2R, 3R-BD and *meso*-BD, the activity was increased to 114.2% and 100.7 % with 1 mM Cd^{2+} and Zn^{2+}, respectively (Fig. 5b and Table 2). In contrast, the activity was decreased remarkably to 52% with 1 mM Mn^{2+} added, and the inhibitory effect of Mn^{2+} on R-BDH from *Rhodococcus erythropolis* was also reported [21].

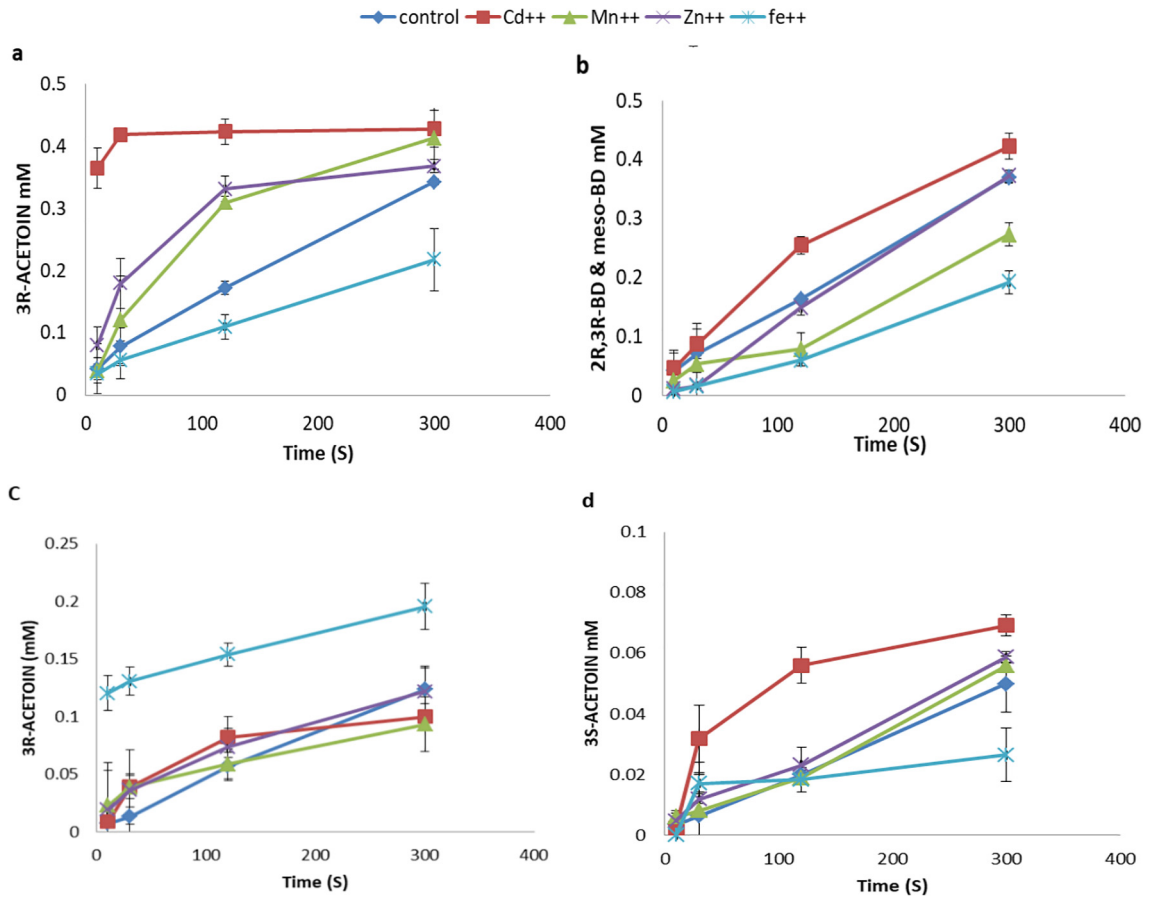

Fig. 5. Dynamics of the R-BDH reactions with metal ions. (a) Catalytic reaction: DA + NADH to 3S-AC + NAD^+. (b) Catalytic reaction: 3R/3S-AC + NADH to 2R,3R-BD + Meso-BD + NAD^+. (c) Catalytic reaction: 2R,3R-BD + NAD^+ to 3R-AC + NADH. (d) Catalytic reaction: Meso-BD + NAD^+ to 3S-AC + NADH. At standard condition, the metal concentration: 1 mM Cd^{2+}, Mn^{2+}, Zn^{2+}, and Fe^{2+}.

Table 2
Effect of metals ions on the activity of R-BDH towards different substrates.

Substrate	Relative activity %										
	Con.	Mg^{2+}	Ca^{2+}	Cd^{2+}	Ni^{2+}	Cu^{2+}	Ba^{2+}	Mn^{2+}	Zn^{2+}	Fe^{2+}	Fe^{3+}
DA[1]	100	43.8	38.3	124.9	32.0	69.5	59.1	120.6	107.4	63.4	15.8
3R/3S-AC[1]	100	46.8	49.1	114.2	31.9	45.1	63.6	59.2	100.7	52.0	ND
2R,3R-BD[2]	100	48.5	47.2	80.8	30.8	55.1	0.00	75.4	98.4	157.9	67.2
Meso-BD[2]	100	72.4	74.2	138.5	32.0	43.9	7.4	80.7	105.2	45.1	ND
2S,3S-BD	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[1] Assay conditions: 100 mM phosphate buffer (pH 7.4) with 12.5 mM substrate DA and 3R/3S-AC, 0.2 mM NADH, and 1 mM metal.
[2] Assay conditions: 100 mM phosphate buffer (pH 7.4), 12.5 mM of 2R,3R-BD and Meso-2,3 BD, 0.2 mM NAD^+, and 1 mM metal. Con. Control. ND: Not detected.

The activity of the R-BDH in the oxidation reaction from 2R, 3R-BD to 3R-AC was increased significantly to 157.9% when supplemented with 1 mM Fe^{2+}, but declined to 98.5%, 75%, and 80% in the presence of Zn^{2+}, Mn^{2+}, and Cd^{2+}, respectively (Fig. 5c and Table 2). And the activity from *meso*-BD to 3S-AC was increased to 138.5% and 105%, respectively, when 1 mM Cd^{2+} and Zn^{2+} were supplemented, but decreased to 80% and 50% in the presence of Mn^{2+} and Fe^{2+}, respectively (Fig. 5d and Table 2).

3.3.4. Kinetic parameters of R-BDH

The kinetic parameters of the R-BDH were determined with different concentrations of substrates DA, 3R/3S-AC, 2R, 3R-BD, and *meso*-BD. The K_m of R-BDH for 3R/3S-AC was 0.25 mM, which was considered the lowest K_m value compared with other substrates (as shown in Table 3), indicating high binding affinity of R-BDH to 3R/3S-AC. Furthermore, K_{cat} and K_{cat}/K_m values showed that racemic 3R/3S-AC was the optimal substrate for the R-BDH, indicating racemic 3R/3S-AC reduced by the R-BDH preferably. K_m values of R-BDH from *Bacillus* sp. DL01 for 3R/3S-AC, 2R, 3R-BD and *meso*-BD was the lowest compared with the other reported R-BDHs (as shown in Table 4). These results indicated high affinity of R-BDH from *Bacillus* sp. DL01 to these substrates and its ability to catalyze the reversible conversion from 3R/3S-AC to 2, 3-BD.

4. Conclusion

In this study, the R-*bdh* gene encoding R-BDH enzyme contributing to 3R/3S-AC and 2,3-BD formation in *Bacillus* sp. DL01 was isolated and identified. The new R-BDH sequence showed about 99.7% identity with R-BDHs from *B. velezensis* and *B. amyloliquefaciens* which were not characterized. The R-BDH showed a significant reduction activity with DA and 3R/3S-AC as substrates,

Table 3
Kinetic parameters of R-BDH from *Bacillus* sp. DL01 towards DA, 3R/3S-AC, R,3R-BD, and Meso-BD.

Substrate	V_{max} (μM/min^{-1})	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} mM^{-1})
DA[1]	8.33 ± 0.38	0.626 ± 0.12	0.65	1.05
3R/3S-AC[1]	27.47 ± 1.25	0.253 ± 0.03	2.16	6.14
2R,3R-BD [2]	3.86 ± 0.21	0.389 ± 0.09	0.30	0.80
Meso-BD [2]	1.98 ± 0.9	0.397 ± 0.11	0.15	0.39
2S,3S-BD	ND	ND	ND	ND

[1] Assay conditions: 100 mM phosphate buffer, 0.2 mM NADH and different concentration of 3R/3S-AC and DA at room temperature.
[2] Assay conditions: 100 mM phosphate buffer, 0.2 mM NAD^+ and different concentration of 2R,3R-BD and Meso-BD at room temperature. ND: Not detection.

Table 4
Comparison of K_m values between the R-BDH from *Bacillus* sp. DL01 and other strains.

Microorganism	Enzyme name (NCBI)	K_m for 3R/3S-AC (mM)	K_m for 2R/3R-BD (mM)	K_m for Meso-BD (mM)	Reference
Bacillus sp. DL01	2R/3R-BDH	0.253 ± 0.03	0.389 ± 0.090	0.397 ± 0.11	This study
Paenibacillus polymyxa	2R,3R-BDH	0.30 ± 0.03	1.76 ± 0.29	5.62 ± 0.81	[15]
Bacillus clausii	2R,3R-BDH	0.529 ± 0.079	2.2 ± 0.5	23.1 ± 2.8	[26]
B. thuringiensis	2R,3R-BDH	0.49 ± 0.08	0.76 ± 0.12	3.67 ± 0.55	[22]
Rhodococcus erythropolis	2R,3R-BDH	ND	0.58 ± 0.05	ND	[21]

as well as oxidization activity with 2R,3R-BD and Meso-BD as substrates. In contrast, R-BDH could not catalyze 2S,3S-BD oxidization. The activity of R-BDH was enhanced significantly by Cd^{2+} when DA, 3R/3S-AC and Meso-BD were used as substrates, and increased remarkably by $Fe^{2+}$for 2R,3R-BD oxidation. The kinetic parameters of K_m, $K_{cat,}$ and K_{cat}/K_m showed that 3R/3S-AC was the optimal substrate for R-BDH, ant that this enzyme had higher affinity towards 2R,3R-BD and Meso-BD compared with other *Bacillus* species. These findings indicated the high reversible conversion rate of 2,3-BD to 3R/3S-AC in *Bacillus* sp. DL01. These results suggested that the R-BDH was highly enantioselective for R-configuration, and provided an approach to regulating single chiral 3R/3S-AC or 2,3-BD accumulation in bacteria.

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Conflict of interest

The authors declare that they have no conflict of interest.

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