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Properties of a 2,3-Butanediol Dehydrogenase from *Taiwanofungus camphorata*

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2,3-Butanediol dehydrogenase (Bdh) plays important roles in reduction of acetoin to 2,3-butanediol, an important platform chemical with many industrial applications. Here, a *Tc*Bdh cDNA (1348 bp, GenBank accession JF896462) encoding a putative Bdh was cloned from *Taiwanofungus camphorata*. The deduced amino acid sequence is similar to the Bdhs from other species. A 3-D structural model of *Tc*Bdh has been constructed based on the known structure of *Pseudomonas putida* formaldehyde dehydrogenase (*Pp*Fdh, PDB code 1KOL). To characterize the *Tc*Bdh protein, the coding region was subcloned into an expression vector pYEX-S1 and transformed into *Saccharomyces cerevisiae*. The recombinant His6-tagged *Tc*Bdh was expressed and purified by Ni^{2+}-nitrilotriacetic acid Sepharose. The purified enzyme showed a single band of 49 kDa on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The Michaelis constant (K_M) value of the recombinant enzyme for acetoin was 8.5 mM. The enzyme's optical pH was 6. The thermal inactivation of the enzyme showed a half-life of 5.3 min at 45 °C.

Keywords: 2,3-Butanediol dehydrogenase; *Taiwanofungus camphorate*; Three-dimension structural model; *Saccharomyces cerevisiae*; Acetoin; 2,3-Butanediol.

INTRODUCTION

2,3-Butanediol (Bd), also known as 2,3-butylene glycol or 2,3-dihyroxybutane, is a very important chemical used in a variety of chemical feedstocks and liquid fuels.[1,2] Its chemical formula is CH_3-CH(OH)-CH(OH)-CH_3 with a molecular mass of 90.12 Da and a high boiling point of 180-184 ^oC and a low freezing point of -60 ^oC. It can be derived from natural resources such as agricultural waste (biomass) through bioconversion.[1,2,3] One of its well-known applications is the formation of methyl ethyl ketone, a liquid fuel additive, through dehydration, Bd can be converted to 1,3-butadiene, which is a substance used in the production of synthetic rubber.[4]

The enzyme 2,3-butanediol dehydrogenase (Bdh) catalyzes a reversible reaction between 2,3-butanediol and acetoin (CH_3-CO-CH(OH)-CH_3, known as acetyl methyl carbinol). The metabolic reactions resulting from the utilization of glucose by *Bacillus polymyxa* can be described by the following equations.[5] Both the forward reaction of acetoin to Bd and its reverse reaction can be catalyzed by Bdh depending on NADH or NAD^+, respectively.

$$\text{Glucose} \rightarrow \text{acetoin} + 2CO_2 + 2ATP + 2NADH$$
$$\text{Glucose} \rightarrow \text{butanediol} + 2CO_2 + 2ATP + NADH$$
$$\text{Acetoin} \leftrightarrow \text{Bd}$$

Taiwanofungus camphorata (T. Camphorata), a unique fungus to Taiwan, is a well-known traditional Chinese medicine used for treating cancer, food and drug intoxication, abdominal pain, and itchy skin.[6,7] Regular consumption of the mushroom is believed to enhance vitality and longevity. *T. camphorata* can be obtained as health supplements formulated from *T. camphorata* mass or from artificial cultivation (http://www.asian-bio.com/) at a high market value. Many studies were aimed to find the exact bioactive compounds of the mushroom.[6] We have established EST (expressed sequence tag) from fruiting bodies of *T. camphorata* and have been using it for gene discovery. Several redox enzymes have been cloned and characterized including a 1-Cys peroxiredoxin,[8] a cambialistic-superoxide dismutase,[9] a catalase,[10] a glutathione-dependent formaldehyde dehydrogenase,[11,12] a phospholipid hydroperoxide glutathione peroxidase,[13] a glutaredoxin,[14] a

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novel 2-Cys peroxiredoxin,[15] a thioredoxin reductase[16] and a nitroreductase.[17] Here we report the first cloning and expression of a Bdh from *T. camphorata*. The coding region of the cDNA was introduced into a *Saccharomyces cerevisiae* expression system. The functional target protein was expressed, and showed activity. The recombinant enzyme has been purified, and its properties studied.

EXPERIMENTAL

Total RNA preparation from *Taiwanofungus camphorata* and cDNA synthesis: Fruiting bodies of *T. camphorata,* a parasitic fungus that occurs in the inner cavity of an endemic tree *Cinnamonum kanehirai*, were obtained from Asian Nova Biotechnology Inc., Taiwan (http://www.asian-bio.com/). Fresh fruiting bodies (wet weight 10 g) were frozen in liquid nitrogen and ground to powder in a ceramic mortar. PolyA mRNA (30 μg) was prepared using Novagen's Straight A's mRNA Isolation System (Gibbstown, NJ, USA). Four micrograms of the mRNA were used in the 5'-RACE-Ready cDNA and 3'-RACE-Ready cDNA synthesis using Clontech's SMART RACE cDNA Amplification Kit (Mountain View, CA).

Isolation of Bdh cDNA: Using the *T. camphorata* 5'-RACE-Ready cDNA as a template and a degenerate primer *Tc*Bdh-1F (5'-GCG SDG GNC ARG GDA TTG-3') & a degenerate primer *Tc*Bdh-1R (5'-GGG GCA TNC ARG CSG CG-3'), a 0.6 kb fragment was amplified by PCR. The degenerate primers were designed based on the conserved sequences of Bdh from *As*Bdh (*Acinetobacter sp*. ADP1, YP_045734), *Bs*Bdh (*Bacillus subtilis subsp.* spizizenii ATCC 6633, ZP_06873103), *Sa*Bdh (*Staphylococcus aureus subsp.* aureus Mu50, NP_370650), *Bc*Bdh (*Bacillus clausii* KSM-K16, YP_174181). The 0.6 kb fragment was subcloned and sequenced. Based on this DNA sequence, a *Tc*Bdh-3F (5'-GCT TGA GAC GGA CGT CTC AG-3') and a *Tc*Bdh-3R (5'-CTG AGA CGT CCG TCT CAA GC-3') were synthesized. The primers allowed sequence extension from 5' end of the 0.6 kb fragment and extension from 3' end of the 0.6 kb fragment. Using the *T. camphorata* 5'-RACE-Ready cDNA as a template and UPM and *Tc*Bdh-3R primer pair, a 0.6 kb fragment was amplified by PCR. Using the *T. camphorata* 3'-RACE-Ready cDNA as a template and *Tc*Bdh-3F and UPM primer pair, a 0.7 kb fragment was amplified by PCR. Both 0.6 and 0.7 kb DNA fragment were subcloned into pCR4 vector and transformed into *Escherichia coli* TOPO10. The nucleotide sequences of these inserts were determined in both strands. Sequence analyses revealed that the combined sequences covered an open reading frame of a putative *Tc*Bdh cDNA (1348 bp, JF896462).

Bioinformatics analysis of *Tc*Bdh sequence: The identity of the *Tc*Bdh cDNA clone was verified by comparing the inferred amino acid sequence in various database using the basic local alignment search tool (BLAST) and DELTA BLAST.[18] Multiple alignments were constructed using ClustalW2 program. Protein secondary structure was predicted by SWISS-MODEL program and represented as α helices and β strands. A 3-D structural model of *Tc*Bdh has been constructed by SWISS-MODEL[19] (http://swissmodel.expasy.org/SWISS-MODEL.html) based on the known structure of *Pp*Fdh (*Pseudomonas putida* formaldehyde dehydrogenase, PDB ID: 1KOL[20]).

Subcloning of *Tc*Bdh cDNA into an expression vector: The coding region of the*Tc*Bdh cDNA was amplified using gene specific flanking primers. The 5' upstream primer contains *Eco*RI recognition site (5' GAA TTC G ATG AAG GCT CTC GCC TGG 3') and the 3' downstream primer contains *Not* I recognition site (5' GCG GCC GC GAC TGT CGC CCA TTC GTC 3'). Using 0.2 μg of 5'-RACE-ready cDNA as a template, and 10 pmole of each 5' upstream and 3' downstream primers, a 1.2 kb fragment was amplified by PCR. The fragment was ligated into pCR4 and transformed into *E. coli*. The recombinant plasmid was isolated and digested with *Eco*RI and *Not* I. The digestion products were separated on a 0.8% agarose gel. The 1.2 kb insert DNA was gel-purified and subcloned into *Eco*RI and *Not* I site of pET-20b(+) expression vector (Novagen). The recombinant DNA (pET-20b(+)-*Tc*Bdh) was then transformed into *E. coli* C43(DE3). The recombinant protein was over-expressed as inclusion body in the *E. coli* expression system. We decided to subclone the gene into a yeast expression system. The coding region of the *Tc*Bdh cDNA was re-amplified by using two gene-specific primers: the 5' upstream primer is contains *Eco*RI recognition site (5' GAA TTC G ATG AAG GCT CTC GCC TGG 3') whereas the 3' downstream primer contains a His6-tag and *Eco*RI recognition site (5' CGT CTC GAA TTC TCA GTG GTG GTG GTG GTG GTG 3'). Using the 0.2 μg recombinant DNA of pET-20b(+)-*Tc*Bdh as a template, and 10 pmol of each 5' upstream and 3' downstream primers, a 1.2 kb fragment was amplified by PCR. The fragment was ligated into pCR4 and transformed into *E. coli*. The recombinant plasmid was isolated and digested with *Eco*RI. The digestion products were separated on a 0.8% agarose gel. The 1.2 kb insert DNA was gel-purified and subcloned into *Eco*RI site of pYEX-S1 expression vector (Clontech) and introduced into *Saccharomyces cerevisiae* (trp^- ura^-). The transformed yeast cells were selected by YNBDT (0.17% yeast nitrogen base, 0.5% ammonium sulfate, and 2% glucose) agar plates containing 20 μg Trp/mL. The presence of *Tc*Bdh cDNA in the selected transformants was verified by PCR using gene specific flanking primers. The recombinant *Tc*Bdh protein was expressed in yeast in YPD medium (1% yeast

extract, 2% peptone, 2% glucose) containing 0.36 mM acetoin. The production of active recombinant *Tc*Bdh was shown by the enzyme assay.

Expression and purification of the recombinant *Tc*Bdh: The transformed yeast containing the *Tc*Bdh gene was grown at 30 °C in 250 mL of YPD medium containing 0.36 mM acetoin for 2 days. The cells were harvested and soluble proteins extracted in 40 mM Tris-HCl, pH 7.0, containing with glass beads as described before.[16] The recombinant *Tc*Bdh was purified by Ni-NTA affinity chromatography (elution buffer: 40 mM Tris-HCl, pH 7.0, containing 5-250 mM imidazole) according to the manufacture's instruction (Qiagen). The purified protein was checked by 12% SDS-PAGE. The purified protein was pooled and centrifuged to remove salt using Amicon membrane (5000 MW), finally the recombinant *Tc*Bdh (0.16 μg/μL) was in 20 mM Tris-HCl containing 2.5 mM imidazole, 45% glycerol. Proteins on gel were detected by staining with Coomassie Brilliant Blue R-250. Protein concentration was determined by a Protein Assay Kit (Bio-Rad, Richmond, CA) using bovine serum albumin as a standard.

Activity assay and kinetic studies of the recombinant *Tc*Bdh: Bdh activity was determined by measuring NADH oxidation.[21] The reaction mixture (100 μL) contained 33 mM potassium phosphate, pH 7.0, 0.2 mM NADH, 25 mM acetoin. The reaction was started by the addition of 2 μg (2 nM) *Tc*Bdh. The reaction was followed by the decrease in A_{340} due to the oxidation of NADH.

The kinetic properties of the *Tc*Bdh (2 μg) was determined by varying the concentrations of acetoin (7 to 25 mM) with fixed amount of 0.2 mM NADH. The change in absorbance at 340 nm was recorded between 10 sec and 40 sec. The molar absorption coefficient of NADH at 340 nm is 6.22 $mM^{-1}cm^{-1}$. The K_M, V_{max} and k_{cat} were calculated from Lineweaver-Burk plots.

Biochemical properties: The stability of *Tc*Bdh under various conditions was studied by assaying its ability to reduce acetoin as described above. Aliquots of the *Tc*Bdh sample were tested for: (1) *Thermal effect*. Enzyme sample (2 μg/24 μL enzyme in 20 mM Tris-buffer, pH 7.0, containing 5% glycerol per reaction) was heated to 45 °C for 2, 4, 8 or 16 min. (2) *pH effect*. Enzyme sample (2 μg/24 μL enzyme in 20 mM Tris-buffer, pH 7.0, containing 5% glycerol per reaction) was adjusted to desired pH by adding a half volume of buffer with different pHs: 0.2 M citrate buffer (pH 4.0), 0.2 M potassium phosphate buffer (pH 6.0, or 8.0) or 0.2 M CAPS buffer (pH 10.0). Each sample was incubated at 37 °C for 30 min. After each treatment, the residual Bdh activity was tested as described above.

RESULTS AND DISCUSSION

Cloning, characterization of a cDNA encoding *Tc*Bdh and a 3-D structural model of *Tc*Bdh

A putative *Tc*Bdh cDNA clone was identified based on its sequence homology to the published Bdhs in NCBI website. The coding region of *Tc*Bdh cDNA was 1224 bp that encodes a protein of 408 amino acid residues with a calculated molecular mass of 49.3 kDa (GenBank accession JF896462). Theoretical pI/Mw is 6.15/49300. A homology search using DELTA-BLAST detected putative conserved domains of medium chain dehydrogenase/reductase (MDR) superfamily. The conserved domains included a catalytic Zn^{2+} binding site at Cys^{38}, Ser^{40}, His^{60}, Asp^{168}, a structural Zn binding site at Cys^{90}, Cys^{93}, Cys^{96}, Cys^{104}, and a NAD^+ binding site at $Cys^{38}Gly^{39}Ser^{40}$, His^{43}, Phe^{86}, Asp^{168}, Thr^{172}, $Trp^{191}Gly^{192}$, $Gly^{194}Pro^{195}Ile^{196}$, $Ile^{215}Asp^{216}Arg^{217}$, Arg^{221}, Phe^{236}, $Cys^{260}Gly^{261}Thr^{262}$, Ile^{286}, $Ile^{303}Ala^{304}Val^{305}$, Gly^{329} $Gln^{330}Ala^{331}$, Phe^{372}. Fig. 1A shows the optimal alignment of the amino acid sequences of *Tc*Bdh with 4 related Bdh sequences from other sources. The *Tc*Bdh shares a 68% identity with R,R-butanediol dehydrogenase of *Sc*Bdh (*Saccharomyces cerevisiae S288c*, NP_009341), a 67% with R,R-butanediol dehydrogenase of *Cn*Bdh (*Cryptococcus neoformans var.* neoformans JEC21, XP_568483), a 66% with R,R-butanediol dehydrogenase of *Cg*Bdh (*Cryptococcus gattii* WM276, accession no. XP_003197328), and a 60% identity with *Cn*Bdh (*Cryptococcus neoformans var.* neoformans JEC21, XP_568483). The N-terminal end of the protein also contains GroES-like domain as identified by InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/). The secondary structure (Fig. 1A, represented as α helices and β strands) and a 3-D structural model (Fig. 1B, represented as solid ribbon) were predicted using SWISS-MODEL program. The 3-D structural model (Fig. 1B, light blue) was constructed based on the known crystal structure of *Pseudomonas putida* formaldehyde dehydrogenase (*Pp*Fdh, PDB ID: 1KOL, white). Red box (Fig. 1A, $Gly^{192}XGly^{194}XXGly^{197}$) indicates the highly conserved Gly-X-Gly-X-X-Gly sequence found in the MDR (medium chain dehydrogenase/reductase, Nordling et al. 2002[22]) family and residues found in the coenzyme-binding pocket. Red denotes structure of NAD^+ (Fig. 1B).

Expression and purification of the recombinant *Tc*Bdh

The coding region of *Tc*Bdh (1,224 bp) was amplified by PCR and subcloned into a yeast expression vector,

pYEX-S1 as described in the Materials and Methods. Positive clones were verified by DNA sequence analysis. The recombinant *Tc*Bdh protein was expressed and analyzed on a 12% SDS-PAGE in the absence a reducing agent and without boiling (Fig. 2). The recombinant *Tc*Bdh was expressed as a His6-tagged fusion protein and was purified by affinity chromatography with nickel chelating Sepharose. The purified *Tc*Bdh protein appears as a single band on SDS-PAGE with molecular mass of ~49 kDa (expected size of *Tc*Bdh) (Fig. 2, lanes 4-6). The Ni-NTA eluted fractions were pooled and characterized further. The yield of the purified His6-tagged *Tc*Bdh was 160 μg from 250 mL of culture. Functional *Tc*Bdh was detected by activity assay as describe below.

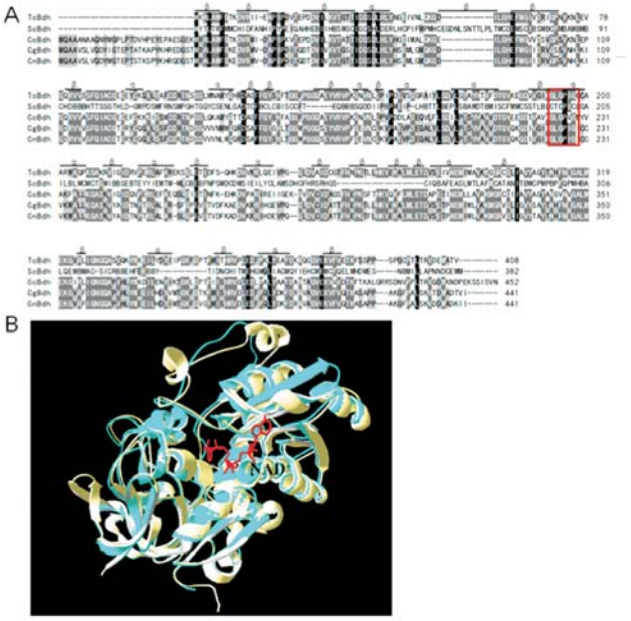

Fig. 1. Alignment of the amino acid sequences of recombinant *Tc*Bdh with other organism's Bdhs and 3-D structural model. (A) Sequence alignment: *Tc*Bdh (this study), ScBdh (*Saccharomyces cerevisiae S288c*, NP_009341), CcBdh (*Coprinopsis cinerea* okayama7#130), CgBdh (*Cryptococcus gattii* WM276), and CnBdh (*Cryptococcus neoformans var.* neoformans JEC21). Conservative replacements are shaded gray. Protein secondary structure was predicted by SWISS-MODEL program and predicted α helices and β strands are indicated. (B) A 3-D structural model of *Tc*Bdh was modeled based on the known X-ray structure of *Pseudomonas putida* formaldehyde dehydrogenase (*Pp*Fdh, PDB code 1KOL) via SWISS-MODEL program and was superimposed to obtain a better structure via SPDBV_4 program. Superimposition of *Tc*Bdh (light blue) and *Pp*Fdh (white) was shown using protein solid ribbons. Red box denotes NAD^+ binding site.

Kinetic studies of the recombinant *Tc*Bdh and Properties

The recombinant *Tc*Bdh was used to catalyze reduction of acetoin to 2,3-butanediol. Fig. 3 shows the acetoin consumption in the presence of NADH (0.2 mM) and the purified *Tc*Bdh (2 μg/0.1 mL). As shown in Fig. 3, the Lineweaver-Burk plot of the velocity (1/V) against 1/[acetoin] gave the K_M = 8.46 mM, *kcat* = 55.15 min^{-1}, k_{cat}/K_M = 6.52 min^{-1} mM^{-1}. We compared the K_M values of

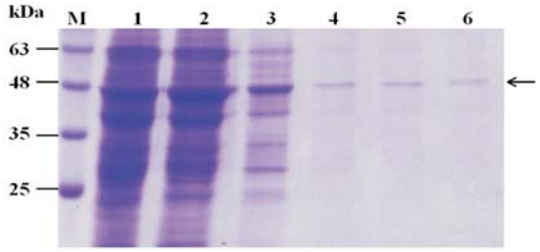

Fig. 2. Expression and purification of recombinant *Tc*Bdh from yeast. Fifteen μL (loading buffer without β-mercaptoethanol and without boiling) of each fraction was loaded into each lane of the 12% SDS-PAGE. Lane 1, crude extract from yeast expressing *Tc*Bdh; 2, flow-through proteins from the Ni-NTA column (2 mL); 3, washed from Ni-NTA column; 4-6, *Tc*Bdh (each fraction was 2 mL) eluted from Ni-NTA column. Molecular masses (in kDa) of standards are shown at left. Arrow indicated the target protein.

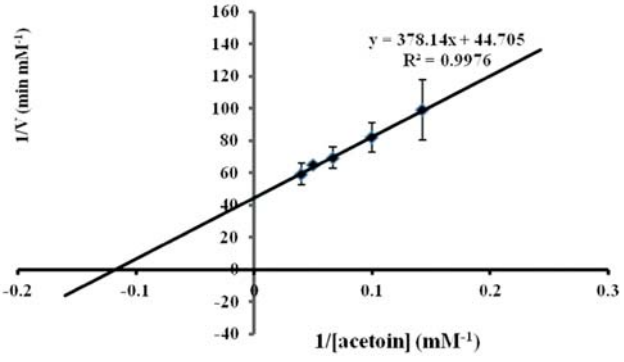

Fig. 3. Double-reciprocal plots of varying acetoin concentrations on *Tc*Bdh activity. The initial rate of the enzymatic reaction was measured at 0.2 mM NADH with the acetoin concentration varied from 7 to 25 mM. The K_M, V_{max}, and k_{cat} were calculated from the Lineweaver-Burk plots.

Table 1. Kinetic analyses of recombinant *Tc*Bdh and the other three Bdhs (from *S. Cerevisiae, K. pneumonia* and *E. aerogenes*). The kinetic parameters were determined as described in the Materials and Methods. The K_M value for acetoin was determined at 7–25 mM acetoin and 0.2 mM NADH. Data represent the mean (±SE) of three separate experiments

Species	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1} mM^{-1})	Ref.
T. camphorata	8.5	55.15	6.52	This study
S. cerevisiae	3.0 [a]	NA	NA	Ehsani *et al.* 2009[25,28]
K. pneumoniae	0.6 [b]	NA	NA	Zhang *et al.* 2012[26]
E. aerogenes	0.4 [c]	NA	NA	Carballo *et al.* 1991[27]

Values are from this work [*T. camphorata* (*Tc*Bdh)] or from the literatures. NA: not available.
[a] (Ehsani et al. 2009b),[25,28] [b] (Zhang et al. 2012),[26] [c] (Carballo et al. 1991).[27]

the *Tc*Bdh for acetoin with those of other organism Bdhs. As shown in Table 1, the K_M value is a little higher than other organism Bdhs. To examine the effect of temperature on the *Tc*Bdh activity, the enzyme was treated as described in the Materials and Methods and then analyzed for the residual Bdh activity. The *Tc*Bdh's half-life of inactivation at 45 °C was 5.3 min (Fig. 4). The *Tc*Bdh has an optimal activity for the reduction of acetoin at pH 6.0 (Fig. 5). It seemed consistent to report that production of R,R-2,3-butanediol may be promoted at pH 6.[23,24]

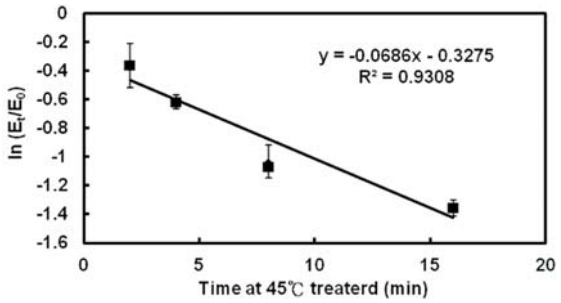

Fig. 4. Effect of temperature on the purified recombinant *Tc*Bdh. The enzyme sample was heated at 45 °C for various time intervals. Aliquots of the sample were taken at 0, 2, 4, 8 or 16 min and analyzed by SDS-PAGE (A) and assayed for Bdh activity. The thermal inactivation kinetics of *Tc*Bdh activity was plotted (B). E_0 and E_t are original activity and residual activity after being heated for different time intervals. Data are means of three experiments.

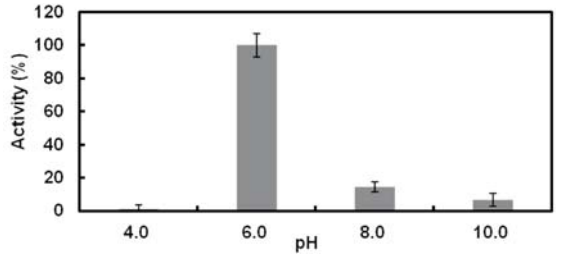

Fig. 5. Effect of pH on the purified recombinant *Tc*Bdh. The enzyme samples were incubated with different pH buffer at 37 °C for 30 min and then assayed for Bdh activity. Data are means of three experiments.

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