J. Microbiol. Biotechnol. (2008), **18**(4), 663–669

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Purification and Characterization of NAD-Dependent *n*-Butanol Dehydrogenase from Solvent-Tolerant *n*-Butanol-Degrading *Enterobacter* sp. VKGH12

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Received: June 27, 2007 / Accepted: September 5, 2007

The solvent-tolerant bacterium *Enterobacter* sp. VKGH12 is capable of utilizing *n*-butanol and contains an NAD^+-dependent *n*-butanol dehydrogenase (BDH). The BDH from *n*-butanol-grown *Enterobacter* sp. was purified from a cell-free extract (soluble fraction) to near homogeneity using a 3-step procedure. The BDH was purified 15.37-fold with a recovery of only 10.51, and the molecular mass estimated to be 38 kDa. The apparent Michaelis-Menten constant (K_m) for the BDH was found to be 4 mM with respect to *n*-butanol. The BDH also had a broad range of substrate specificity, including primary alcohols, secondary alcohols, and aromatic alcohols, and exhibited an optimal activity at pH 9.0 and 40°C. Among the metal ions studied, Mg^{2+} and Mn^{2+} had no effect, whereas Cu^{2+}, Zn^{2+}, and Fe^{2+} at 1 mM completely inhibited the BDH activity. The BDH activity was not inhibited by PMSF, suggesting that serine is not involved in the catalytic site. The known metal ion chelator EDTA had no effect on the BDH activity. Thus, in addition to its physiological significance, some features of the enzyme, such as its activity at an alkaline pH and broad range of substrate specificity, including primary and secondary alcohols, are attractive for application to the enzymatic conversion of alcohols.

Keywords: *n*-Butanol, *Enterobacter* sp., solvent tolerance, NAD^+-dependent dehydrogenase

A potential problem for the biodegradation of chemical contaminants in a natural environment is that many compounds are also very toxic to the degrading microorganisms [20]. Although organic solvents are toxic to bacteria, several solvent-tolerant bacteria have devised various novel adaptive mechanisms that enable them to thrive in the presence of supersaturating amounts of toxic organic solvents [17]. Thus, understanding the physiological consequences of solvent-degrading microorganisms will help to improve the biodegradation efficiency of the strain.

n-Butanol is a versatile organic solvent that is used for various purposes in industry and thus released into the environment [10]. Although alcohol metabolism has already been studied in alkane- and alcohol-degrading bacteria, reports on *n*-butanol degradation are limited. Recently, we have shown that a solvent-tolerant *Enterobacter* sp., VKGH12, was able to degrade a high concentration of *n*-butanol in batch, repeated batch, and continuous modes [24]. This bacterium also exhibited a unique adaptive mechanism under *n*-butanol-stressed conditions. Cells growing on glucose intoxicated with *n*-butanol have been shown to increase their size and thus decrease the surface-to-volume ratio. This relative reduction of the cell surface has been discussed as an adaptive response against the membrane-active action of these toxic compounds [16]. Conversely, cells completely adapted to *n*-butanol as a growth substrate decrease their size, which ultimately increases their relative surface, their membrane area, to allow a better uptake and transformation of the substrate *n*-butanol [23]. Accordingly, since *n*-butanol dehydrogenase (BDH) is a key enzyme involved in the first step of the degradation of *n*-butanol, we have examined the possibility of the presence of an efficient alcohol dehydrogenase (ADH) in this strain. Here, we found that *Enterobacter* sp. VKGH12 is able to produce an NAD^+-dependent butanol dehydrogenase. To date, there would appear to be no other reports on an NAD^+-dependent butanol dehydrogenase from *n*-butanol-degrading bacteria. Therefore, this is the first report on the purification and characterization of the NAD^+-dependent

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butanol dehydrogenase from the *n*-butanol-degrading bacterium *Enterobacter* sp. VKGH12.

Materials and Methods

Bacterial Strain and Culture Conditions

The *Enterobacter* sp. VKGH12 was cultivated in a mineral salt medium, as described earlier [23]. The medium was supplied with 0.8% *n*-butanol as the carbon and energy source, and the culture maintained on a synthetic agar plate prepared from an MM2 medium containing 2% (w/v) agar overlaid with *n*-butanol. Unless otherwise stated, the inoculum used in this experiment was prepared by subculturing the bacterium in an MM2 medium containing 0.8% (v/v) *n*-butanol as the carbon and energy source. The cells were grown in 250-ml shake flasks in a horizontally shaking water bath at 30°C.

Preparation of Cell-free Extract

Fresh cells during early logarithmic growth were harvested by centrifugation at 8,000 rpm for 20 min at 4°C. The pellet (5 g) was then washed twice with a 50 mM potassium phosphate buffer (PPB, pH 7.0) and resuspended in 100 ml of 50 mM PPB containing 10 mM β-mercaptoethanol. The disruption of the cells was carried out with a Vibra Cell Ultrasonicator (model 375, U.S.A., with 3/4-inch flat tip). Complete disruption of the cells was achieved by giving 10 pulses of 60-sec sonic bursts at a nominal power of 70 W with 15-sec intervening pauses. After each interval, the disrupted cells and oscillator probe were cooled in ice for 1 min. The unbroken cells and cell debris were removed by centrifugation at 15,000 rpm for 30 min at 4°C. The supernatant thus obtained was then used for the purification studies.

Purification of *n*-Butanol Dehydrogenase

The isolation and purification procedures were carried out at 4°C, unless otherwise stated. During the purification of the BDH, 10 mM β-mercaptoethanol was added to the cell-free extract immediately after the sonication.

The cell-free extract obtained as described above was used for ammonium salt precipitation, where 40% ammonium sulfate was added to the cell-free extract, and the mixture kept on a magnetic stirrer for 2 h at 4°C. The resulting precipitate was removed by centrifugation at 14,000 rpm for 30 min. Additional ammonium sulfate was then added to the supernatant to reach 60% saturation. The resulting precipitate was collected by centrifugation, and redissolved in 50 mM PPB (pH 7.0). The resulting solution was then dialyzed overnight against 50 mM PPB at 4°C. Thereafter, the dialyzed sample was placed on a Q-Sepharose column (25×2.0) pre-equilibrated with 50 mM PPB (pH 7.0). After washing with the same buffer, the bound BDH was eluted with 200 ml of an increasing linear gradient of 0–1 M NaCl in PPB (pH 7.0) at a flow rate of 25 ml/h and collected as 3-ml fractions. The active fractions from the above step (40–47) were pooled and precipitated by the addition of ammonium sulfate to 60% saturation, and the resulting precipitate was dialyzed against 50 mM PPB (pH 7.0) at 4°C. The dialyzed sample was then loaded onto a Sephadex G75 column (80×1.5 cm) previously equilibrated with 50 mM PPB. The BDH was then eluted with the same buffer at a flow rate of 15 ml/h and collected as 2-ml fractions. The fractions containing BDH activity were pooled and used for checking the homogeneity and characterization.

Enzyme Assay

The enzyme activity of the NAD^+-dependent *n*-butanol dehydrogenase was measured by the increase in A_{340} due to the formation of NADH, in a reaction mixture (3 ml) consisting of a 50 mM Tris-HCl buffer of pH 8.8, 2 mM NAD^+, 2 mM DTT, and appropriately diluted cell-free extract. The reaction was initiated by the addition of 20 mM *n*-butanol and the increase in the absorbance (340 nm) was monitored for 5 min at 30°C. The endogenous rate of NAD^+ reduction obtained without the substrate was subtracted from the rate observed with the substrate. The activity was then calculated based on the extinction coefficient of NADH (ε=6,221 M^{-1} cm^{-1}), and the protein concentration determined using the method of Lowry *et al.* [13] with bovine serum albumin as the standard.

Electrophoresis

The electrophoresis was carried out using a vertical slab gel electrophoresis unit (Bangalore Genei Pvt Ltd). An 8% SDS-PAGE followed by Coomassie brilliant blue G250 staining was used to analyze the homogeneity of the purified enzyme.

Nondenaturing Gel and Activity Staining

A crude extract prepared from *n*-butanol-grown cells of *Enterobacter* sp. VKGH12 was applied to a nondenaturing gel prepared as described above, but without SDS. After the electrophoresis, the gel was incubated for 5 min in the dark with a reaction mixture containing 0.01% phenazine methosulfate (PMS), 0.02% nitroblue tetrazolium (NBT), and 0.13% NAD^+. Twenty mM of *n*-butanol was added to the same reaction mixture, which was then incubated with gentle rocking for another 20 min to develop the color. The reaction was stopped by rinsing the gel with water.

Molecular Mass Determination

The active fraction from Sephadex G-75 fractionations was used for gel elution. The preparative nondenaturing PAGE was carried out as described above. A strip of the gel was cut and stained for BDH activity, as described earlier. The BDH bands on the corresponding gel strip were cut out and crushed in a glass tissue homogenizer. The gels were extracted with 50 mM PPB (pH 7.0) overnight and removed by centrifugation. The supernatant was then used for molecular mass determination of the BDH using SDS-PAGE.

MS Analysis of BDH

The purified BDH was excised from the gel and digested with trypsin, as described by Shevchenko *et al.* [18]. The resulting peptide mixture was analyzed by MALDI-MS and ESI Q-TOF MS/MS using a 4700 Proteomic Analyzer (Applied Biosystems, U.S.A.). The protein was first identified by searching the NCBI nonredundant database using the MASCOT Peptide Mass Fingerprint software (Matrixscience, London). The MALDI mass spectra of the tryptic peptide mixtures were searched for among all entries in the database. The partial peptide sequences were compared with those of the proteins from a MASCOT or BLAST similarity search (http://www.ncbi.nlm.nih.gov/BLAST/) to obtain identifications with a higher confidence.

Substrate Specificity

The substrate specificity of the primary BDH was tested with C1-C8 alcohols, some secondary alcohols, and aromatic alcohols. The

activity obtained with *n*-butanol was considered as 100% and compared with that of the other alcohols.

Effect of pH and Temperature on BDH

The effect of pH on the BDH activity was determined using the purified enzyme with *n*-butanol as the substrate. The reaction mixture (1 ml) consisted of an appropriate dilution of the enzyme, 20 mM *n*-butanol, and different 50 mM buffers (Phosphate buffer pH 6.5 to 7.5, Tris-HCl buffer pH 8 to 8.8, and Glycine-NaOH buffer pH 8.6 to 10.0). The effect of temperature on the BDH activity was measured by incubating the reaction mixture at different temperatures (30 to 50°C). The enzyme activity was expressed as described previously.

Effect of Certain Metal Ions and Chemical Reagents on BDH

The effect of various inhibitors and activators on the BDH activity was determined using the purified enzyme. The assay mixture (1 ml) consisted of an appropriate dilution of the enzyme, a 50 mM Tris-HCl buffer (pH 8.8), 20 mM *n*-butanol, and different concentrations of activators/inhibitors/metal ions. The BDH activity was determined as described previously and expressed as a percentage of the activity of the enzyme under identical conditions in the absence of activators/inhibitors/metal ions.

RESULTS

Induction of 1-Butanol Dehydrogenase in *Enterobacter* sp. VKGH12

Cell-free extracts prepared from either glucose- or *n*-butanol-grown cells of *Enterobacter* sp. VKGH12 were examined for BDH activity under nondenaturing conditions using *n*-butanol as the substrate. A single distinct activity band was identified on the gel stained for BDH activity when *n*-butanol was used as the substrate (Fig. 1A). However, the glucose-grown cells did not exhibit any activity band when *n*-butanol was used as the substrate (data not shown). Therefore, these results suggested that *Enterobacter* sp. VKGH12 may contain one BDH for *n*-butanol oxidation, although the presence of more than one protein with a similar molecular mass cannot be ruled out. Furthermore, although most alcohol dehydrogenases (ADH) produced from microorganisms exhibit NAD^+-dependent ADH activity during growth on different alcohols [12, 14], no BDH activity was found when using artificial electron acceptors, like phenazine methosulfate (PMS), in place of NAD^+ and 2,6-dichlorophenolindophenol (DCPIP), indicating that the BDH from this strain required NAD^+ for activity and should be considered as an NAD^+-dependent BDH. The results also suggested that the NAD^+-dependent BDH was primarily responsible for *n*-butanol oxidation. In contrast, Alisa and Arp [1] reported that an enzyme responsible for *n*-butanol oxidation in butane-grown *Pseudomonas butanovora* was an NAD^+-independent primary ADH [1, 8]. It was previously shown that *Ralstonia eutropha* (formerly known as *Alcaligenes eutrophus*) strains could induce different

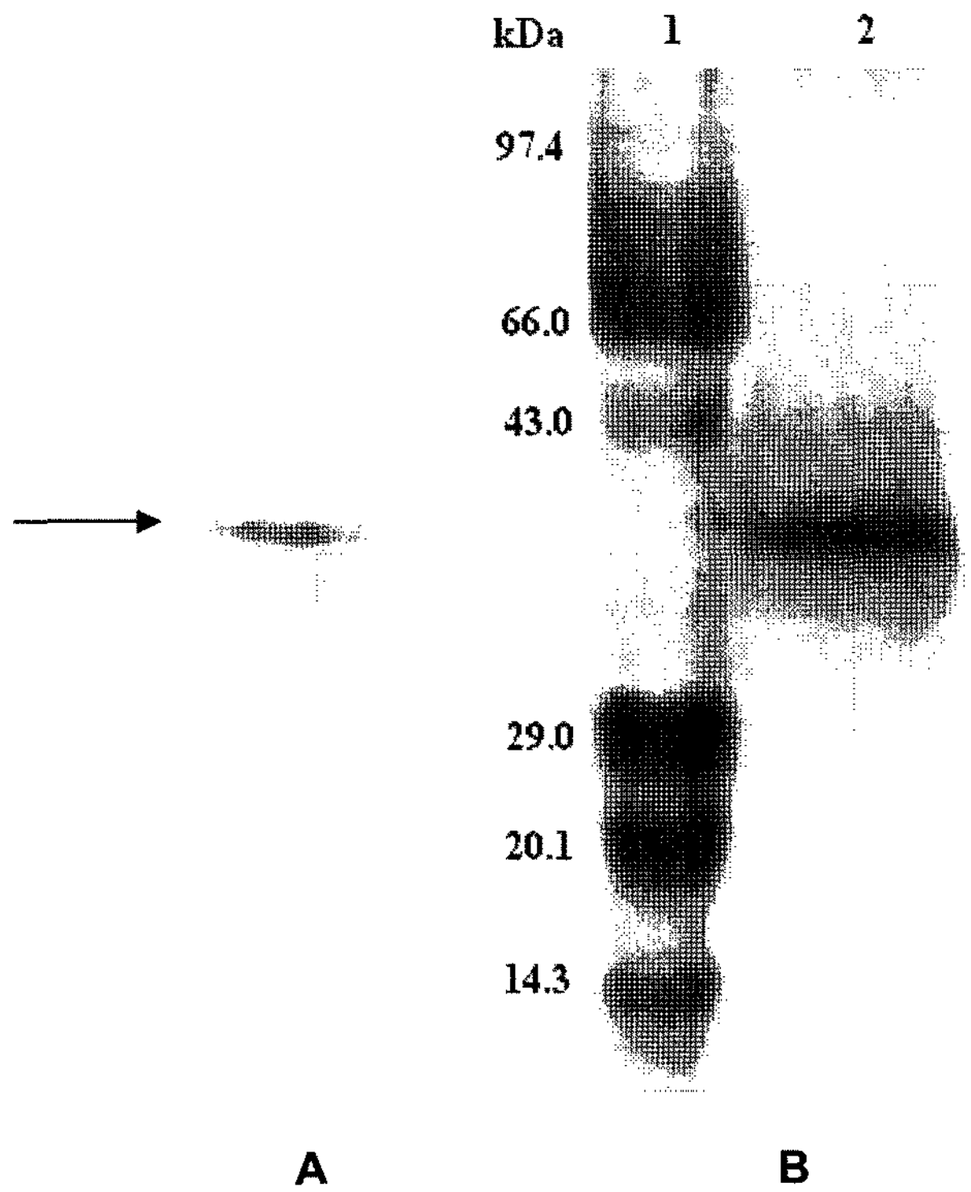

Fig. 1. A. An 8% Nondenaturing gel stained for BDH activity. The activity staining band corresponding to the BDH found in the crude extracts is indicated by an arrow. **B**. An 8% SDS-PAGE and Coomassie brilliant blue staining of BDH from *n*-butanol-grown *Enterobacter* sp. VKGH12 after purification steps.
Lanes: 1. Molecular mass markers; 2. Purified BDH.

NAD(P)-dependent ADHs, depending on the growth conditions [12, 14]. For example, under conditions of a restricted oxygen supply, the wild-type strains produced an unspecific NAD(P)-dependent ADH as a typical fermentation enzyme [22], whereas Madyastha and Gururaja [14] isolated a secondary ADH from a strain of *Alcaligenes eutrophus* that was unable to oxidize short-chain alcohols, like ethanol or isopropanol. However, *Enterobacter* sp. VKGH12 grown on different alcohols was still able to produce an NAD^+-dependent BDH.

Purification and Characterization of BDH

For the purification of the BDH, the *Enterobacter* sp. was grown in an MM2 medium supplemented with 0.8% *n*-butanol as the sole carbon and energy source. The BDH from the *n*-butanol-grown *Enterobacter* sp. was then purified from a cell-free extract (soluble fraction) to near homogeneity (Table 1). Many contaminating proteins were removed by 40–60% ammonium sulfate precipitation. The dialyzed sample was then subjected to Q-Sepharose column chromatography, and the active fractions from the Q-Sepharose column were used for Sephadex G-75 fractionation. Thereafter, the BDH was purified 15.37-fold with a recovery of only 10.51%, although the Sephadex G-

Table 1. Purification steps and specific activity of BDH from *n*-butanol-grown *Enterobacter* sp. VKGH12.

Purification step	Total protein activity (mg)	Total activity (U*)	Specific (U/mg)	Purification (-fold)	Recovery (%)
Crude supernatant	382	133	0.35	1	100
Ammonium sulfate precipitation (10–60%)	129	102	0.79	2.26	76.69
Q-Sepharose fractionation	16.3	53.14	3.26	9.31	40.0
Sephadex G-75 fractionation	2.6	13.98	5.38	15.37	10.51

*One unit (U) of enzyme activity is defined as the amount of enzyme catalyzing the conversion of 1 μmol of the substrate into a product per min under the standard assay conditions.

75 active fractions still contained a few contaminating proteins. Therefore, the active fractions from the Sephadex G-75 column were treated with gel elution, and the resulting sample was used for the molecular mass determination by SDS-PAGE and an MS analysis, where the molecular mass of the BDH was estimated to be 38 kDa (Fig. 1B). However, butane-grown "*Pseudomonas butanovora*" expressed two soluble alcohol dehydrogenases (ADHs), an NAD^+-dependent secondary ADH and NAD^+-independent primary ADH [1], where the latter was primarily responsible for *n*-butanol oxidation in the butane metabolic pathway. In this case, the BDH was synthesized as a monomer of approximately 66 kDa. ADHs from propane-grown *Rhodococcus rhodochrous* PNKb1, *Mycobacterium vaccae* JOB5, and *Pseudomonas fluorescens* NRRL B-1244 have also been purified and characterized as NAD^+-dependent secondary ADH(s) [5, 6, 9].

MS Characterization

The peptide mass fingerprint of the purified BDH was analyzed using a Mascot database search. Three peptides, *m/z* 1370.9, 1415.7, and 1523.8, were further analyzed with ESI Q-TOF MS/MS spectrometry and the amino acid sequence of peptides was found to be TTICGTDLHIIK, AAIESGVATLPYPK, and IVNVASTHGLVASAGK, respectively. The peptide with $[M+H]^+$=1,370.9 showed the highest identity (90%) with the putative alcohol dehydrogenase of *Streptococcus pneumoniae* R6 (gi 15459556) and *Staphylococcus epidermidis* RP62A (gi 57865543). The same peptide also showed an 88% identity with the alcohol dehydrogenase of *Staphylococcus haemolyticus* JCSC1435 (gi 68446247). The remaining two peptides did not exhibit any identity with alcohol dehydrogenases, but rather with dehydrogenases. For example, the peptide with $[M+H]^+$=1,415.7 showed a 72% identity with the malate dehydrogenase of *Pseudomonas syringae* pv. syringae B728a (gi 66043668), whereas the peptide with $[M+H]^+$=1,523.8 showed a 93% identity with the hydroxybutyrate dehydrogenase of *Pseudomonas aeruginosa* (gi 46911452). Therefore, these results indicated that the BDH purified from *Enterobacter* sp. VKGH12 has a unique structure compared with the alcohol dehydrogenases of other bacteria.

Kinetic Studies of Purified Butanol Dehydrogenase

The BDH obtained from the Sephedex G-75 column was also used to determine the kinetic constants. The kinetics of the purified BDH from *Enterobacter* sp. VKGH12 was studied with various concentrations of *n*-butanol. The apparent Michaelis-Menten constant (K_m) and V_{max} for the purified BDH from a double reciprocal plot were found to be 4 mM and 5.53 U/mg, respectively (Fig. 2). Earlier reports on the BDH from *Pseudomonas butanovora* showed the lowest K_m, 7 mM [1].

Substrate Specificity of *n*-Butanol Dehydrogenase

The BDH from *Enterobacter* sp. VKGH12 exhibited a broad substrate specificity towards various primary and secondary alcohols. The activities obtained for different substrates were compared with the activity obtained for *n*-butanol. In addition to *n*-butanol, the enzyme showed a relatively high activity with *n*-propanol among the tested primary alcohols (Table 2). The relative activities among the primary alcohols decreased with an increase in the chain length. Very little activity was detected with methanol as a substrate, which was consistent with the observation that *Enterobacter* sp. cannot grow on methanol as the sole carbon and energy source. Similar observations were also previously reported for the BDH purified from *Pseudomonas*

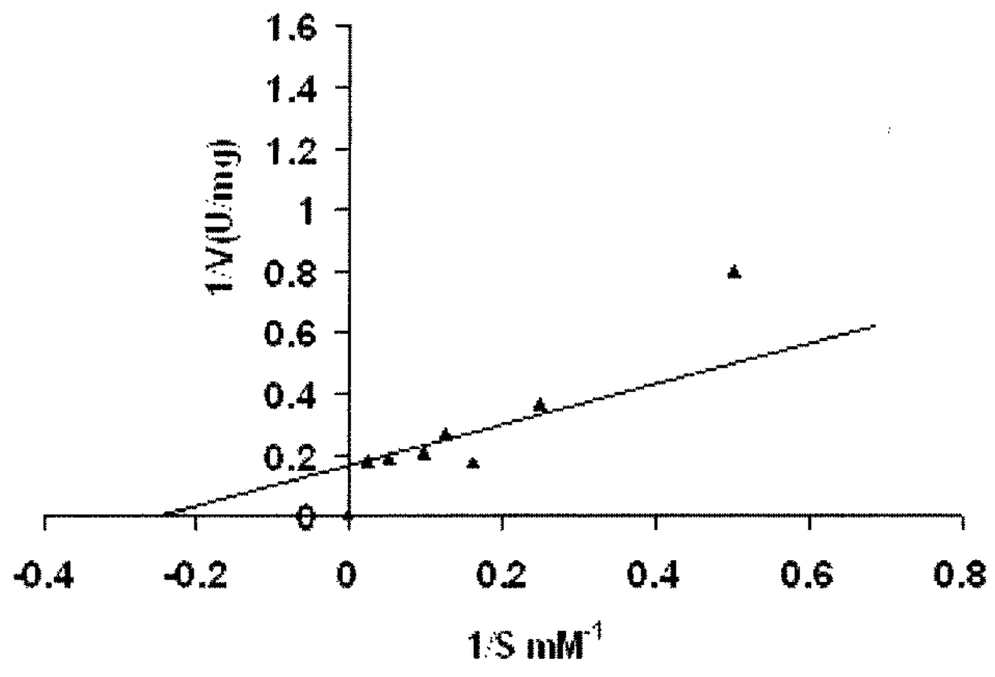

Fig. 2. Lineweaver-Burk plot of BDH activity using *n*-butanol as substrate.

The K_m and V_{max} for the BDH were 4.33 mM and 5.61 U/mg, respectively.

Table 2. Substrate specificity of purified BDH from *Enterobacter* sp. VHGK12.

Substrate	Relative activity (%)*
Primary alcohols	
Methanol	1.9
Ethanol	22.3
n-Propanol	130.0
n-Butanol	100.0
n-Pentanol	88.3
n-Octanol	12.1
Secondary alcohols	
Iso-propanol	202.0
Iso-butanol	16.0
Iso-pentanol	2.9
Aromatic alcohols	
Benzyl alcohol	13.8
Phenol	16.9

* BDH activity obtained with *n*-butanol as the substrate was considered as 100%.

butanovora [1]. Among the secondary alcohols tested, the BDH exhibited less preference towards iso-butanol, and the activity gradually decreased with long-chain secondary alcohols (data not shown). The BDH exhibited a distinct high activity against 2-propanol. Similar observations were also previously reported for methanol with the ADH from *Ralstonia eutropha*. Although methanol did not support the growth of *Ralstonia eutropha,* the purified ADH exhibited a distinct activity towards methanol [11]. In the present study, although the BDH purified from *n*-butanol-grown *Enterobacter* sp. VKGH12 was able to exhibit a high activity towards iso-propanol, the culture was unable to use iso-propanol as the sole carbon and energy source. Thus, it was presumed that the ADH purified from *Enterobacter* sp. VKGH12 was a BDH rather than an iso-propanol dehydrogenase. The activity of the BDH towards aromatic alcohols, such as benzyl alcohol and phenol, was relatively low.

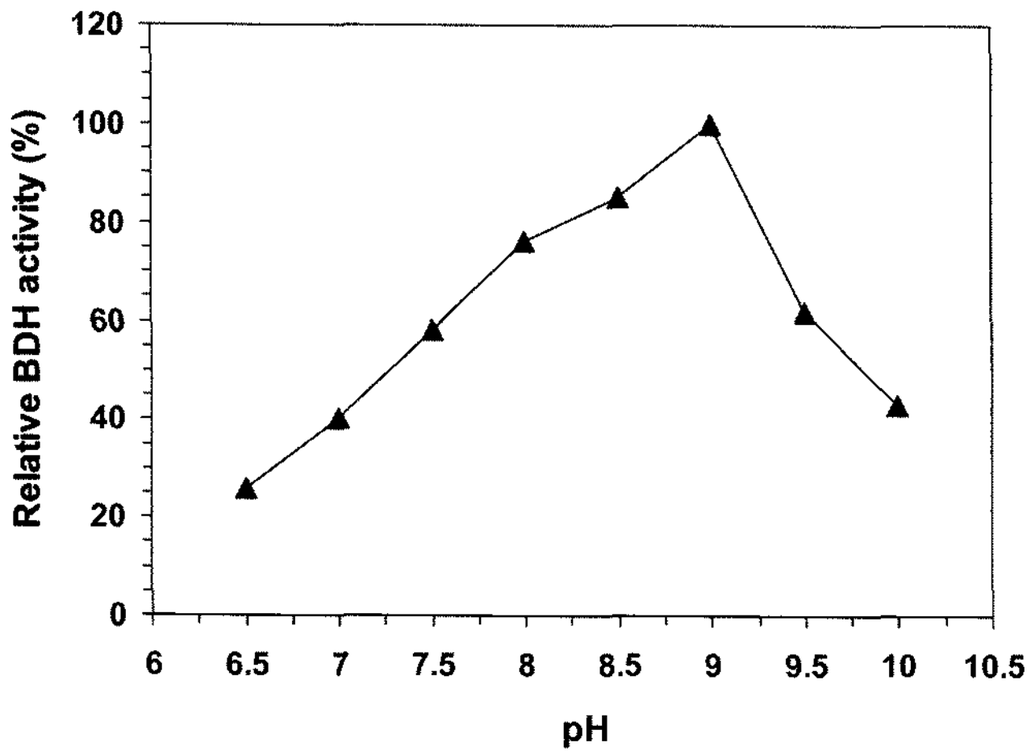

Fig. 3. Effect of pH on the activity of BDH from *Enterobacter* sp. VKGH12.
The BDH activity at pH 9.0 (5.33 U/mg) was considered as 100%. The buffers used in the assay are described in Materials and Methods.

Effect of pH and Temperature on Purified *n*-Butanol Dehydrogenase

The effect of pH and temperature on the purified BDH was investigated. The enzyme showed an optimal activity at pH 9.0 (Fig. 3). In contrast, the BDH purified from *Pseudomonas butanovora* exhibited an optimum pH of 8.0 [1]. Nonetheless, although the optimum activity of the BDH was found at pH 9.0, the BDH did not remain stable when stored at pH 9.0. Therefore, the crude extracts were made at pH 7.0 for all the experiments. The BDH exhibited an optimal activity at 40°C when tested at different temperatures from 25 to 50°C (Fig. 4), and retained about 55% of its original activity at 45°C. The enzyme was completely inactivated at 50°C when incubated for 5 min. The activity of the BDH remained stable for more than 10 days when stored in 50 mM PPB (pH 7.0) at 4°C, and had a half-life of 15 days. In contrast, the BDH purified from *Pseudomonas butanovora* exhibited an optimum activity at 60°C [1].

Effect of Metal Ions, Sulfhydryl, and Other Compounds on BDH Activity

The effect of different divalent cations and various chemical reagents on the activity of the purified BDH was studied (Table 3). Among the metal ions studied, the enzyme activity was inhibited by Cu^{2+}, Zn^{2+}, and Fe^{2+} at a 1 mM concentration, whereas Mg^{2+} and Mn^{2+} had no effect on the BDH activity. Reducing agents, like cysteine, β-mercaptoethanol, and dithiothreitol (DTT), enhanced the BDH activity 2-, 4-, and 6-fold, respectively. The activity of the BDH was not inhibited by EDTA, even at a 10 mM

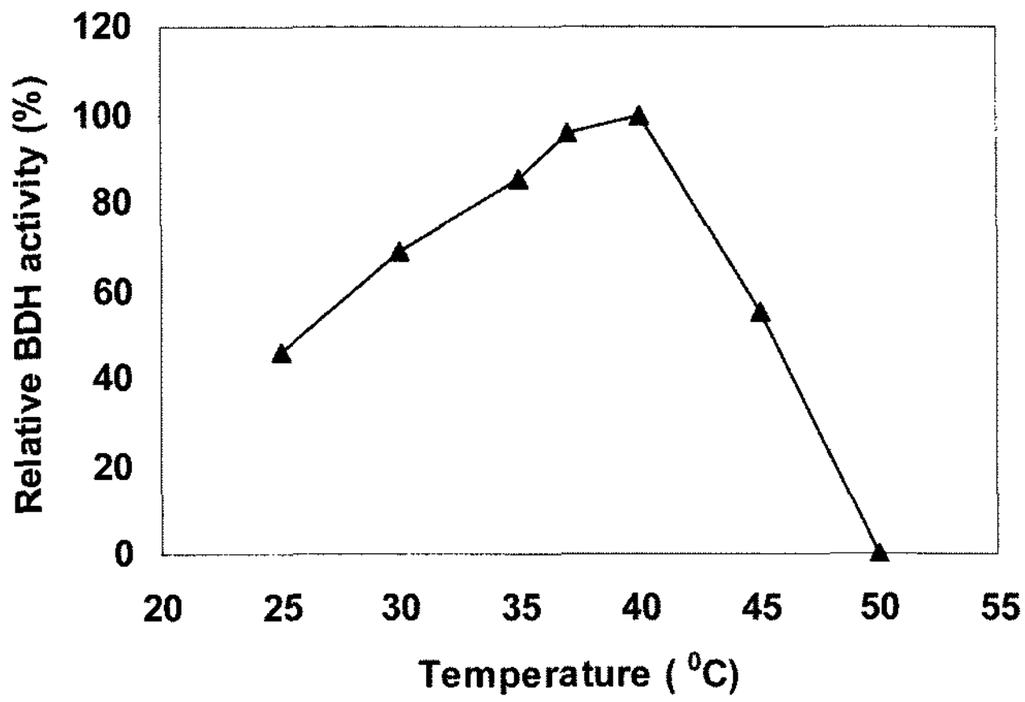

Fig. 4. Effect of temperature on the activity of BDH from *Enterobacter* sp. VKGH12.
The BDH activity at 40°C was considered as 100%.

Table 3. Effects of metal ions, sulfhydryl, and other compounds on BDH activity.

Compound	Concentration (mM)	Relative activity (%)*
Control (none)	–	100
$MgSO_4$	1	100
$MnSO_4$	1	96
$CuSO_4$	1	0
$ZnSO_4$	1	0
$FeSO_4$	1	0
Cysteine	10	230
β-Mercaptoethanol	10	409
Dithiothreitol	2	625
NH_4Cl	5	159
EDTA	10	96
PMSF	2	100
Sodium azide	1%	92

* BDH assay without activator/inhibitor was considered as 100%.

concentration, suggesting that the metal ion was not required for the catalytic property of the BDH. In addition, the known serine proteinase inhibitor, phenylmethylsulfonyl fluoride (PMSF), did not have any effect on the BDH activity, suggesting that serine was not involved in the catalytic mechanism of the BDH. However, the BDH activity was increased approximately 0.5-fold when ammonium ions (as 10 mM NH_4Cl) were present. Similar results were previously obtained for the BDH from *Pseudomonas butanovora* and tetrahydrofurfuryl ADH from *Ralstonia eutropha*, where the activity was increased 2.5-fold in the presence of ammonium sulfate [1, 11]. We have also tested BDH activity in the presence of common organic solvents, and no activity was found in the presence of 1% DMF or DMSO (data not shown).

Discussion

The metabolism of alcohols has already been studied in both Gram-negative and Gram-positive alkane- and alcohol-degrading bacteria, and these bacteria were shown to exhibit mainly two kinds of ADH for the efficient conversion of alcohol. In the first group, the ADHs use $NAD^+/NADP^+$ as the H^+ acceptor from the OH group in alcohol, and thus are called $NAD^+/NADP^+$-dependent ADHs. In the second group, the ADHs do not use $NAD^+/NADP^+$, but rather pyrroloquinoline quinone (PQQ) or a *c*-type cytochrome (cytochrome c_{QEDH} or cytochrome c_{550}) as the electron acceptor from alcohol, and thus are designated as $NAD^+/NADP^+$-independent ADHs [3, 4, 19]. In addition, $NAD^+/NADP^+$-independent ADHs have been further classified into three groups (types I, II, and III) on the basis of their molecular properties, catalytic properties, and localization [15]. NAD^+-dependent secondary ADHs have already been purified and characterized from propane-grown *Rhodococcus rhodochrous* PNKb1 and *Mycobacterium vaccae* JOB5 [5, 6, 9]. Moreover, in the present investigation, *Enterobacter* sp. VKGH12 was able to produce an NAD^+-dependent *n*-butanol dehydrogenase from *n*-butanol-grown cells. The BHD of *Enterobacter* sp. VKGH12 had an unusually high K_m for *n*-butanol (Fig. 2), which may reflect the solvent-tolerant ability of VKGH12. In contrast, butane-grown *Pseudomonas butanovora* has been shown to be able to express two distinct NAD^+-independent PQQ-containing *n*-butanol dehydrogenases, BOH (a quinoprotein) and BDH (a quinohemoprotein), where BOH is a 64-kDa type I quinoprotein located in the periplasm, whereas BDH is a soluble, periplasmic, type II quinohemoprotein that contains 1.0 mol of PQQ and 0.25 mol of heme *c* as prosthetic groups and exists as a monomer with an apparent molecular mass of 67 kDa [1, 2]. Furthermore, when *Pseudomonas putida* HK5 is grown on ethanol, *n*-butanol, and 1,2-propanediol, it produces three different quinoprotein ADHs: one type I ADH and two type II ADHs (ADH IIB and ADH IIG), respectively [22].

In *Rhodococcus rhodochrous* PNKb1, NAD^+-dependent ADH activities specific for either 1-propanol or iso-propanol have been demonstrated [6]. In the present study, although the *Enterobacter* sp. could not use iso-propanol as its sole carbon and energy source, the BDH from *n*-butanol-grown *Enterobacter* sp. VKGH12 exhibited a relatively high activity towards iso-propanol. One possible explanation is that the formation of acetone, a product of iso-propanol oxidation, may be toxic to *Enterobacter* sp. VKGH12. Multiple NAD^+- and $NADP^+$-dependent ADHs have also been reported from *Acinetobacter* sp. strain HO1-N, where ADH-A was required for growth on ethanol and short-chain alcohols, ADH-B was specified for mid-chain-length alcohols, and a hexadecanol dehydrogenase was induced specifically during growth on hexadecane and hexadecanol [21].

In conclusion, solvent-tolerant *Enterobacter* sp. VKGH12 was able to produce an NAD^+-dependent butanol dehydrogenase, the presence of which facilitated the survival of the bacteria under high butanol stress conditions. In addition to its physiological significance, other features of the enzyme, such as its activity at an alkaline pH and broad range of substrate specificity, including primary alcohols and secondary alcohols, make it attractive for application in the enzymatic conversion of alcohols.

Acknowledgment

One of the authors, Y.V., would like to thank Gulbarga University, Gulbarga, for providing a research fellowship.

REFERENCES

1. Alisa, V. S. and J. D Arp. 2001. An inducible 1-butanol dehydrogenase, a quinohaemoprotein, is involved in the oxidation of butane by *Pseudomonas butanovora*. *Microbiology* **147:** 745–756.
2. Alisa, V. S., J. D. Arp, and L. A. Sayavedra-Soto. 2002. Two distinct alcohol dehydrogenases participate in butane metabolism by *Pseudomonas butanovora*. *J. Bacteriol.* **184:** 1916–1924.
3. Anthony, C. 1993. Methanol dehydrogenase in Gram-negative bacteria, pp. 17–45. *In* V. L. Davidson (ed.), *Principles and Applications of Quinoproteins*. Marcel Dekker, Inc., New York, N.Y.
4. Anthony, C. 1998. The pyrroloquinoline quinone (PQQ)-containing quinoprotein dehydrogenases. *Biochem. Soc. Trans*. **26:** 413–417.
5. Ashraf, W. and J. C. Murrell. 1990. Purification and characterization of a NAD^+-dependent secondary alcohol dehydrogenase from propane-grown *Rhodococcus rhodochrous* PNKb1. *Arch. Microbiol*. **153:** 163–168.
6. Ashraf, W. and J. C. Murrell. 1992. Genetic, biochemical and immunological evidence for the involvement of two alcohol dehydrogenases in the metabolism of propane by *Rhodococcus rhodochrous* PNKb1. *Arch. Microbiol*. **157:** 488–492.
7. Beardmore-Gray, M. and C. Anthony. 1986. The oxidation of glucose by *Acinetobacter calcoaceticus*: Interaction of the quinoprotein glucose dehydrogenase with the electron transport chain. *J. Gen. Microbiol*. **132:** 1257–1268.
8. Beers, P. J. 1988. The diversity of alcohol dehydrogenases in *Pseudomonas butanovora* and their role in alkane metabolism. MSc thesis, University of Warwick.
9. Coleman, J. P. and J. J. Perry. 1985. Purification and characterization of the secondary alcohol dehydrogenase from propane-utilizing *Mycobacterium vaccae* strain JOB5. *J. Gen. Microbiol*. **131:** 2901–2907.
10. EPA. 1994. *Chemicals in the Environment: 1-Butanol*. Environmental Protection Agency, Office of Pollution Prevention and Toxics, Washington DC.
11. Grit, Z., T. Schraderr, and J. R. Andreesen. 1997. Degradation of tetrahydrofurfuryl alcohol by *Ralstonia eutropha* is initiated by an inducible pyrroloquinoline quinone-dependent alcohol dehydrogenase. *Appl. Microbiol. Biotechnol*. **63:** 4891–4898.
12. Jendrossek, D., N. Kruger, and A. Steinbuchel. 1990. Characterization of alcohol dehydrogenase genes of derepressible wild-type *Alcaligenes eutrophus* H16 and constitutive mutants. *J. Bacteriol*. **172:** 4844–4851.
13. Lowry, O. H., M. J. Rosebrough, A. Farr, and R. J. Randall. 1951. Protein measurement with folin phenol reagent. *J. Biol. Chem*. **193:** 265–275
14. Madyastha, K. M. and T. L. Gururaja. 1995. Purification and some properties of a novel secondary alcohol dehydrogenase from *Alcaligenes eutrophus*. *Biochem. Biophys. Res. Commun*. **211:** 540–546.
15. Matsushita, K., H. Toyama, and O. Adashi. 1994. Respiratory chains and bioenergetics of acetic acid bacteria. *Adv. Microbiol. Physiol*. **36:** 247–301.
16. Neumann, G., Y. Veeranagouda, T. B Karegoudar, O. Sahin, I. Mäusezahl, N. Kabelitz, U. Kappelmeyer, and H. J. Heipieper. 2005. Cells of *Pseudomonas* and *Enterobacter* sp. adapt to the presence of toxic organic compounds by increasing their cell size. *Extremophiles* **9:** 63–68.
17. Ramos, J. L., M. T. Gallegos, S. Marques, M. I. Ramos-Gonzalez, M. Espinosa-Urgel, and A. Segura. 2001. Responses of Gram-negative bacteria to certain environmental stresses. *Curr. Opin. Microbiol*. **4:** 166–171.
18. Shevchenko, A., M. Wilm, O. Vorm, and M. Mann. 1996. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Anal. Chem*. **68:** 850–858.
19. Shim, E. J., J. Sang-hoon, and K. Kwang-Hoon. 2003. Overexpression, purification, and biochemical characterization of the thermostable NAD-dependent alcohol dehydrogenase from *Bacillus stearothermophilus*. *J. Microbiol. Biotechnol*. **13:** 738–744.
20. Sikkema, J., B. J. De Bont, and B. Poolman. 1995. Mechanisms of membrane toxicity of hydrocarbons. *Microbiol. Rev*. **59:** 201–222.
21. Singer, M. E. and W. R. Finnerty. 1985. Alcohol dehydrogenase in *Acinetobacter* sp. strain H01-N: Role in hexadecane and hexadecanol metabolism. *J. Bacteriol*. **164:** 1017–1024.
22. Toyama, H. A., K. Fujii, E. Matsushita, M. Shinagawa, and A. O. Ameyama. 1995. Three distinct quinoprotein alcohol dehydrogenases are expressed when *Pseudomonas putida* is grown on different alcohols. *J. Bacteriol*. **177:** 2442–2450.
23. Veeranagouda, Y., T. B. Karegoudar, G. Neumann, and H. J. Heipieper. 2006. *Enterobacter* sp. VKGH12 growing with *n*-butanol as sole carbon source and cells to which the alcohol is added as pure toxin show great differences in their adaptive responses. *FEMS Microbiol. Lett*. **254:** 48–54.
24. Veeranagouda, Y., M. H. Vijaykumar, P. K. Neelakanteshwar, S. N. Anand, and T. B. Karegoudar. 2006. Degradation of 1-butanol by solvent tolerant *Enterobacter* sp. VKGH12. *Int. Biodeterior. Biodegradation* **57:** 186–189.