Pollution, 3(4): 613-622, Autumn 2017
DOI: 10.22059/POLL.2017.62777
Print ISSN: 2383-451X Online ISSN: 2383-4501
Web Page: https://jpoll.ut.ac.ir, Email: jpoll@ut.ac.ir

Biochemical characterization of recombinant benzyl alcohol dehydrogenase from *Rhodococcus ruber* UKMP-5M

Tavakoli, A.[1*] and Hamzah, A.[2]

1. Department of Nursing, Faculty of Nursing, Islamic Azad University, Eghlid Branch, Eghlid, Iran

2. School of Biosciences and Biotechnology, Faculty of Science and Technology, National University of Malaysia, Selangor, Malaysia

Received: 2 Apr. 2017 Accepted: 23 Jun. 2017

ABSTRACT: Benzyl Alcohol Dehydrogenase (BADH) is an important enzyme for hydrocarbon degradation, which can oxidize benzyl alcohols to aldehydes, while being capable of catalyzing a reversible reaction by reducing benzaldehyde. BADH is a member of medium chain alcohol dehydrogenases, in which zinc and NAD are essential for enzyme activity. This paper describes the expression, purification, and characterization of recombinant benzyl alcohol dehydrogenase, encoded by *xyl*B gene from *Rhodococcus ruber* UKMP-5M. The gene has been amplified and cloned into *E. coli*, and the recombinant plasmid pGEMT-xylB has been digested by *Nde*I and *Hind*III to construct plasmid pET28b-xylC and then ligated into *E. coli* BL21 (DE3), itself induced by 0.3 mM isopropyl *β-D*-thiogalactoside (IPTG) at 25°C. The expressed BADH has been 38 kDa, and is purified by affinity chromatography, in which the specific activity was 30 U/mg after 17 folds purification, leading to a NAD-dependent enzyme that uses benzyl alcohol as a substrate for enzyme characterization. The final metabolite is benzaldehyde, identified by gas chromatography mass spectrometry (GC-MS). The BADH activity has been 0.7 U/mL and the optimum pH and temperature, 9.5 and 30ºC, respectively. Also the Michaelis constant (K_m) and maximum velocity (V_{max}) have accounted to 705 µM and 1.3 U/mL, respectively. Benzyl alcohol dehydrogenase from *R. ruber* UKMP-5M can be used for hydrocarbon biodegradation in contaminated sites.

Keywords: alcohol dehydrogenase, expression, purification, recombinant protein.

INTRODUCTION

Alcohol Dehydrogenase (ADH) is a class of enzymes that catalyze the oxidation of alcohols to corresponding aldehydes or ketones. Its enzymes are determined in many prokaryotes and eukaryotes, playing an important role in physiological processes such as biodegradation and fermentation (Machielsen et al., 2006; Abbasian et al., 2016). Based on cofactor requirements and molecular structure, ADH is classified into three subfamilies: Type I, the medium-chain zinc-dependent ADH (370 amino acids), Type II, the short-chain zinc-independent ADHs (250 amino acids), and Type III, the long-chain iron-activated ADHs (385-900 amino acids) (Kulig et al., 2013; Wang et al., 2011). Benzyl Alcohol Dehydrogenase (BADH) is a zinc-containing medium chain alcohol dehydrogenase, encoded by *xyl*B gene (Raj et al., 2014). The enzyme belongs to

* Corresponding author Email: a_tavakoli@iaueghlid.ac.ir

oxidoreductase family that is able to catalyze the reversible oxidation of benzyl alcohol to benzaldehyde reduction (Shaw et al., 1992) with Nicotinamide Adenine Dinucleotide (NAD) essential as a cofactor for BADH activity (Shaw et al., 1993).

BADH is a key enzyme in xyl pathway for biodegradation of many aromatic hydrocarbon such as toluene and xylene (Ken et al., 2014; Ying et al., 2014). Several enzymes are involved in xyl pathway such as Benzyl Alcohol Dehydrogenase (BADH) and benzaldehyde dehydrogenase (BZDH) (Chalmers et al., 1991; Tavakoli & Hamzah, 2017). The present research identifies a benzyl alcohol dehydrogenase from *Rhodococcus ruber* UKMP-5M, which can degrade crude oil and toluene to less toxic compounds by xyl pathway (Hamzah et al., 2011). Benzyl alcohol dehydrogenase was determined in many bacteria such as *Pseudomonas* sp (Paliwal et al., 2014; Shrivastava et al., 2011), *Acinetobacter calcoaceticus* (Chalmers et al., 1990; Gillooly & Fewson, 1998), and *Yokenella sp* (Ying et al., 2014). All BADH enzymes are zinc-containing medium-chain alcohol dehydrogenase, dependent on NAD as a cofactor (Guzmán-Rondríguez & Santos, 2017; Wang et al., 2011). BADH from *A. calcoaceticus* and *P. putida* are quite similar to each other in terms of overall properties, but the BADH from *A. calcoaceticus* is encoded by chromosome, whereas BADH from *P. putida* is encoded by plasmid (Paliwal et al., 2014; MacKintosh & Fewson, 1988a). This study aims to determine a benzyl alcohol dehydrogenase from *Rhodococcus ruber* UKMP-5M. Subsequently cloning, expression, purification of recombinant protein, and kinetic characters have been carried out and as a final point the metabolites of BADH is determined.

MATERIALS AND METHODS

Rhodococcus ruber UKMP-5M was isolated from crude oil, contaminating the soil in Malaysia. Genomic DNA from *R. ruber* UKMP-5M was prepared, using Wizard genomic DNA purification kit (Promega). The extract was added to a 1% agarose gel to check DNA purity with its concentration and quantity being measured with spectrophotometer (Wpa Biowave II) (Sambrook & Russell, 2006).

PCR amplification was performed in an automated thermal cycler (Bio-rad). The PCR primers xylB (Forward and Reverse) were designed, based on genome sequence from *R. ruber* UKMP-5M and synthesized by First Base Company (Malaysia).

Forward xylB:
5'CATATGATGACCGACCCGGTCGAG GCGATAG3´

Reverse xylB:
5'AAGCTTTCAGCTGCCCCGGCGGAT CCACTCCT3´

The PCR reaction mixture contained distilled water 12.45 µL, Taq polymerase 5.0 µL, $MgCl_2$ 2.0 µL, dNTP 1.5 µL, Forward and Reverse primers (250 pmol) each one 1.0 µL, DMSO 0.3 µL, Template DNA 1.5 µL, and Taq polymerase (Promega) 0.25 µL. The total volume was 25 µL. The PCR program consisted of initial denaturation step for 5 min at 94 °C, followed by 35 cycle denaturation for 1 min at 94 °C, annealing for 1 min at 64 °C by 35 cycle, extension for 1 min at 72 °C by 35 cycle, and at last final extension for 10 min at 72 °C. The amplified product with the expected size was separated on 1% agarose gel and purified afterwards from the gel, using QIA Miniprep gel extraction kit (Qiagen). The purified product was ligated into pGEM-T Easy vector (Promega), using T4 DNA ligase for 16 hours incubation at 4 °C and the ligation product was transformed into competent cells of *Escherichia coli* DH5α, using heat shock method at 42 °C. Transformed colonies, carrying *xyl*B gene, were screened by culture on LB agar, supplemented with 50 µg/mL ampicillin (Sigma), 50 µg/mL 5-bromo-4 chloro-3-indoyl-β-*D*-Galactopyranoside (X-Gal)

(Promega), and 100 mM isopropyl β-*D*-1-thiogalacto pyranoside (IPTG) (Sigma). Several white colonies were selected for PCR colony analysis after an overnight incubation at 37 °C. The recombinant plasmid pGEMT-xylB was extracted by QIA Miniprep kit (Promega) from the positive transformants with the size of the extract estimated by agarose gel electrophoresis, using supercoil ladder as standard. The DNA purity and concentration were determined prior to sequencing. The presence of insert DNA in the isolated plasmids were confirmed by PCR with M13 primers and specific primers xylB, which sequenced from both directions in First Base Company (Malaysia) and the sequence data were analyzed, using Vect Screen and BLAST software.

The xylB fragment and pET-28b expression vector (Novagen) were double-digested with *Nde*I and *Hind*III restriction endonucleases so that complementary sticky ends could be generated. The products were analyzed and separated by electrophoresis. The purified fragment xylB ligated into digested pET28b by means of T4 DNA ligase that make up the xylB fragment with C-terminal His-tagged. The reaction mixture contained 4.0 µL 5X ligation buffer, 7 µL of linearized pET 28b (150 ng), 7.5 µL purified insert (250 ng), 0.5 µL ATP, and 5U T4 DNA ligase. It was then incubated at different temperatures (14-16 °C) to stop the reaction after 14-17 hours. The recombinant plasmid pET28b-xylB was made and then transformed into the competent cell *E. coli* DH5α via heat shock method. The recombinant *E. coli*, containing xylB fragment (1.1 kb), was grown on LB agar plates with 50 µg/mL kanamycin (Sigma) and positive transformants, screened by PCR colony with specific primers xylB. The plasmid pET28b-xylB was extracted from transformed *E. coli*, and verified by PCR. In order to confirm the orientation and presence of the insert in proper frame, the recombinant plasmid pET28-xylB was sequenced from both directions with T7 and T7 PBR rev Bam and specific primers xylB with the data being analyzed by bioinformatics software programs, such as Clustal W and BLAST P and N.

The recombinant plasmid pET28-xylB was transformed into *E. coli* BL21 (DE3) as a protein expression host by heat shock. The recombinant cells were initially grown in 50 mL LB broth at 37 °C at 150 rpm. The overnight culture was centrifuged at 4000 rpm for 15 min and the pellet was washed and re-suspended with 50 mM phosphate buffer to give an optical density of ~0.5 at 550_{nm}. A pre-culture, using 10% of the standard inoculum, was diluted in Minimal salt medium (MSM) (Hamzah et al., 2011), which was supplemented with toluene or benzyl alcohol as inducer at the range of (0.05-1 mM). The culture was incubated at 30 °C, 150 rpm for three days and the optical density was measured at the wavelength of 550_{nm}. The control was run in parallel condition with *E. coli* BL 21 (DE3) without recombinant plasmid.

In order to express the recombinant benzyl alcohol dehydrogenase, a single colony of *E. coli* BL21 (DE3), containing *xyl*B gene, was inoculated into 10 mL LB broth, supplemented with kanamycin (50 µg/mL). It got incubated at 37 °C, being shaken at 250 rpm for 16 hours. The culture was centrifuged at 4000 rpm and the pellet was washed and diluted 5 folds (50 mL) to grow and reach an optical density 550 nm of ~0.5-0.7 prior to induction with 0.05-1 mM IPTG. Cells were pelleted 2, 4, 6, and 16 hours after induction at 25 °C and 37 °C. Un-induced expression (leaky expression) of BADH was also tried, aiming to slow down the expression process so that the recombinant protein would have enough time to fold. Subsequently, the cells were dissolved in 1-2 mL lysis buffer (300 mM NaCl and 50 mM NaH_2PO_4 pH 8.0) and then

centrifuged to separate the cells debris from the supernatant.

The BADH expression was analyzed with 12% sodium dodecyl sulphate-poly acryl amide gel that ran at 150 V for an hour (Sambrook & Russell, 2006). The presence of His-tag protein was demonstrated by western blotting when the protein was transferred from the gel onto a nitrocellulose membrane at a constant voltage of 15 V for 45 minutes, using Trans-Blot SD semi-dry electrophoretic transfer cell (Bio-rad). The target protein was determined after the reaction with a monoclonal antibody. After the small scale optimization, the expression of BADH was scaled up with the following conditions. Then, His-tag BADH from *R. ruber* UKMP-5M was overexpressed in *E. coli* BL21 in at least 3L of LB broth.

The cells grew to an optical density 550_{nm}~ 0.6, induced with 0.3 mM IPTG with the incubation, itself, continuing for 16 hours at 25 °C. The bacterial cells were harvested by centrifugation at 6000 rpm, 4 °C for 45 min. In general, the protein was expressed in the inclusion body so the cells were dissolved in 30 mL lysis buffer, supplemented with 1 µg/mL lysozyme and 0.1 mM phenyl methyl sulfonyl fluoride (PMSF) (Sigma) and the diluted cells got incubated in ice for 30 min. The cells were ruptured by sonicator (sonic-vibra cells) for six cycles of 20 sec functioning followed by 5 min intervals for a total duration of 30 min. The crude lysate was centrifuged at 12000 rpm for 60 min at 4 °C and the supernatant (soluble fraction) and pellet (inclusion bodies) were separated and stored at -20 °C. The crude lysate was filtered with filter membrane (0.22 µm) prior to purification, which took place by affinity chromatography, using nickel-nitriloacetic acid (Ni-NTA) column (i.d×h 0.7×2.5 cm) (GE Healthcare).

At the beginning of purification the machine and column were washed with buffer A, consisted of 300 mM NaCl, 50 mM NaH_2PO_4 and 20 mM imidazole (pH 8.0). Then the lysate was loaded onto the column that had been equilibrated with buffer A. BADH was eluted with buffer B (buffer A, supplemented with 400-420 mM imidazole), applied at the rate of 0.8 mL/min. The possible fractions containing BADH were collected and concentrated, using vivspin or Amicon (Millipore) protein column. The existence and purity of the desired protein on the collected fractions were verified by SDS-PAGE and further analyzed, using western blotting. Protein concentrations were determined by means of bicinchoninic acid (BCA) method.

To characterize the recombinant BADH, Nicotine amide adenine dinucleotide (β-NAD) was used as a cofactor that was converted to reduced form of NADH. The reaction was monitored by spectrophotometer at OD_{340nm} (Wang et al., 2011) with all assay measurements performed in triplicate.

BADH activity was assayed by NADH production. The reaction mixture contained 50 mM sodium pyrophosphate buffer 125.7 µL, zinc sulphate $ZnSO_4$ (10 mM) 4 µL, *β*-Nicotineamide dinucleotide phosphate (Sigma) (1 mM) 40 µL, and benzyl alcohol with a final concentration of 0.3 mM. The reaction was initiated by adding 30 µL purified benzyl alcohol dehydrogenase into the mixture with a final volume of 200 µL. The mixture was incubated at 25 °C. During the first 10 min (at 1 min interval) in linear range, the rate of increase at absorbance at 340 nm was recorded by spectrophotometer. The mixture without substrate was used as the blank. An extinction coefficient of 6.22 mM^{-1} cm^{-1} for NADH was used to calculate enzyme activity. One unit is the amount of enzyme that catalyzes the conversion of 1.0 µmol of substrate to the expected product per minute at the standard assay condition. The enzyme's specific-activity calculation formula is as follows:

$$\left(A_{340} \times X \times DF\right) / \left(6.22 \times V\right)$$

where A340 is the change in absorbance at 340 nm per; X, the final reaction volume; DF, the dilution factor; and V, the amount of BADH in the reaction system (Wang et al., 2011).

Optimal pH for enzyme was determined by measuring BADH activity under standard enzyme assay conditions at various pH levels, ranging from 6 to 11. The following 50 mM buffer systems of varying pH were used: K_2HPO_4 for pH between 6.0 and 8.0, Tris buffer for pH between 8.0 and 10, and $NaHCO_3$ for pH between 10 and 11. The reaction mixture was incubated for 3 min at 25C and OD_{340nm} was measured. The effect of temperature on the enzyme activity was determined at temperatures from 4.0 to 70 °C at standard assay condition. The reaction mixture got incubated at different temperature and the reaction was stopped after 3 minutes of incubation, with OD_{340nm} being measured with a spectrophotometer.

Apparent values of K_m (Michaelis constant) and maximum velocity (V_{max}) were calculated based on the velocity at varying concentrations of benzyl alcohol (0.05-3 mM), using Lineweaver Burke plot.

The end metabolite was identified via GC-MS. The benzyl alcohol dehydrogenase reaction was carried out for 5 min in the standard assay conditions and stopped after adding 300 µL of 0.1 M HC1, with the protein removed by vivspin 500 (Sartorius Germany). After 300 µL, diethyl ether was added then 10 µL was extracted from volatile phase, the aliquot of which was analyzed by GC-MS. The standard was prepared in the same manner, though without adding the substrate.

RESULTS AND DISCUSSION

Rhodococcus ruber was isolated from oil-contaminated soil, degraded into many hydrocarbons such as crude oil and toluene (Hamzah et al., 2011). This study purified and characterized the recombinant benzyl alcohol dehydrogenase.

BADH from *Rhodococcus ruber* UKMP-5M, encoded by *xyl*B gene, was amplified at 64°C so that the PCR product was 1.1 kb (Fig. 1A). This was ligated into the pGEM-T vector successfully and transformed into *E. coli* DH5α to extract recombinant plasmid pGEMT-xylB which was 4.2 kb (Fig. 1B). The insert fragment xylB was purified after double digestion, using *Nde*I and *Hind*III restriction enzymes (Fig. 1C) and the sequence accuracy was confirmed by sequencing data and PCR. The recombinant plasmid pET28b-xylB was formed (6.5 kb) and extracted from *E. coli*, consisting pET 28b (5.4 kb) and xylB fragment (1.1 kb) after 15 hours of incubation at 16 °C (Figure 1D). DNA sequencing and PCR amplification results verified the accurate product, constructed with 367 amino acid residues. The nucleotides sequence of xylB from *R. ruber* UKMP-5M was closely related to *Rhodococcus* member's specifically *R. opacus* B4. The amino acid analysis of BADH from *R. ruber* UKMP-5M showed the overall identity to other zinc-containing medium alcohol dehydrogenase bacteria, such as *Rhodococcus* and *Pseudomonas*. The amino acids alignment of BADH from *R. ruber* UKMP-5M showed all characteristics of medium-chain zinc-dependent alcohol dehydrogenases, including the structural and catalytic domains, as well as the binding sites for NAD. Benzyl alcohol dehydrogenases bind to metal ion (zinc) and NAD whose link is essential for enzyme activity (Shaw & Harayama, 1990; Ying et al., 2014).

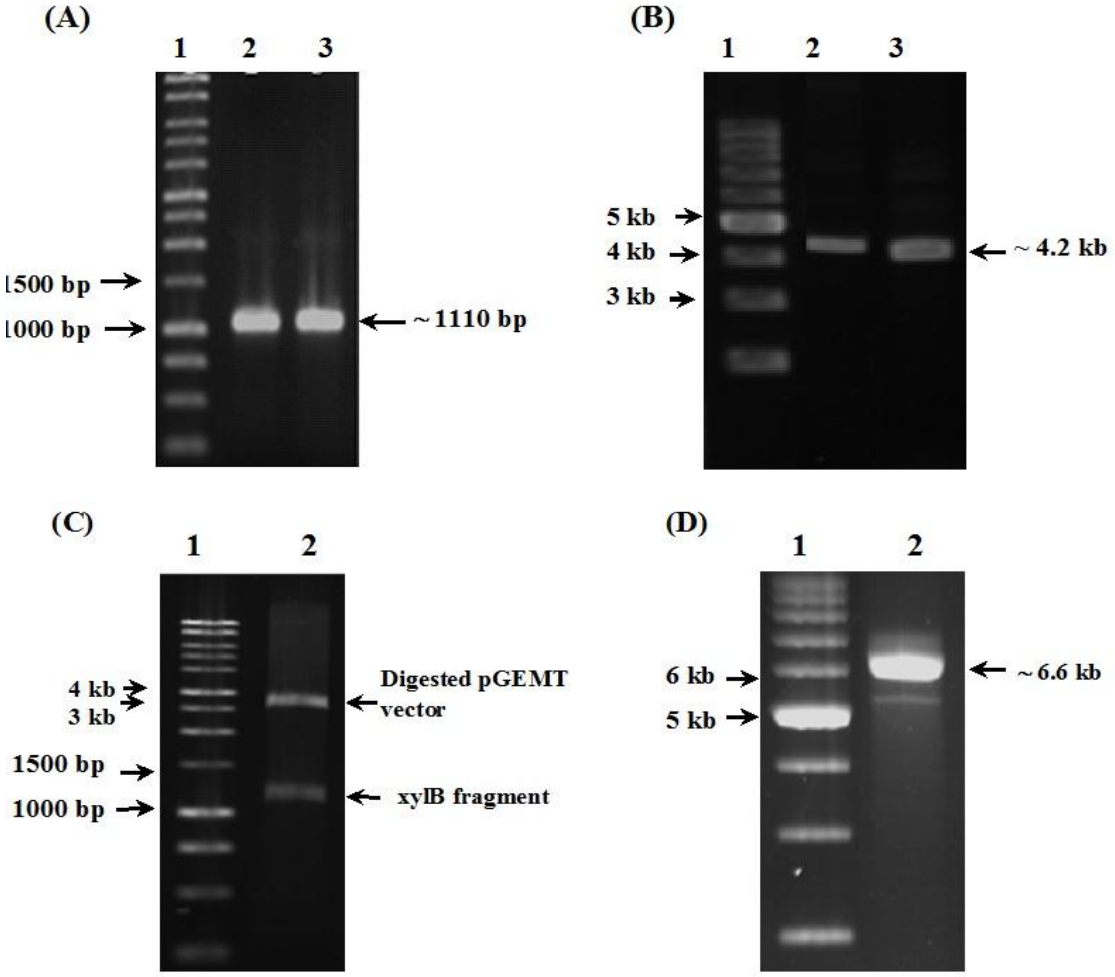

Fig. 1. Agarose gel electrophoresis analysis of *xyl*B gene; (A) DNA amplification; Lane 1: 1 kb DNA Ladder, Lane 2, 3: PCR product before and after DNA purification, (B) Analysis of plasmid pGEMT-xylB. Lane 1: DNA Super-coiled ladder, Lane 2-3: Extracted plasmid of pGEMT-xylB, (C) Double digestion of plasmid pGEMT-xylB. Lane 1: 1 kb DNA Ladder, Lane 2: DNA fragment xylB after being cleaved with *Nde*I and *Hind*III, (D) Analysis of plasmid pET 28b-xylB; Lane 1: DNA Supercoiled ladder, Lane 2: Extracted plasmid pET 28b-xylB

The maximum growth was determined when the cells got incubated in 0.1 mM benzyl alcohol for 24 hours, as compared to higher concentrations (0.5 and 1 mM) of benzyl alcohol. The recombinant His-tag BADH was expressed in *E. coli* BL21 (DE3) harboring pET28b-xylB after induction by 0.3 mM IPTG and 16 hours incubation. The temperature was important for BADH expression, with the protein being expressed both at 25 °C and 15 °C, while at 15 °C the product was low owing to little cell biomass. The overexpressed BADH was prone to aggregate, and the formation of inclusion bodies could be partly avoided by high sonication and lowering of the IPTG concentration as well as induction temperature. The estimated molecular BADH mass, using SDS-PAGE, was 38 kDa, also confirmed by western blotting (Fig. 2A, B). The molecular mass of purified BADH from *R. ruber* UKMP-5M (38 kDa) corresponded to other BADHs from *P. putida* CSV86 (Shrivastava et al., 2011), *A. calcoaceticus* (38.9) (Gillooly & Fewson, 1998), *Thermoplasma acidophilum* (36.2 kDa) (Guzmán-Rondríguez & Santos, 2017), *Yokenella* sp. Strain WZY002 (36.5 kDa) (Ying et al., 2014), and 42 kDa from *P.*

putida (Shaw & Harayama, 1990). The isoelectric point (pI) of BADH from *R. ruber* UKMP-5M turned out to be 5.15, determined by protein calculator software, similar to *A. calcoaceticus* (5.0) (MacKintosh & Fewson, 1988a).

The purified BADH was eluted with 27% elution buffer, containing 400-420 mM imidazole. A major band with molecular mass of ~38 kDa was detected in eluted fractions (11-12) by SDS-PAGE and western blot (Fig. 2C, D, Lanes 1, 2).

Addition of 0.5-1 mM dithiothreitol (DTT) or mercaptoethanol (ME) improved protein stability. Table 1 summarizes the results of BADH purification, using affinity chromatography.

The specific activity of purified BADH from *R. ruber* UKMP-5M rose to 30 U/mg with 87% yield after 17-fold purification that was very closed by BADH from *P. putida* CSV86 (32 U/mg, 94% yield) (Shrivastava et al., 2011). The reported specific activity for another *P. putida* was 214 U/mg with 22% yield (Shaw & Harayama, 1990).

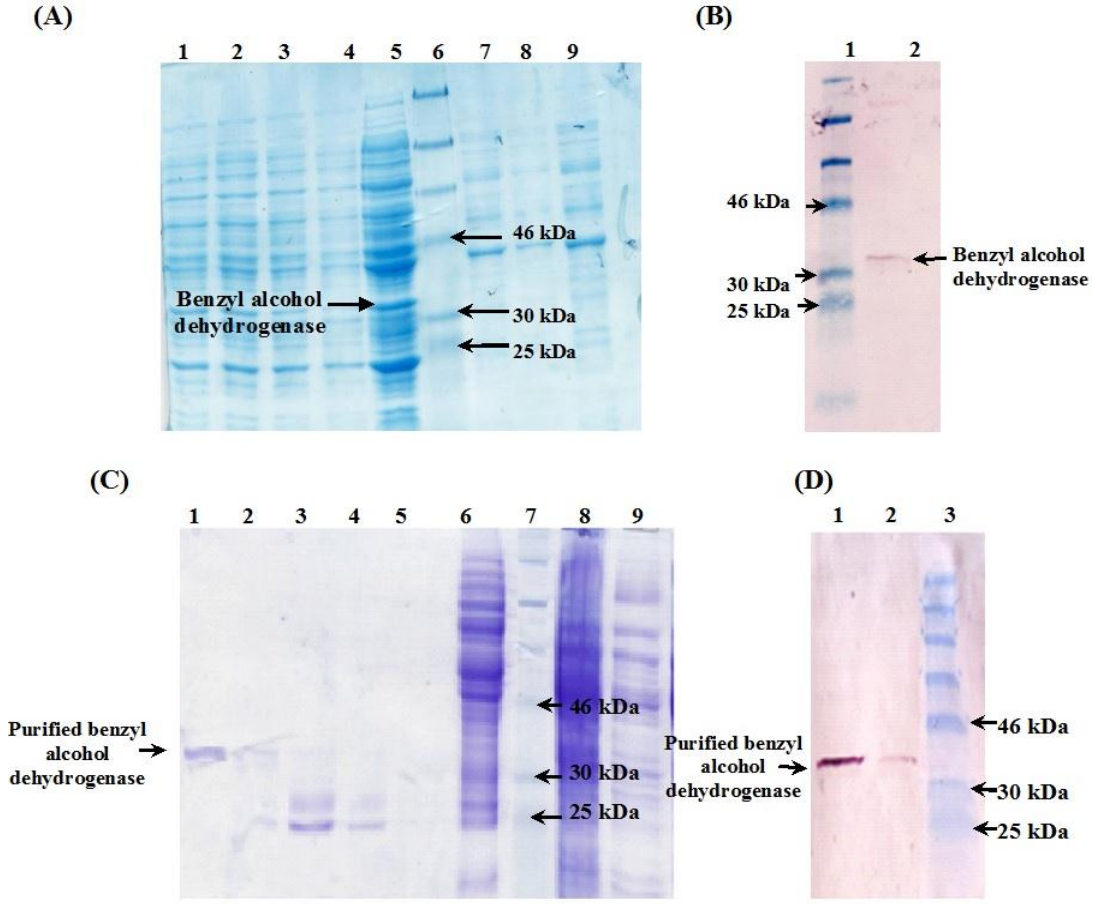

Fig. 2. Analysis of BADH before and after purification; (A) SDS-PAGE analysis after BADH expression: Lane 1: Non-induced sample (Negative control), Lanes 2-5: Cell free soluble extract (Supernatant) induced at 2, 4, 6, and 16 h, Lane 6: Molecular mass markers, Lanes 7-9: Insoluble extract (Pellet) induced at 4, 6, 16 h, (B) Western blot analysis; Lane 1: Molecular mass marker, Lane 2: Expression of benzyl alcohol dehydrogenase induced at 0.3 mM IPTG, 25ºC for 16 h, (C) SDS-PAGE analysis after BADH purification; Lanes 1-6: Bound fraction (elution step), Lane 7: Molecular mass marker, Lanes 8-9: Unspecific bound fraction (wash step). The purified protein was demonstrated in lanes 1-2, (D) Western blot analysis after BADH purification: Lanes 1-2: Purified protein that was determined in SDS-PAGE analysis (C) in lanes 1- 2, Lane 3: Molecular mass marker

Table 1. Purification of benzyl alcohol dehydrogenase from *R. ruber* UKMP-5M

Purification stage	Volume (mL)	Total activity (Unit)	Total protein (mg)	Specific activity (U/mg)	Yields (%)	Purification folds
Cell-free extract	30	931.4	515	1.80	100	1
Affinity chromatography	10	811.2	27.1	30	87	17

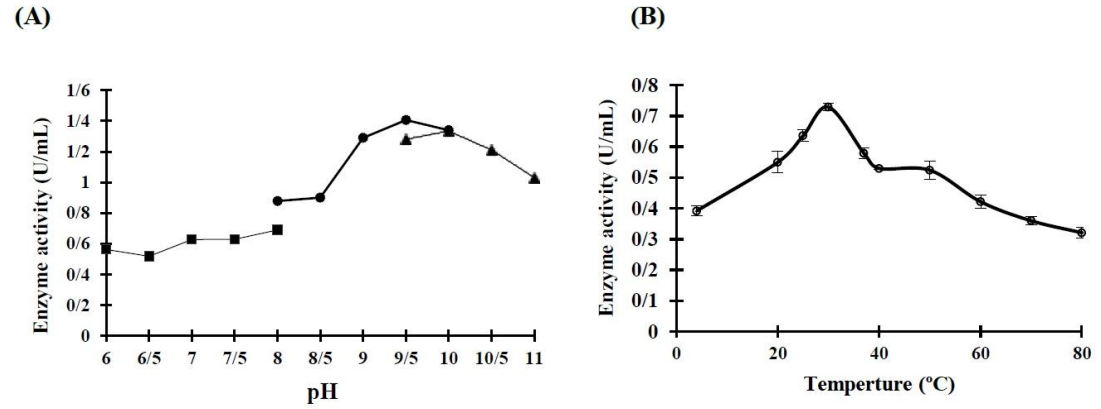

Fig. 3. Effect of physical parameters on BADH activity; (A) Effect of different pH on BDH activity. The following buffers were used: (■) 50 mM K2HPO4 (pH 6-8), (●) 50 mM Tris-buffer (pH 8-10), (▲) 50 mM NaHCO3 (pH 10-11). The incubation time was three minutes, (B) Effect of different temperature on BADH activity

The effect of time on BADH activity was determined by measuring the absorbance at 340 nm due to the formation of NADH. The highest activity, concerning the use of benzyl alcohol, was 0.7 U/mL after 9 minutes and 3.2 U/mL after 7. It was determined when toluene was used. The highest activity for BADH appeared at pH 9.5 in 50 mM Tris buffer (Fig. 3A). The optimum pH BADH by *R. ruber* UKMP-5M was a value close to other studies, being 9.0 for *P. putida* CSV86 (Shrivastava et al., 2011), 9.2 for *A. calcoaceticus* (MacKintosh & Fewson, 1988b), and 9.4 for *P. putida* (Shaw & Harayama, 1990) and *P. putida* MT53 (Chalmers et al., 1990). Since BADH is a NAD-dependent enzyme. Dependency on pH is an important factor for NAD-binding. In general, affinity for NAD^+ was increased when pH increased to 10 (Reid & Fewson, 1994). But the reversible reaction of BADH occurred during benzaldehyde reduction at pH below 7.0 (Shrivastava et al., 2011).

The optimal temperature value for benzyl alcohol oxidation by BADH was 30 °C (Fig. 3B). Enzyme activity decreased when the incubation temperature reached 50 °C with 30% of maximum activity decreased. The optimum temperature for BADH from *R. ruber* UKMP-5M was close to *Lactobacillus plantarum* WCFS1 at 30 °C (Landete et al., 2008). But the optimum activity BADH by *A. calcoaceticus* was at 40 °C (Uthoff & Steinbüchel, 2012).

The maximum velocity (V_{max}) and Michaelis constant (K_m) for the recombinant BADH from *R. ruber* UKMP-5M was 1.3 U/mL and 705 µM, respectively. The k_m value for BADH from *R. ruber* UKMP-5M was 705 µM, being higher than similar studies, i.e. 220-240 µM for *P. putida* (Shaw & Harayama, 1990), 62 µM for *P. putida* (Shrivastava et al., 2011), 19 µM for *A. calcoaceticus* (Gillooly et al., 1998), and 17.3 µM for *A. calcoaceticus* (Shaw & Harayama, 1990). The greater K_m value of BADH for *R. ruber* UKMP-5M than other

homologues BADH may indicate higher tolerance to substrate inhibition. The V_{max} for *R. ruber* UKMP-5M was 1.3 U/mL, being lower than reported V_{max} of 81 U/mg from *A. calcoaceticus* (Gillooly et al., 1998) and 231 U/mg from *P. putida* (MacKintosh & Fewson, 1988b). In *P. putida* CSV86, BADH activity decreased after the substrate concentration increased (Shrivastava et al., 2011) that may clarify the low BADH activity from *R. ruber* UKMP-5M.

BADH used benzyl alcohol, resulting in benzaldehyde at a retention time of 9 minutes, as determined by GC-MS. The product from benzyl alcohol oxidation by BADH from *R. ruber* UKMP-5M, *P. putida* CSV 86 (Shrivastava et al., 2011), and *P. putida* (Shaw & Harayama, 1990) was benzaldehyde, determined by GC-MS.

Benzyl alcohol dehydrogenase in different bacteria showed a similar function, although they differed with respect to one or more of the following features such as cofactor or substrate specificity, location within the cell, and genetic regulation (MacKintosh & Fewson, 1988b). The different substrate specifity in *R. ruber* UKMP-5M showed lower activity for benzyl alcohol than toluene as a substrate. Another report showed that the favored substrate for benzyl alcohol dehydrogenase did not necessarily correspond to higher BADH activity than other substrates (Shaw et al., 1992).

CONCLUSION

The protein homology analysis showed the recombinant BADH from *R. ruber* UKMP-5M, belonging to zinc dependent alcohol dehydrogenase. The experiment also confirmed Zn^{+2} dependency, with its results suggesting that BADH from *R. ruber* UKMP-5M was capable of being used for biodegradation in hydrocarbon-contaminated area, as this enzyme is a key enzyme for degradation of many aromatic hydrocarbons.

Acknowledgments

This work was supported by grant number STGL-003-2007 and 02-01-02-SF0408.

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