Journal of Basic Microbiology
Environment · Health · Techniques

Research Paper

Characterization of two novel butanol dehydrogenases involved in butanol degradation in syngas-utilizing bacterium *Clostridium ljungdahlii* DSM 13528

Yang Tan[1,2], Juanjuan Liu[1], Zhen Liu[1] and Fuli Li[1]

[1] Key Laboratory of Biofuels, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, Qingdao, 266101, China
[2] University of Chinese Academy of Sciences, Beijing, 100039, China

Syngas utilizing bacterium *Clostridium ljungdahlii* DSM 13528 is a promising platform organism for a whole variety of different biofuels and biochemicals production from syngas. During syngas fermentation, *C. ljungdahlii* DSM 13528 could convert butanol into butyrate, which significantly reduces productivity of butanol. However, there has been no any enzyme involved in the degradation of butanol characterized in *C. ljungdahlii* DSM 13528. In this study two genes, CLJU_c24880 and CLJU_c39950, encoding putative butanol dehydrogenase (designated as BDH1 and BDH2) were identified in the genome of *C. ljungdahlii* DSM 13528 and qRT-PCR analysis showed the expression of *bdh1* and *bdh2* was significantly upregulated in the presence of 0.25% butanol. And the deduced amino acid sequence for BDH1 and BDH2 showed 69.85 and 68.04% identity with *Clostridium acetobutylicum* ADH1, respectively. Both BDH1 and BDH2 were oxygen-sensitive and preferred $NADP^+$ as cofactor and butanol as optimal substrate. The optimal temperature and pH for BDH1 were at 55 °C and pH 7.5 and specific activity was 18.07 ± 0.01 μmol min^{-1} mg^{-1}. BDH2 was a thermoactive dehydrogenase with maximum activity at 65 °C and at pH 7.0. The specific activity for BDH2 was 11.21 ± 0.02 μmol min^{-1} mg^{-1}. This study provided important information for understanding the molecular mechanism of butanol degradation and determining the targets for gene knockout to improve the productivity of butanol from syngas in *C. ljungdahlii* DSM 13528 in future.

Keywords: *Clostridium ljungdahlii* / syngas / butanol degradation / butanol dehydrogenase / qRT-PCR

Received: January 16, 2013; accepted: April 24, 2013

DOI 10.1002/jobm.201300046

Introduction

As the world becomes more and more industrialized, fossil fuels such as oil and coal diminished faster and faster. Biological fuels such as ethanol, butanol, and biodiesel, as important alternatives, are receiving increasing public and scientific attention [1–3]. The technology that fermenting syngas into the biofuels is considered to be more attractive than others [4–6].

Syngas can be inexpensively produced from several resources including natural gas, coal, oil, biomass, municipal waste. Synthesis gas has been used to generate chemicals through chemical catalysts for decades [7]. However, biological process showed its advantages over catalytic processes such as higher yields, higher specificity, generally greater resistance to catalyst poisoning, and lower energy cost [8]. Until now, a number of syngas-utilizing microbial organisms including *Clostridium ljungdahlii*, *Clostridium autoethanogenum*, *Acetobacterium woodii*, *Clostridium carboxidivorans*, *Butyribacterium methylotrophicum*, *Peptostreptococcus productus* have been isolated [9]. Many studies related to syngas fermentation by these bacteria are undergoing, most of which focus on the factors influencing the efficiency of syngas conversion to bioethanol, such as medium-pH, nutrients availability, gas composition and total pressure, the partial pressure of substrate gases, and mass transfer [6, 10–15]. Until

Correspondence: Fuli Li, Institute of Bioenergy and Bioprocess Technology, CAS, Songling Road No. 189, Qingdao 266101, China
E-mail: lifl@qibebt.ac.cn
Phone: +86 532 80662655
Fax: +86 532 80662778

now, it has been reported that *C. ljungdahlii* DSM 13528 produced the highest ethanol concentration of 48 g L^{-1} through syngas fermentation in continuous reactor studies [16].With the advantage of high efficiency of fermenting syngas into ethanol and short generation time *C. ljungdahlii* attracted wider range of attention [16]. And recently, the publication of *C. ljungdahlii* DSM 13528 genome and the establishment of its genetic transformation system have made *C. ljungdahlii* DSM 13528 become tailor-made strains, suitable for whole variety of different production processes [17].

However, when using ethanol as fuel there exist many problems such as difficulty to distribute, effects of corrosiveness on the pipeline and engine, and low intersolubility with diesel fuels. Compared with ethanol, butanol will not bring troubles mentioned above, but also owns the following inherent merits such as higher heating value, lower volatility, and higher viscosity. Butanol is naturally formed by a number of clostridia such as *Clostridium acetobutylicum* and *Clostridium beijerinckii*. The substrate for butanol production mainly includes starch-based packaging materials [18], soy molasses [19], fruit processing industry waste [18], and corncob residual [20].

Recently, a modified *C. ljungdahlii* DSM 13528 with plasmid pSOBPptb could produce butanol from syngas [17]. At the end of growth, most of the butanol was converted into butyrate. And wild-type *C. ljungdahlii* DSM 13528 was also able to convert butanol into butyrate. It was predicted that butanol was uptaken and degraded into butyrate through the four-step pathway including butanol dehydrogenase, aldehyde dehydrogenase, phosphotransbutyrylase, and butyrate kinase [17]. Butanol dehydrogenase is one key enzyme in that pathway, which has been identified and characterized in butanol-producing bacteria and butanol-utilizing bacteria [21–23].

It is necessary to identify and characterize the butanol dehydrogenases, which is helpful for elucidating the mechanism of butanol degradation in *C. ljungdahlii* DSM 13528 and determining the targets for gene knockout to improve the butanol productivity in future. After screening the genomes, three putative genes encoding butanol dehydrogenases (CLJU_c24880, CLJU_c39950 and CLJU_c23460) were identified. The mRNA abundances for CLJU_c24880 and CLJU_c39950 were significantly increased in the presence of butanol in the medium, which indicated that these two genes are more likely to participate in butanol degradation. And these two genes were heterologously expressed in *Escherichia coli* BL21 (DE3) and the enzymatic properties of the corresponding enzymes were characterized.

Materials and methods

Bacterial strains and growth conditions

C. ljungdahlii DSM 13528 was purchased from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and grown anaerobically in PETC medium (DSMZ 879) at 37 °C. *E. coli* BL21(DE3 (TransGen Biotech Co., Ltd, Beijing, China) was grown in Luria–Bertani (LB) medium with shaking 200 rpm at 37 °C. When necessary, 100 mg L^{-1} ampicillin was added to the LB medium. Growth was monitored by measuring the optical density at 600 nm ($OD_{600\ nm}$).

Real-time RT-PCR

When the growth of cells are at the middle exponential phase (OD_{600nm} = 0.5–0.6), the cells were centrifuged anaerobically at 3000*g* for 10 min under 4 °C. The cells were resuspended in the same volume of PETC medium supplemented with 0.25% butanol. The cells samples were harvested after 0, 0.5, 1, 1.5, and 2 h treatment with butanol. All the samples were immediately frozen in liquid nitrogen, and stored at −80 °C. Total RNA was extracted using RNAprep pure Cell/Bacteria Kit (Tiangen Biotech Co., Ltd, Beijing, China) and treated by the DNase. Quantitative RT-PCR was performed with RNA samples to confirm the absence of DNA contamination using 16S primers [24]. The concentration and the purity of RNA were determined using the NanoDrop™ spectrophotometer ND-1000 (Thermo Fisher Scientific, Inc., Massachusetts, USA). The cDNA synthesis was performed according to the instruction (Tiangen Biotech Co., Ltd). Real-time PCR for quantification of cDNA was performed using the SYBR Green PCR master mix (Roche Applied Science, Indianapolis, USA) and the Roche real-time PCR system. Reaction mixtures were incubated for 10 min at 95 °C, followed by 40 amplification cycles for 15 s at 95 °C and 60 s at 60 °C. All reactions were carried out on 96-well reaction plate. The *gyrA* (gryrase subunit A) was used as reference gene for normalization. The primers used for qRT-PCR were listed in Table 1. The length of expected products for *16S rRNA*, *gyrA*, *bdh1*, *bdh2*, and CLJU_c23460 were 173, 157, 168, 201, and 115 bp. Comparative C_t method was used to quantify relative expression levels of *bdh1* and *bdh2*.

Cloning of *bdh1* and *bdh2* gene from *C. ljungdahlii* DSM 13528

Genomic DNA of *C. ljungdahlii* DSM 13528 was extracted as described [25]. The *bdh1* and *bdh2* genes were amplified by PCR from *C. ljungdahlii* DSM 13528 genomic DNA. The oligonucleotide primers for cloning were listed in Table 1. KOD-Plus-Neo DNA polymerase of high fidelity (Toyobo

Table 1. Primers for qRT-PCR and cloning.

Primers	Primer sequences (5′–3′)
16S rRNA	TACTAGGTGTAGGAGGTATCG
	GTCAAGTCCAGGTAAGGTTC
gyrA	GGGGAAAGGGAATACAAGCA
	TTAGCCGTTCTACCCGCATC
bdh1-RT	CTTACTCACGCAATAGAAGCA
	TGCCATCCCAGCTAGACATTG
bdh2-RT	TTGTTCCGTTTGGATTACCAG
	TAGGTGGCATTGTCTGGGCTA
CLJU_c23460-RT	TGCTTAGTGCGGGTAGAATA
	ATCCATGCAGGAAATACAAT
bdh1	ATGGGAAGATTTACTTTGC
	CTAGAAATTAACCTTTTGC
bdh2	ATGGAGAGATTTACGTTGC
	TTAAAAAGTTACCTTTTTTCC

Life Science, Shanghai, China) was used. And dATP was added to the 3′ terminus of two PCR products for *bdh1* and *bdh2* by the Taq polymerase (TransGen Biotech Co., Ltd). The two target fragments were cloned into the Peasy-E1 vector carrying an N-terminal histidine tag (TransGen Biotech Co., Ltd). The constructed plasmids, Peasy-E1-*bdh1* and Peasy-E1-*bdh2*, were used to transform into *E. coli* DH5a for replication. Both of two target plasmids were extracted from *E. coli* DH5a with TIANprep Mini Plasmid kit (Tiangen Biotech Co., Ltd). Then, the pure plasmids carrying *bdh1* and *bdh2* were used to transform into *E. coli* BL21(DE3) for expression, respectively.

Expression and purification of BDH1 and BDH2

The colonies of *E. coli* BL21(DE3) carrying Peasy-E1-*bdh1* and Peasy-E1-*bdh2* were cultured in LB containing 100 mg L^{-1} ampicillin. When the cells optical density ($OD_{600\ nm}$) reached 0.5, 0.5 mM IPTG was added into the medium to induce the expression of two *bdhs* at 16 °C for 10 h. Cells were harvested by centrifugation at 3000*g* for 10 min and washed with phosphate buffer (300 mM NaCl, 50 mM NaH_2PO_4, 1 mM DTT, pH 8.0). All steps in the following procedures were carried out at 4 °C anaerobically. And the cells were resuspended in the same buffer and subjected to the sonication (30% amplitude, 150 cycles of 10 s on, 15 s off) by VCX500 ultrasonic processor (Sonics&Materials, Inc., Newtowns, USA). The lysates were centrifuged at 14,000*g* for 10 min at 4 °C to remove the cell debris. Crude extracts obtained were subjected to the Ni^{2+} column according to the manufacturer's instruction (Sangon Biotech Co., Ltd, Shanghai, China). Unbound proteins were washed out with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 50 mM imidazole, 1 mM DTT, pH 8.0). His-tagged fusion proteins were eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 1 mM DTT, pH 8.0) and dialyzed against dialysis buffer (50 mM Tris–HCl buffer, 1 mM DTT, pH 8.0). Protein concentration was determined by the Bradford method [26]. SDS–PAGE was performed according to the description by Laemmli [27].

Enzymatic assays

The assay was carried out in 1 mL mixture containing 50 mM Tris–HCl (pH 8.0), 0.25 mM $NADP^+$, 1 mM DTT, and 10 mM butanol and the appropriate amount of enzyme samples (6.9 μg for BDH1 and 8.8 μg for BDH2) for butanol oxidation 1 min at 37 °C, anaerobically. The increment in absorbance at 340 nm due to NADPH generation was monitored by DU800 Spectrophotometer (Beckman, Maryland, USA). One unit of enzyme activity was defined as the amount of enzyme required for the generation of 1 μmol of NADPH per min.

Effects of temperature, pH, metal ions, Cys, NH_4Cl, EDTA, SDS, and Triton X-100 on BDH1 and BDH2 activity

The effect of temperature on the activity of BDH1 and BDH2 was analyzed by measuring the activity of enzymes over the range of 25–75 °C at pH 7.5. Also the optimal pH for the two butanol dehydrogenases was determined by analyzing the activity of enzymes over the range of pH 6–9.5 at their optimal temperature. Metal ions were added into the reaction mixture for studying their effect on the activity of enzymes with a final concentration of 2 mM. To determine the effects of Cys, NH_4Cl, EDTA, SDS, and TritonX-100, 5 mM Cys, 10 mM NH_4Cl, 1 mM EDTA, 0.05% SDS, or 1% TritonX-100 was included in the standard reaction mixture.

Substrate specificity

In order to determine the substrate specificity of the two BDHs, C1-C8 alcohols, several secondary alcohols, polyhydric alcohols, and aromatic alcohols were used for the reaction. The activity with *n*-butanol as substrate was considered as 100%.

Protein database search

The amino acids of the related enzymes in the study were obtained by performing BLAST program (National Centre for Biotechnology Information [http://www.ncbi.nlm.nih.gov/BLAST]) using the default parameters. The multiple sequence alignment was performed with ClustalW program using the standard parameters. The phylogenetic tree was constructed with the neighbor-joining method by the Mega 5.0 using the default parameters. Bootstrap values were calculated based on 1000 replicates.

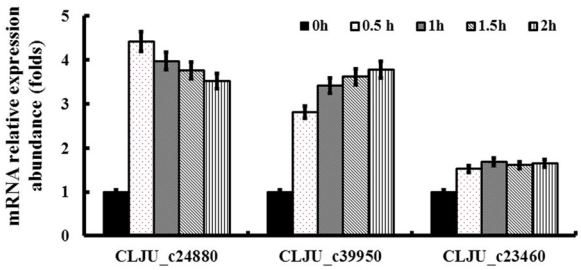

Figure 1. The relative expression of CLJU_c24880, CLJU_c39950 and CLJU_c23460 for *C. ljungdahlii* DSM 13528 grown under the PETC medium with 0.25% butanol. All reactions were performed in triplicates.

Results

Expression analysis of CLJU_c24880, CLJU_c39950, and CLJU_c23460 by qRT-PCR

In the genome of *C. ljungdahlii* DSM 13528, three genes, CLJU_c24880, CLJU_c39950, and CLJU_c23460, were annotated as putative butanol dehydrogenases. The qRT-PCR analysis was conducted to determine the transcriptional levels of these three genes within 2 h after the cells exposed to 0.25% butanol (Fig. 1). The mRNA abundance for CLJU_c24880 increased by 3.4 folds after 0.5 h treatment and kept constant. Similarly, the expression of CLJU_c39950 was significantly upregulated in the presence of butanol. The mRNA transcripts for CLJU_c39950 increased gradually within 2 h as the time went forward and its expression levels reached 3.8 folds after 2 h. By comparison, the mRNA abundance of CLJU_c23460 kept 1.5∼1.7 folds within 2 h than at 0 h and that showed the expression of CLJU_c23460 was not significantly induced. Apparently, it was validated that both CLJU_c24880 and CLJU_c39950 have close relationship with butanol degradation in *C. ljungdahlii* DSM 13528.

Phylogenetic tree analysis and sequence alignment

The deduced amino acid sequences of BDH1 and BDH2 were compared with other 15 ADHs including five Zn-dependent ADHs, four SDRs and six type III ADHs which are available in the GenBank database. The phylogenetic tree showed both BDH1 and BDH2 were clustered into the type III ADH group (Fig. 2).

BDH1 and BDH2 shared 40.20–69.85% identity and 35.23–68.04% identity with BMMDH and CAADH1, two characterized members of type III ADH group, respectively. BDH1 and BDH2 shared 78.25% identity with each other. Amino acid sequence alignments of the deduced polypeptides of BDH1, BDH2 and these two ADHs were shown in Fig. 3. The four marker regions of type III ADH were included in both BDH1 and BDH2 [28]. Conserved structural iron-coordinating residues were also observed

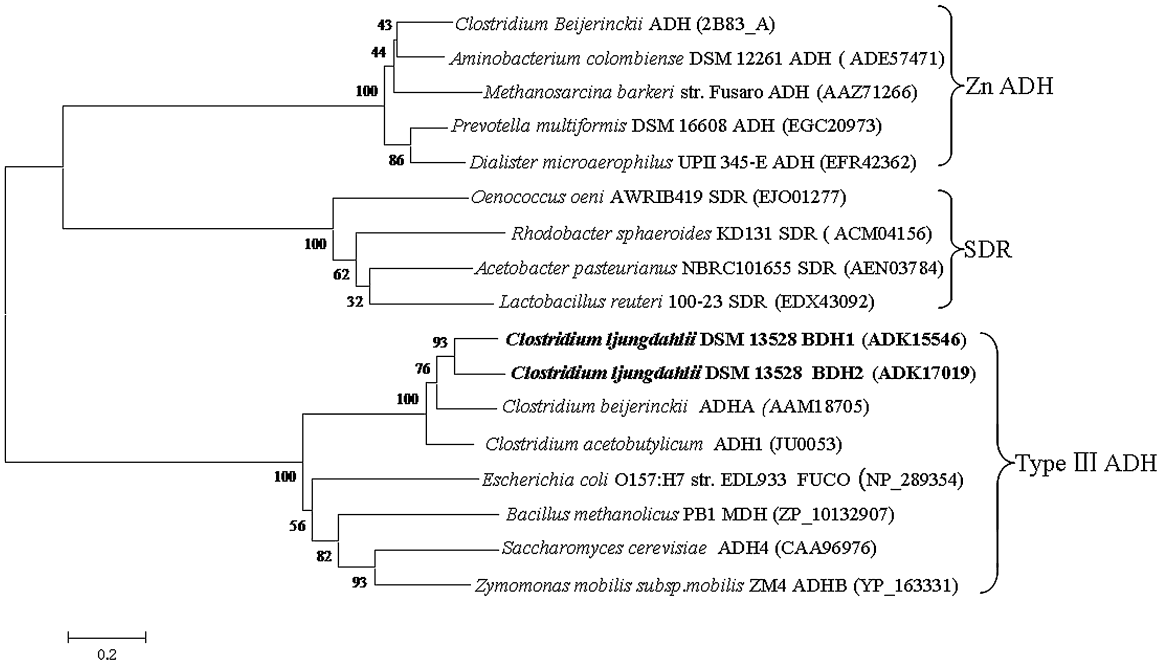

Figure 2. A phylogenetic tree derived from the amino acid sequences of NAD(P)$^+$-dependent ADHs from *C. ljungdahlii* DSM 13528 and related bacteria. Zn ADH, Zn-dependent alcohol dehydrogenase; SDR, short-chain dehydrogenase/reductase; Fe ADH, iron-containing alcohol dehydrogenase. The analysis was based on the neighbor-joining method with bootstrap value 1000 and performed by MEGA 5.0 software. The amino acid sequences were obtained from the GenBank database. The two BDHs in this study were in bold.

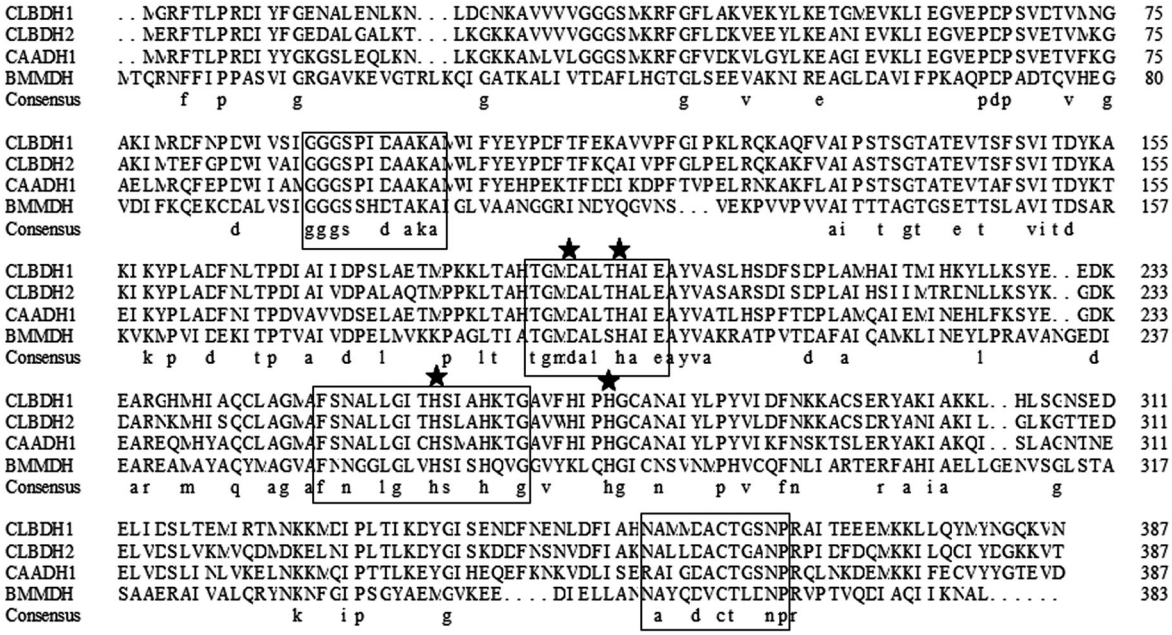

Figure 3. Amino acid sequence alignment of type III alcohol dehydrogenases. CLBDH1: *C. ljungdahlii* DSM 13528 BDH1; CLBDH2: *C. ljungdahlii* DSM 13528 BDH2. BMMDH: *Bacillus methanolicus* MDH; CAADH1: *Clostridium acetobutylicum* ADH1. The sequence alignment was performed using ClustalW. The marker region of type III alcohol dehydrogenases were enclosed by black boxes and the conserved structural iron-coordinating residues were indicated by five stars.

in the deduced amino acid sequence of BDH1 and BDH2 at Asp-191, His-195, His-258, and His-272, respectively.

Expression of *bdh1* and *bdh2* in *E. coli* BL (DE3) and purification of the recombinant enzymes

The expression of recombinant proteins was successfully induced by IPTG. Both of total proteins and soluble proteins (30–70 μg) and purified recombinant proteins (8 μg) were subjected to the SDS–PAGE analysis, two induced bands corresponding to 44.3 kDa were detected (Fig. 4). BDH1 and BDH2 were also successfully purified and the molecular weights were determined to be 45.5 and 43.5 kDa by SDS–PAGE analysis, respectively.

Effects of different substrates, metal ions, Cys, EDTA, SDS, and Triton X-100 on the activity of BDH1 and BDH2

When $NADP^+$ or NAD^+ was added into the reaction mixture, respectively, only in the presence of $NADP^+$, BDH1 and BDH2 showed activity, indicating the two butanol dehydrogenases are restrictedly dependent on $NADP^+$. Furthermore, substrate specificity of the two ADHs was also investigated. Both of the two enzymes are sensitive to oxygen and they lost 65 and 75% activity for BDH1 and BDH2, respectively, when exposed to air for 1 h (Fig. 5). Their $t_{1/2}$-values were about 0.5 h. As shown in Table 2, butanol was the most appropriate substrate for both of the two enzymes. Under the optimal assay conditions (55 °C, pH 7.5 for BDH1; 65 °C, pH 7.0 for BDH2), the kinetic constants for BDH1 and BDH2 were determined (Table 3). All of the primary alcohols used in this study can be oxygenated by BDH1 and BDH2, the activities range from 10.1 to 74.5% of that with butanol

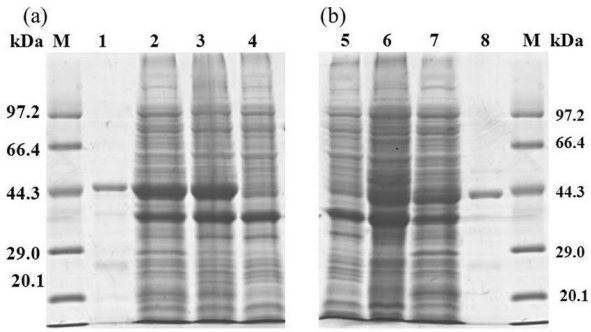

Figure 4. SDS–PAGE analysis of BDH1 and BDH2 expression in recombinant *E. coli* BL21(DE3) containing plasmid Peasy-E1-*bdh1* (a) and Peasy-E1-*bdh2* (b). Lane M, molecular weight standards; Lane 1, purified His-tagged BDH1; Lane 2, crude extract of *E. coli* carrying Peasy-E1-*bdh1* after IPTG induction; Lane 3, total proteins of *E. coli* carrying Peasy-E1-*bdh1* after IPTG induction; Lane 4, total proteins of *E. coli* carrying Peasy-E1-*bdh1* before IPTG induction; Lane 5, total proteins of *E. coli* carrying Peasy-E1-*bdh2* before IPTG induction; Lane 6, total proteins of *E. coli* carrying Peasy-E1-*bdh2* after IPTG induction; Lane 7, crude extract of *E. coli* carrying Peasy-E1-*bdh2* after IPTG induction; Lane 8, purified His-tagged BDH2.

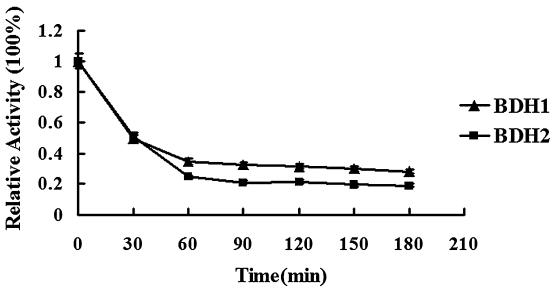

Figure 5. Oxygen sensitivity of BDH1 and BDH2: (▲) BDH1, (■) BDH2. The relative activity of 100% corresponds to 18.07 ± 0.01 μmol min^{-1} mg^{-1} for BDH1 and 11.21 ± 0.02 μmol min^{-1} mg^{-1} for BDH2, respectively.

as the substrate. BDH2 showed its adaptability to the wider range of substrates than BDH1 such as 2-butanol (4.8%), isopropanol (17.9%), ethylene glycol (9.5%), 1,4-butanediol (27.7%), 2,3-butanediol (8.7%), and glycerol (7.9%). Benzyl alcohol as the typical member of aromatic alcohol can provide 8.1 and 3.6% activity for BDH1 and BDH2, respectively.

The effects of different metal ions and various chemical reagents on the activities of the purified BDHs were investigated (Table 2). It was shown that to different extents (19.1–71.9%) the activity of BDH1 was inhibited by Ba^{2+}, Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+}, and Li^{+} at 2 mM concentration. And Ba^{2+}, Ca^{2+}, Co^{2+}, Ni^{2+}, and Li^{+} were able to stimulate the activity of BDH2 (28.8–62.5% increment), and especially Co^{2+} and Ni^{2+} increased the activity of the enzyme by 62.5 and 40.6%. Mg^{2+} and Mn^{2+} did not almost affect the activity of BDH2. Zn^{2+} and NH_4^{+} can decrease the activity of BDH2 by 33.3 and 41.9%. Both Cu^{2+} and Fe^{2+} completely inhibited the activity of BDH2 and BDH1 at 2 mM concentration. The activities of BDH1 and BDH2 were inhibited by Cys, EDTA, Triton X-100, and SDS.

Effects of pH, temperature, on the activity of BDH1 and BDH2

Butanol oxidation activity of BDH1 and BDH2 were tested under different pH ranging from 6 to 9.5 (Fig. 6). At pH 6.0, the activities of two enzymes showed 31–37%

Table 2. Substrate spectrum of BDH1 and BDH2 and the effects of metal ions and chemical reagents on the activity of BDH1 and BDH2.

	Relative activity (%)			Relative activity (%)	
Substrate	BDH1	BDH2	Metal ion/chelator	BDH1	BDH2
n-Butanol	100	100	None	100	100
Methanol	40.1	30.6	Ba^{2+}	46.5	128.8
Ethanol	71.3	74.5	Ca^{2+}	61.8	136.1
n-Propanol	63.6	51.1	Co^{2+}	49.6	162.5
n-Pentanol	61.9	52.6	Cu^{2+}	0	0
n-Octanol	13.3	10.1	Fe^{2+}	0	0
2-Butanol	0	4.8	Mg^{2+}	70.1	95.2
Isopropanol	0.4	17.9	Mn^{2+}	80.9	97.8
Ethylene glycol	0	9.5	Zn^{2+}	46.8	66.7
1,4-Butanediol	7.2	27.7	Ni^{2+}	28.1	140.6
2,3-Butanediol	0.0	8.7	Li^{+}	44.9	136.8
Glycerol	2.2	7.9	NH_4Cl	47.1	58.1
Benzyl alcohol	8.1	3.6	Cys	33.5	32.9
			EDTA	89.1	80.7
			SDS	4.8	6.5
			TritonX-100	87.6	81.3

The activities of the two enzymes under different substrates, metal ions and chemical reagents were measured in triplicates. The relative activity of 100% corresponds to 18.07 ± 0.01 μmol min^{-1} mg^{-1} for BDH1 and 11.21 ± 0.02 μmol min^{-1} mg^{-1} for BDH2, respectively.

Table 3. Kinetic constants for BDH1 and BDH2.[a]

Enzyme	Apparent K_m (mM)	Apparent V_{max} (μmol min^{-1} mg^{-1})	Apparent K_{cat} (s^{-1})	Apparent K_{cat}/K_m (mM^{-1} S^{-1})
BDH1	6.4 ± 0.1	18.07 ± 0.01	13 ± 1	2.03 ± 0.02
BDH2	3.1 ± 0.1	11.21 ± 0.02	8 ± 1	2.61 ± 0.01

[a]The reaction was performed under the optimal conditions for BDH1 and BDH2 anaerobically, respectively. $NADP^{+}$ concentration used in the reaction is 0.25 mM.

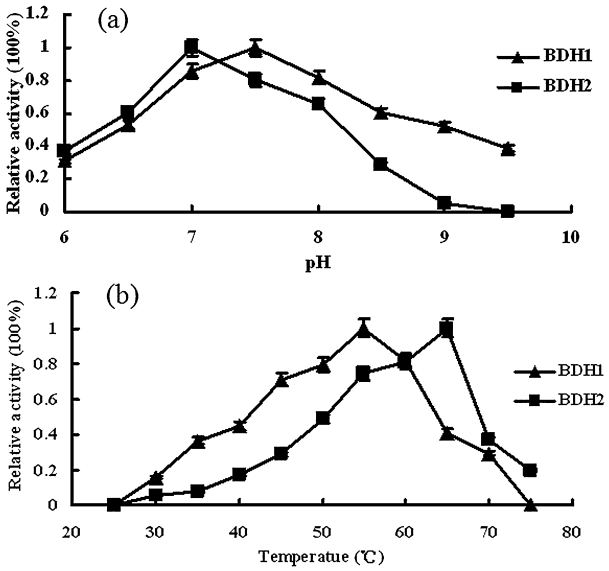

Figure 6. The effects of pH (a) and temperature (b) on the activities of BDH1 and BDH2: (▲) BDH1, (■) BDH2. The activities of the two enzymes under different pH were measured in triplicates. The relative activity of 100% corresponds to 18.07 ± 0.01 μmol min^{-1} mg^{-1} for BDH1 and 11.21 ± 0.02 μmol min^{-1} mg^{-1} for BDH2, respectively.

of the maximum activity. As the pH gradually increased the enzymatic activity also went up. The optimal pH for BDH1 and BDH2 were determined to be pH 7.5 and 7.0, respectively. No activity of BDH2 was detected and only 38% activity of BDH1 remained at pH 9.5.

The activity of both BDH1 and BDH2 was detected at temperatures ranging from 25 to 80 °C (Fig. 6). The activity of two enzymes increased as the temperature increased. The activity of BDH1 reached at the maximum value at 55 °C, while 65 °C is the optimal temperature for BDH2. No activity of BDH1 was observed and about 20% activity of BDH2 remained under 75 °C.

Discussion

When *C. ljungdahlii* DSM 13528 was grown in PETC medium supplemented with 0.25% butanol, 0.05% butyrate was detected at the end of growth. In contrast, no butyrate was detected during the whole period of growth without adding butanol. It is validated that *C. ljungdahlii* DSM 13528 is capable of converting the butanol into the butyrate. In this study BDH1 and BDH2 belonging to type III family ADH were verified to be able to oxygenate butanol into butyraldehyde. In addition, qRT-PCR analysis showed the expression of *bdh1* and *bdh2* was significantly induced to upregulate in the presence of butanol, indicating the important role of *bdh1* and *bdh2* in the conversion of butanol into butyrate. Although BDH1 and BDH2 showed 66.9 and 71.5% of maximum activity with ethanol as substrate, both of them are more likely to take part in conversion between butanol and butyraldehyde. Therefore, *bdh1* and *bdh2* can be considered as the targets for gene knockout to solve the problem of butanol degradation in *C. ljungdahlii* DSM 13528.

According to the result of phylogenetic analysis and homologous sequence alignment it is suggested that BDH1 and BDH2 belong to type III ADH family including *Zymononas mobilis* ADHB [29], *S. cerevisiae* ADH4 [30], *E. coli* POR [31], *C. acetobutylicum* ADH1 [32], *Bacillus methanolicus* MDH [28]. These members of type III ADH family required $NAD(P)^+$ as cofactor. The $NAD(P)^+$-binding site of all dehydrogenases have a conserved glycine-rich region consisting of a GxGxxG or a GxxGxxG consensus sequence within the β-α-β Rossmann fold [33]. However, the highly conserved GxGxxG or GxxGxxG consensus sequence did not appear at the N-terminal of $NADP^+$-dependent BDH1 and BDH2. The first glycine residue involved in the forming of a tight first β–α turn is present in both BDH1 and BDH2 at position 13. The second Gly, the most important residue, is responsible for binding dinucleotide in the consensus sequence. The second Gly was conserved in all other five members of type III ADH family. $NADP^+$-dependent dehydrogenases often feature a corresponding Gly or Arg residue [34, 35]. However, in the consensus sequence the Gly was replaced by the Asn and Asp in the BDH1 and BDH2 at position 15, respectively. That showed the $NADP^+$-dependent BDH1 and BDH2 may represent special examples which exhibited unique cofactor binding form.

The two recombinant dehydrogenases were also purified aerobically and the specific activities were 7.48 ± 0.01 and 2.73 ± 0.02 μmol min^{-1} mg^{-1} for BDH1 and BDH2. Both of the two butanol dehydrogenases were oxygen-sensitive. It is predicted that the irreversible inactivity might be related to the oxidation of important amino acids such as cysteine residues or oxidation of ferrous to ferric, and/or loss of ferrous ion, and replacement with other metals such as zinc [36].

Most thermoactive ethanol dehydrogenases were present in thermophilic bacteria [37, 38]. And in some mesophilic organisms thermoactive ethanol dehydrogenases were also identified [39–41]. Thermoactive ADHs belonging to type III ethanol dehydrogenase were also characterized, which are mostly derived from genus thermophilic *Thermococcus* [36, 42, 43]. The optimal temperature for BDH2 was at 65 °C and BDH2 was the first thermoactive member of type III ethanol dehydrogenase, which was characterized in mesophilic bacteria.

Although BDH1 and BDH2 shared 77.58% identity with each other, the optimal temperature (55 °C) for BDH1 was 10 °C lower than BDH2.

In this study, two butanol-dehydrogenase-encoding genes (CLJU_c24880 and CLJU_c39950) genes (CLJU_c24880 and CLJU_c39950) were identified in the genome of *C. ljungdahlii* DSM 13528 and their expressions were significantly upregulated in the presence of butanol. That showed these two genes were closely related to butanol degradation in this organism. In addition, *bdh1* and *bdh2* were heterologously expressed in the *E. coli* BL21 (DE3) and recombinant enzymes were oxygen-sensitive and capable of converting butanol into butyraldehyde with $NADP^+$ as cofactor. This study provided information for the improvement of butanol productivity from syngas fermentation by genetically modified *C. ljungdahlii* DSM 13528 in the future.

Acknowledgments

This study was supported by the National Basic Research Program of China (2011CB707404) and the Natural Science Foundation of China (30974409, 31111120078).

Conflict of interest

All of the authors declare no financial/commercial conflicts of interest.

References

[1] Manzer, L.E., 2010. Recent developments in the conversion of biomass to renewable fuels and chemicals. Top. Catal., **53**, 1193–1196.

[2] Gong, Y., Guo, X., Wan, X., Liang, Z. et al., 2013. Triacylglycerol accumulation and change in fatty acid content of four marine oleaginous microalgae under nutrient limitation and at different culture ages. J. Basic Microbiol., **53**, 29–36.

[3] Yang, T., Rao, Z., Zhang, X., Lin, Q. et al., 2011. Production of 2,3-butanediol from glucose by GRAS microorganism *Bacillus amyloliquefaciens*. J. Basic Microbiol., **51**, 650–658.

[4] Bredwell, M.D., Srivastava, P., Worden, R.M., 1999. Reactor design issues for synthesis-gas fermentations. Biotechnol. Prog., **15**, 834–844.

[5] Heiskanen, H., Virkajarvi, I., Viikari, L., 2007. The effects of syngas composition on the growth and product formation of *Butyribacterium methylotrophicum*. Enzyme Microb. Technol., **41**, 362–367.

[6] Klasson, K.T., Ackerson, M.D., Clausen, E.C., Gaddy, J.L., 1991. Bioreactor design for synthesis gas fermentation. Fuel, **70**, 605–614.

[7] Kini, K.A., Lahiri, A., 1975. Mechanism of Fischer-Tropsch hydrocarbon and higher alcohol syntheses. J. Sci. Ind. Res. India, **34**, 97–99.

[8] Klasson, K.T., Ackerson, M.D., Clausen, E.C., Gaddy, J.L., 1992. Bioconversion of synthesis gas into liquid or gaseous fuels. Enzyme Microb. Technol., **14**, 602–608.

[9] Henstra, A.M., Sipma, J., Rinzema, A., Stams, A.J., 2007. Microbiology of synthesis gas fermentation for biofuel production. Curr. Opin. Biotechnol., **18**, 200–206.

[10] Guo, Y., Xu, J., Zhang, Y., Xu, H. et al., 2010. Medium optimization for ethanol production with *Clostridium autoethanogenum* with carbon monoxide as sole carbon source. Bioresour. Technol., **101**, 8784–8789.

[11] Saxena, J., Tanner, R.S., 2011. Effect of trace metals on ethanol production from synthesis gas by the ethanologenic acetogen, *Clostridium ragsdalei*. J. Ind. Microbiol. Biotechnol., **38**, 513–521.

[12] Hurst, K.M., Lewis, R.S., 2010. Carbon monoxide partial pressure effects on the metabolic process of syngas fermentation. Biochem. Eng. J., **48**, 159–165.

[13] Cotter, J.L., Chinn, M.S., Grunden, A.M., 2009. Influence of process parameters on growth of *Clostridium ljungdahlii* and *Clostridium autoethanogenum* on synthesis gas. Enzyme Microb. Technol., **44**, 281–288.

[14] Sakai, S., Nakashimada, Y., Yoshimoto, H., Watanabe, S. et al., 2004. Ethanol production from H_2 and CO_2 by a newly isolated thermophilic bacterium, *Moorella* sp. HUC22-1. Biotechnol. Lett., **26**, 1607–1612.

[15] Younesi, H., Najafpour, G., Mohamed, A.R., 2005. Ethanol and acetate production from synthesis gas via fermentation processes using anaerobic bacterium, *Clostridium ljungdahlii*. Biochem. Eng. J., **27**, 110–119.

[16] Klasson, K.T., Ackerson, M.D., Clausen, E.C., Gaddy, J.L., 1993. Biological conversion of coal and coal-derived synthesis gas. Fuel, **72**, 1673–1678.

[17] Köpke, M., Held, C., Hujer, S., Liesegang, H. et al., 2010. *Clostridium ljungdahlii* represents a microbial production platform based on syngas. Proc. Natl. Acad. Sci. USA, **107**, 13087–13092.

[18] Jesse, T.W., Ezeji, T.C., Qureshi, N., Blaschek, H.P., 2002. Production of butanol from starch-based waste packing peanuts and agricultural waste. J. Ind. Microbiol. Biotechnol., **29**, 117–123.

[19] Qureshi, N., Lolas, A., Biaschek, H.P., 2001. Soy molasses as fermentation substrate for production of butanol using *Clostridium beijerinckii* BA101. J. Ind. Microbiol. Biotechnol., **26**, 290–295.

[20] Zhang, W.L., Liu, Z.Y., Liu, Z., Li, F.L., 2012. Butanol production from corncob residue using *Clostridium beijerinckii* NCIMB 8052. Lett. Appl. Microbiol., **55**, 240–246.

[21] Petersen, D.J., Welch, R.W., Rudolph, F.B., Bennett, G.N., 1991. Molecular cloning of an alcohol (butanol) dehydrogenase gene cluster from *Clostridium acetobutylicum* ATCC 824. J. Bacteriol., **173**, 1831–1834.

[22] Hiu, S.F., Zhu, C.X., Yan, R.T., Chen, J.S., 1987. Butanol-ethanol dehydrogenase and butanol-ethanol-isopropanol dehydrogenase: different alcohol dehydrogenases in two strains of *Clostridium beijerinckii* (*Clostridium butylicum*). Appl. Environ. Microbiol., **53**, 697–703.

[23] Veeranagouda, Y., Benndorf, D., Heipieper, H.J., Karegoudar, T.B., 2008. Purification and characterization of NAD-dependent n-butanol dehydrogenase from solvent-tolerant n-butanol-degrading *Enterobacter* sp. VKGH12. J. Microbiol. Biotechnol., **18**, 663–669.

[24] Metcalf, D., Sharif, S., Weese, J.S., 2010. Evaluation of candidate reference genes in *Clostridium difficile* for gene expression normalization. Anaerobe, **16**, 439–443.

[25] Köpke, M., Mihalcea, C., Liew, F., Tizard, J.H. et al., 2011. 2,3-Butanediol production by acetogenic bacteria, an alternative route to chemical synthesis, using industrial waste gas. Appl. Environ. Microbiol., **77**, 5467–5475.

[26] Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem., **72**, 248–254.

[27] Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, **227**, 680–685.

[28] de Vries, G.E., Arfman, N., Terpstra, P., Dijkhuizen, L., 1992. Cloning, expression, and sequence analysis of the *Bacillus methanolicus* C1 methanol dehydrogenase gene. J. Bacteriol., **174**, 5346–5353.

[29] Conway, T., Sewell, G.W., Osman, Y.A., Ingram, L.O., 1987. Cloning and sequencing of the alcohol dehydrogenase II gene from *Zymomonas mobilis*. J. Bacteriol., **169**, 2591–2597.

[30] Williamson, V.M., Paquin, C.E., 1987. Homology of *Saccharomyces cerevisiae* ADH4 to an iron-activated alcohol dehydrogenase from *Zymomonas mobilis*. Mol. Gen. Genet., **209**, 374–381.

[31] Conway, T., Ingram, L.O., 1989. Similarity of *Escherichia coli* propanediol oxidoreductase (*fucO* product) and an unusual alcohol dehydrogenase from *Zymomonas mobilis* and *Saccharomyces cerevisiae*. J. Bacteriol., **171**, 3754–3759.

[32] Youngleson, J.S., Jones, W.A., Jones, D.T., Woods, D.R., 1989. Molecular analysis and nucleotide sequence of the *adh1* gene encoding an NADPH-dependent butanol dehydrogenase in the Gram-positive anaerobe *Clostridium acetobutylicum*. Gene, **78**, 355–364.

[33] Wierenga, R.K., Terpstra, P., Hol, W.G.J., 1986. Prediction of the occurrence of the ADP-binding βαβ-fold in proteins, using an amino acid sequence fingerprint. J. Mol. Biol., **187**, 101–107.

[34] Lesk, A.M., 1995. NAD-binding domains of dehydrogenases. Curr. Opin. Struct. Biol, **5**, 775–783.

[35] Wierenga, R.K., Demaeyer, M.C.H., Hol, W.G.J., 1985. Interaction of pyrophosphate moieties with alpha-helixes in dinucleotide binding-proteins. Biochemistry, **24**, 1346–1357.

[36] Ying, X., Grunden, A.M., Nie, L., Adams, M.W. et al., 2009. Molecular characterization of the recombinant iron-containing alcohol dehydrogenase from the hyperthermophilic Archaeon, *Thermococcus* strain ES1. Extremophiles, **13**, 299–311.

[37] Holt, P.J., Williams, R.E., Jordan, K.N., Lowe, C.R. et al., 2000. Cloning, sequencing and expression in *Escherichia coli* of the primary alcohol dehydrogenase gene from *Thermoanaerobacter ethanolicus* JW200. FEMS Microbiol. Lett., **190**, 57–62.

[38] Jeon, Y.J., Fong, J.C.N., Riyanti, E.I., Neilan, B.A. et al., 2008. Heterologous expression of the alcohol dehydrogenase (*adhI*) gene from *Geobacillus thermoglucosidasius* strain M10EXG. J. Biotechnol., **135**, 127–133.

[39] Cao, Y., Liao, L., Xu, X.W., Oren, A. et al., 2008. Characterization of alcohol dehydrogenase from the haloalkaliphilic archaeon *Natronomonas pharaonis*. Extremophiles, **12**, 471–476.

[40] Timpson, L.M., Alsafadi, D., Mac Donnchadha, C., Liddell, S. et al., 2012. Characterization of alcohol dehydrogenase (ADH12) from *Haloarcula marismortui*, an extreme halophile from the Dead Sea. Extremophiles, **16**, 57–66.

[41] Timpson, L.M., Liliensiek, A.K., Alsafadi, D., Cassidy, J. et al., 2013. A comparison of two novel alcohol dehydrogenase enzymes (ADH1 and ADH2) from the extreme halophile *Haloferax volcanii*. Appl. Microbiol. Biotechnol., **97**, 195–203.

[42] Ma, K., Robb, F.T., Adams, M.W., 1994. Purification and characterization of NADP-specific alcohol dehydrogenase and glutamate dehydrogenase from the hyperthermophilic archaeon *Thermococcus litoralis*. Appl. Environ. Microbiol., **60**, 562–568.

[43] Li, D., Stevenson, K.J., 1997. Purification and sequence analysis of a novel NADP(H)-dependent type III alcohol dehydrogenase from *Thermococcus* strain AN1. J. Bacteriol., **179**, 4433–4437.

