

Recombinant expression, characterization and application of a dihydrolipoamide dehydrogenase with diaphorase activity from *Bacillus sphaericus*

Anvarsadat Kianmehr^{1,2} · Rahman Mahdizadeh³ · Morteza Oladnabi⁴ · Javad Ansari⁵

Received: 2 November 2016 / Accepted: 2 February 2017 / Published online: 8 June 2017
© Springer-Verlag Berlin Heidelberg 2017

Abstract Diaphorases are flavin-containing enzymes with potential applications in biotransformation reactions, biosensor design and in vitro diagnostic tests. In this communication, we describe recombinant expression, characterization and application of a lipoamide dehydrogenase (DLD) with diaphorase activity from a strain of *Bacillus sphaericus*. The DLD gene consisting of 1413 bp encoding a protein of 470 amino acids was expressed in *Escherichia coli* BL21 (DE3) and the recombinant enzyme was characterized. *B. sphaericus* DLD catalyzed the reduction of NAD⁺ by dihydrolipoamide and exhibited NADH-dependent diaphorase activity. The molecular weight of purified enzyme was about 50 kDa, and determined to be a monomeric protein. Diaphorase was active and stable from pH 7.0 to 9.0 with an optimal activity at pH 8.5. It showed its maximal activity at temperature of 30 °C and was almost stable at temperatures between 25 and 30 °C. Different metal ions and inhibitors showed no influence on the activity of target enzyme. The K_m and V_{max} values for NADH were estimated to be 0.33 mM and

200.0 U/ml, respectively. Moreover, recombinant *B. sphaericus* diaphorase exhibited considerable potential to be used as a component of diagnostic tests for the quantification of metabolites. In conclusion, considering the properties of diaphorase from *B. sphaericus* PAD-91, it can have potential application as a diagnostic enzyme.

Keywords *Bacillus sphaericus* · Characterization · Lipoamide dehydrogenase (DLD) · Diaphorase

Introduction

Dihydrolipoamide dehydrogenase (DLD; EC: 1.8.1.4), also known as lipoamide dehydrogenase, is a FAD-dependent enzyme that catalyses the reversible oxidation of dihydrolipoamide to lipoamide (Vaubel et al. 2011). DLD is a flavoenzyme oxidoreductase that contains a reactive disulfide bridge and a FAD cofactor per subunit. It has been extensively isolated from a variety of organisms belonging to prokaryotes, eukaryotes and archaeobacteria (Serrano 1992; Benen et al. 1989). Some of DLD enzymes are also termed diaphorase for their NAD(P)H dehydrogenase activities (Madiagan and Mayhew 1993). Diaphorase activity may have an antioxidant role through its ability to scavenge nitric oxide and to reduce ubiquinone to ubiquinol (Xia et al. 2001). The first such enzyme has been purified from pig heart muscle (Rlyachko et al. 2006). Other enzymes have been identified from various sources such as *Clostridium kluyveri*, *Bacillus stearothermophilus*, *Thermus aquaticus* and *Thermus thermophilus* (Cakraborty et al. 2008; Antiochia et al. 1997). Diaphorases are a group of flavin-bound enzymes that catalyse the pyridine nucleotide-dependent reduction of electron-accepting molecules such as O₂, nitric oxide and ubiquinone

✉ Rahman Mahdizadeh
R.Mahdizadeh@iauba.ac.ir

¹ Biochemistry and Metabolic Disorders Research Center, Golestan University of Medical Sciences, Gorgan, Iran
² Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Golestan University of Medical Sciences, Gorgan, Iran
³ Bandar Abbas Branch, Department of Nursing, Islamic Azad University, Bandar Abbas, Iran
⁴ Gorgan Congenital Malformations Research Center, Golestan University of Medical Sciences, Gorgan, Iran
⁵ Cellular and Molecular Research Center, Qazvin University of Medical Sciences, Qazvin, Iran

(Tedeschi et al. 1995; Adams and Jia 2006; Yan et al. 2013). These enzymes have potential application to be used as a component of assay systems for the determination of different substances such as leucine, phenylalanine and ethanol. They have been used in biotransformation reactions (Bhushan et al. 2002), biosensor design (Antiochia et al. 1997) and in vitro diagnostic tests such as phenylketonuria (PKU) (Dilipkumar et al. 2013; Shahbazmohammadi and Omidinia 2011), maple syrup urine diseases (MSUD) and galactosemia as well (Kianmehr et al. 2016). Due to industrial applications of diaphorases, these enzymes have attracted researcher's attentions for screening of novel sources and cost-benefit production. In this literature, we report a strain of *B. sphaericus* which produced a DLD with NADH-dependent diaphorase activity and examined its potential application as a diagnostic enzyme.

Materials and methods

Chemicals

NAD⁺ was obtained from Sigma (St. Louis, MO, USA) and NADH was from Roche (Germany). Galactose and galactose-1-phosphate were obtained from Merck (Darmstadt, Germany). INT (2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride) was purchased from Sigma-Aldrich Corp (St. Louis, MO, USA). *Escherichia coli* BL21 (DE3) and pET-28b (+) were purchased from Novagen (Philadelphia, PA).

Screening of strains producing enzyme

One gram of each soil sample was suspended in selective liquid medium that contained (per liter) 0.5% lipoamide, 1 g NaCl, 2 g K₂HPO₄, 0.5 g MgSO₄·7H₂O, 5 g yeast extract, 5 g polypeptone in 1 l of tap water, pH 7.0 and then incubated with shaking at 37 °C at 180 rpm for 24 h (Shahbazmohammadi et al. 2007). From each culture, 0.05 ml was taken and placed on agar plates and incubated at 37 °C for 24 h. The strains that grew under such conditions were selected for enzyme production analysis. Production of enzyme was done in the above mentioned medium at 37 °C at 180 rpm for 24 h. After the completion of growth, the fermentation broth was centrifuged at 8000 rpm for 15 min at 4 °C. The harvested cells were washed twice with 0.9% NaCl solution, suspended in buffer A (50 mM NaH₂PO₄, 300 mM NaCl, pH 7.4) and disrupted by ultrasonic oscillator for 10 min. Cells and insoluble materials were removed by centrifugation at 10,000 rpm for 20 min at 4 °C. The supernatant solution was used as crude extract for DLD activity assay (Mashayekhi Mazar et al. 2012). DLD activity was

determined by the oxidation of dihydrolipoamide in the presence of NAD⁺ (Argyrou et al. 2003). The reaction mixture (1 ml) contained 100 mM potassium phosphate buffer (pH 7.8), 1.0 mM EDTA, 0.4 mM dihydrolipoamide and 0.3 mM NAD⁺. A solution containing all assay components except dihydrolipoamide was used as the blank. The assay was monitored by the increase in absorbance at 340 nm for 3 min. The isolates that showed DLD activity were selected for diaphorase assay. Diaphorase activity was measured using thiazolyl blue tetrazolium bromide (MTT) assay as a terminal electron acceptor. The standard reaction mixture was composed of 0.1 M potassium phosphate buffer (pH 7.6), 0.3 mM NADH, 0.4 mM MTT and the enzyme in a total volume of 0.7 ml. The increase in absorbance at 560 nm for 1 min was estimated and corrected for blank values lacking enzyme. One unit (U) of diaphorase activity was defined as the quantity of enzyme, which transfers electrons from 1 μmol of NADH to MTT per minute at 25 °C. All assay experiments were done in triplicate and the average results were used for data analysis (Boething and Weaver 1979).

Isolate identification

Identification of bacterium was done by 16S rRNA sequencing. Polymerase chain reaction (PCR) of 16S rRNA gene was performed with universal primer pair 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (AAGGAGGTGATCCAGCCGCA) (Ki et al. 2009). The PCR program was as follows: 95 °C for 5 min, 30 cycles of 95 °C for 1 min, 45 °C for 1 min, and 72 °C for 2 min and final extension at 72 °C for 10 min. DNA sequencing was performed by the commercial services of MacroGen Co. Ltd. (Seoul, Korea). The 16S rRNA genes of the isolate and *Bacillus* type strains were aligned using the Molecular Evolution Genetic Analysis (MEGA) program, version 5.0 (Tamura et al. 2011). Phylogenetic tree was constructed using the neighbor-joining algorithm using the MEGA version 5.0.

Expression and purification

To amplify the *DLD* gene, forward and reverse primers were designed based on the consensus sequences among the *Bacillus* species using DNASIS MAX software (DNASIS version 3.0, Hitachi Software Engineering Co., Ltd., Tokyo, Japan). The primers used were DLDFw (5'-CCCGGATCCATGGTAGTAGGAGA-3') and DLDRev (5'-CCCGTCTGACTTATTTTACAATGTG-3'), which contained the restriction sites for *Bam*HI and *Sal*I, respectively (underlined). PCR conditions were as follows: initial denaturation at 95 °C for 5 min, 30 cycles at 95 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min and a final

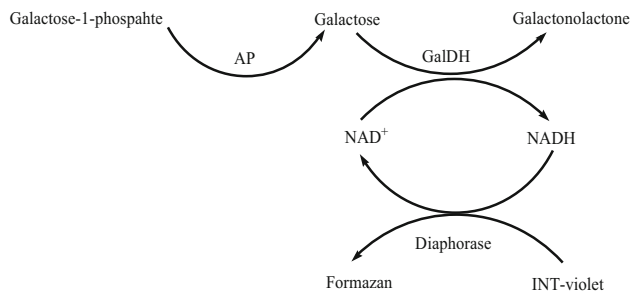


Fig. 1 Schematic representation of diaphorase role in galactose determination test

extension step at 72 °C for 7 min. The construct bearing the desired gene (pET28aDLD) was transformed into *E. coli* strain BL-21 (DE3) competent cells for expression. *E. coli* strain BL21 (DE3) cells bearing pET28aDLD were cultivated overnight in 100 ml of Luria–Bertani (LB) medium containing 40 µg/ml of kanamycin at 37 °C and 180 rpm. 100 ml of precultured medium was transferred into 1 l of LB medium in culture flasks and incubated at 37 °C and 250 rpm. When cell density reached an OD₆₀₀ of 0.6–0.8, DLD enzyme was expressed by the addition of 1 mM sterile isopropyl-β-D-thiogalactopyranoside (IPTG). After 5 h of induction at 37 °C, cells were harvested, dissolved in lysis buffer and disrupted by sonication. To purify the recombinant protein, supernatant of cell lysis was applied to a Ni–NTA affinity column (Qiagen, Germany) according to the manufacture’s instruction. The column was washed with 3 column volumes of the 50 mM Tris–HCl buffer (pH 7.0) containing 50 mM imidazole, and then recombinant enzyme was eluted with an elution buffer (50 mM Tris–HCl, 50 mM NaCl, 10 mM EDTA, 300 mM imidazole, pH 7.0). Purity of enzyme sample was studied by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed

using discontinuous gels (10 × 10 cm) with a 12% separating gel and a 6% stacking gel (Sambrook et al. 1994). Protein bands were visualized by staining with 0.25% Coomassie brilliant Blue R-250 in the mixture of 50% methanol and 10% acetate.

Biochemical characterization

Determination of molecular mass

The molecular mass of purified enzyme was determined by gel filtration on a Sephadex G-200 column gel filtration (1.6 × 60 cm), which had been equilibrated with buffer A. The column was equipped with a high-performance liquid chromatography (HPLC) system. The flow rate was maintained at 1 ml/min. Fractions of 2 ml each were collected and absorbance at 280 nm was recorded.

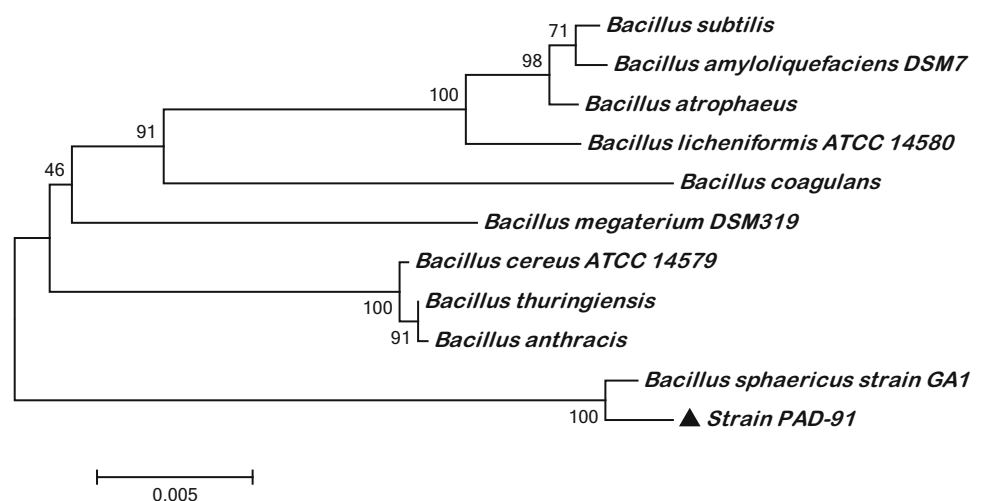
Effect of pH on the activity and stability

The effect of pH was examined by measuring the activity in the following buffers: 0.1 M sodium acetate (pH 3.0–5.0), 0.1 M potassium phosphate (pH 6.0–7.5), 0.1 M Tris–HCl (pH 8.0–9.0), 0.1 M glycine–NaOH (pH 9.0–11.0) and 0.1 M sodium carbonate (pH 11.5–12.0). The pH stability was studied by incubating the enzyme in the above mentioned buffers at a ratio of 1:1 at 4 °C for 24 h. Aliquots were withdrawn at time intervals of 4 h and the residual activity was determined. All experiments were done in triplicate and repeated at least twice.

Effect of temperature on the activity and stability

The effect of temperature on the enzymatic activity was analyzed by performing the enzyme assay for 20 min at

Fig. 2 Phylogenetic tree of the 16S rDNA sequences of strain PAD-91 associated with the other members of *Bacillus* species. The scale bar indicates 0.005 nucleotide substitution per position



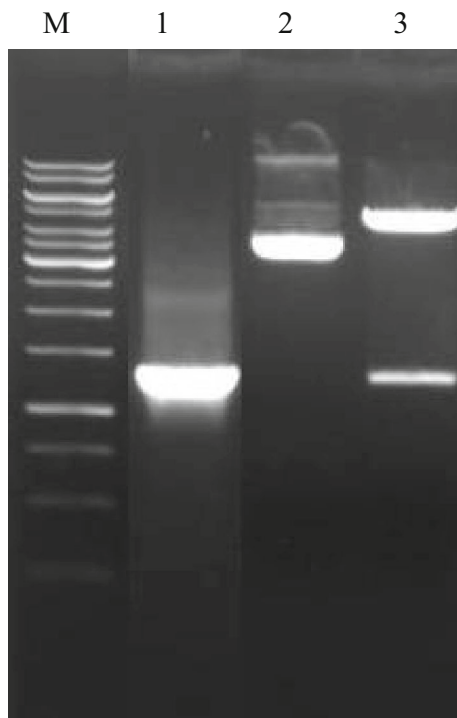


Fig. 3 PCR amplification of *DLD* gene and restriction analysis of pET28aDLD recombinant plasmid. Lane *M* DNA molecular weight marker, lane *1* PCR product, lane *2* undigested pET28aDLD, lane *3* digested pET28aDLD with *Bam*HI and *Sal*I

different temperatures ranging from 30 to 70 °C. The thermal stability was examined by incubating the enzyme at different temperatures ranging from 30 to 50 °C for 60 min and then cooling on ice-cold water. Residual activity was measured at every 10-min interval under standard assay conditions. The non-heated enzyme was used as control.

Effect of metal ions and inhibitors

The effect of various metal ions at concentration of 5 mM was studied by incubating the enzyme at 4 °C for 20 min. The influence of enzyme inhibitors such as ethylene-diaminetetraacetic acid (EDTA) and phenylmethylsulfonyl fluoride (PMSF) on diaphorase activity was examined in a final concentration of 5 mM. Enzyme was pre-incubated with inhibitors at 37 °C for 30 min in 0.1 M potassium phosphate (pH 7.6) and afterwards the residual activity was determined. The activity of the enzyme (without any additives) was taken as 100%.

Kinetic parameters

The kinetic constants (K_m and V_{max}) were determined using GraphPad Prism version 7.00, (GraphPad Software, La Jolla California USA) with varying concentrations of

NADH as a substrate. For the determination of K_{cat} and K_{cat}/K_m , the value of V_{max} was expressed in terms of U/ml.

Evaluation of enzyme application

To illustrate the effectiveness of target diaphorase as a diagnostic reagent, its application in a diagnostic assay, e.g., galactose quantification test was investigated. In this assay, galactose-1-phosphate is dephosphorylated to galactose by alkaline phosphates (AP), which activates a coupled redox cycle involving a galactose dehydrogenase (GalDH) and diaphorase for the production of formazan product (Shahbazmohammadi et al. 2015) (Fig. 1). In the assay procedure, 5-mm (diameter) dried blood spot (DBS) from calibrator specimens were punched into a 96-well microplate and left for 15 min at 95 °C in a bain-marie. Afterwards, 150 μ l of 0.1 M phosphate buffer saline (PBS) (pH 7.4) was added to each well and the plate was shaken for 60 min at 25 °C. The extracts were transferred to a microplate and the mixture of enzymes, coenzyme and INT reagent, was then added. To evaluate the role of *B. sphaericus* enzyme in improving of test performance, it was used in the second step in concentration of 4 U/ml. After 30 min of shaking, the absorbance was read bichromatically at 490/630 nm. The absorbance of a control sample (without diaphorase) was also read.

Results and discussion

Phylogenetic analysis of isolate PAD-91

To identify the enzyme producing microorganism, bacterial isolates were analyzed for *DLD* activity and NADH dehydrogenase ability. From these bacteria, only strain PAD-91 was found to have diaphorase activity. Strain identification was conducted by comparative sequence analysis of the 16S rDNA of isolate and other bacteria in the Genbank database. The 16S rRNA nucleotide was analyzed with the BLAST program and showed 97% homology with *B. sphaericus*. According to the created phylogenetic tree (Fig. 2), it was inferred that the PAD-91 strain was closely related to *B. sphaericus*.

Recombinant expression

The DNA fragment containing *DLD* gene was obtained by PCR with primers designed on the basis of conserved sequences from other *Bacillus* strains (Fig. 3). The 1413-bp open reading frame (ORF) had a coding capacity of 470 amino acids (Fig. 4). The DNA sequence showed 90.37 and 88.24% similarity to the gene encoding from *B. anthracis* and *B. subtilis*, respectively. Also, the deduced

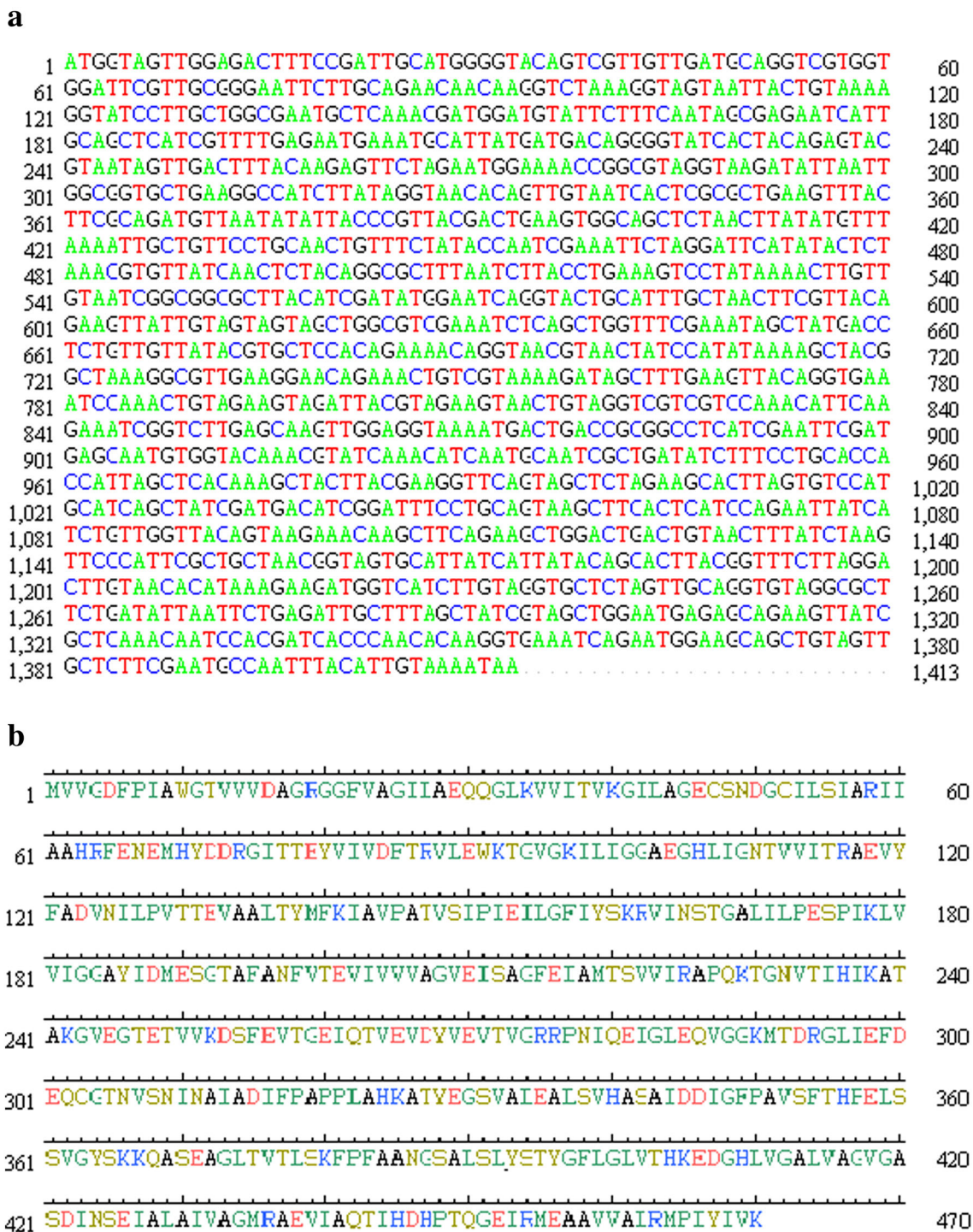


Fig. 4 Nucleotide (a) and protein (b) sequences of diaphorase from *B. sphaericus*. The numbers on the left and right are nucleotide accounts

amino acid sequence of *DLD* gene had 68.30 and 54.89% similarity with *B. anthracis* and *B. subtilis*, respectively. The restriction pattern of expression plasmid with *Bam*HI and *Sal*I confirmed gene cloning. An insert of 1413 bp along with a 3.6-kb vector band was observed after digestion (Fig. 3).

Enzymatic characterization

The native molecular mass of diaphorase enzyme was estimated to be about 50 kDa by gel filtration chromatography. The subunit structure was examined by SDS-PAGE. SDS-PAGE gel analysis showed a single band indicating

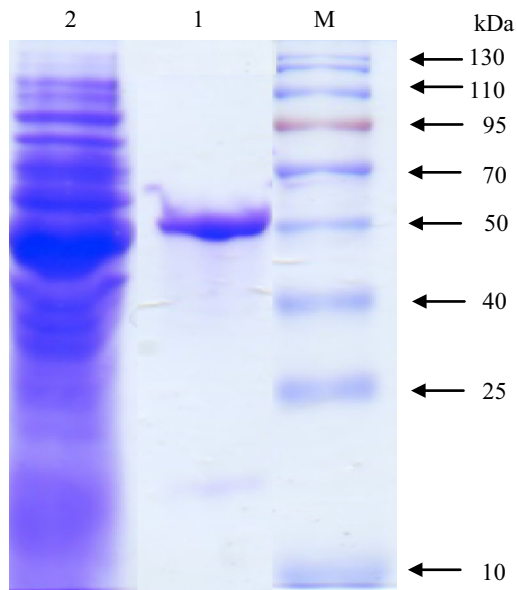


Fig. 5 SDS-PAGE analysis of the purified *B. sphaericus* recombinant enzyme. Lane M protein molecular weight marker, lane 1 purified enzyme, lane 2 whole cell lysate

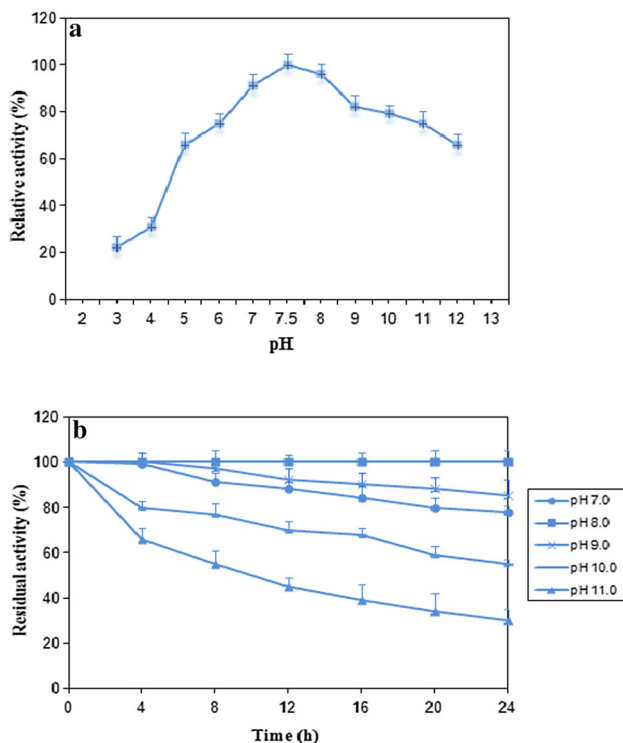


Fig. 6 Influence of pH on the activity (a) and stability (b) of diaphorase reaction. Each value represents mean \pm SD ($n = 3$)

that the enzyme was a monomeric protein (Fig. 5). This finding was in agreement with the previous reports in which many diaphorases have been shown to be monomers with MW values ranging from 50 to 55 kDa (Kaplan et al.

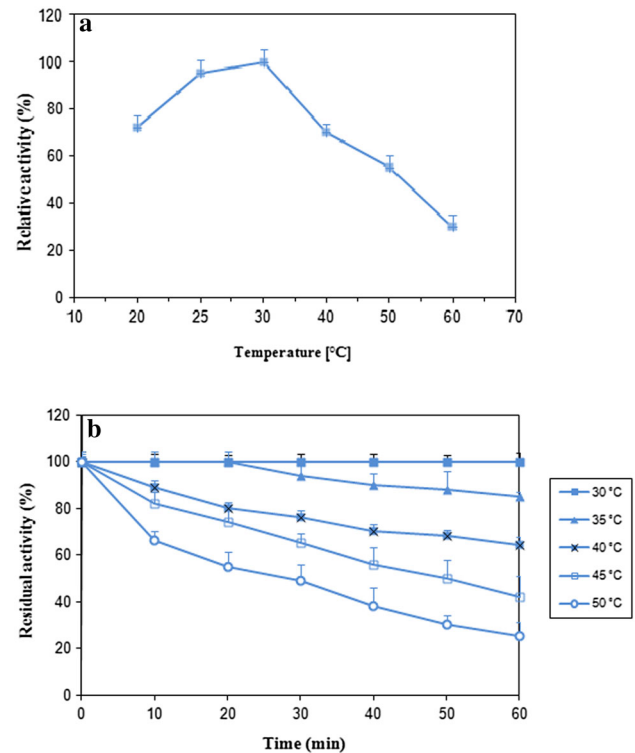


Fig. 7 a Influence of temperature and b time on the activity of diaphorase reaction. Each value represents mean \pm SD ($n = 3$)

1969). The effect of various pH values on the enzymatic reaction of diaphorase was evaluated in the pH range from 3.0 to 12.0 at 30 °C. The optimum pH of the diaphorase reaction was approximately 8.5 and showed high activity in a pH range of 8.0–9.0 (Fig. 6a). The pH stability was also tested by measuring residual activity after incubation at different pH values for 24 h. The enzyme was stable between pH 7.0 and pH 9.0, while at pH 10 it only retained 55% of its original activity during the same period incubation (Fig. 6b). The diaphorase reaction exhibited its maximal activity at 30 °C (Fig. 7a). As seen, a sharp decrease in enzyme activity was observed above 30 °C and was completely inactivated at 70 °C. For examination of the temperature effect on enzyme stability, the residual activity of diaphorase incubated at different temperatures (25–50 °C) for a period of 1 h was measured (Fig. 7b). The enzyme was almost stable at temperatures between 25 and 30 °C for 1 h, but lost 15, 36 and 58% of its initial activity after incubation for 1 h at 35, 40 and 45 °C, respectively. At 50 °C, target enzyme was completely inactivated after 50 min. The pH and temperature profiles of *B. sphaericus* diaphorase correlated with reports of other diaphorases from *C. kluyveri* (Cakraborty et al. 2008) and *B. subtilis* (Bhushan et al. 2002). The effect of metal ions and inhibitors was studied to determine which metal ions or inhibitors could enhance or reduce diaphorase activity. As seen

Table 1 Effect of metal ions and inhibitors on the activity of *B. sphaericus* diaphorase

Reagents	Relative activity (%)
None	100.0
Zn ²⁺	98.5 ± 3.7
Cu ²⁺	99.3 ± 1.5
Mg ²⁺	97.6 ± 1.8
Ca ²⁺	99.2 ± 2.1
Fe ²⁺	98.4 ± 3.2
Mn ²⁺	97.1 ± 2.7
Co ²⁺	99.6 ± 1.9
Ba ²⁺	100.2 ± 1.3
Hg ²⁺	97.7 ± 3.2
EDTA	96.7 ± 2.2
PMSF	97.4 ± 1.2

The enzyme activity without any reagents was taken as 100%. All metal ions and inhibitors were used at 5 mM. Data are given as mean ± SD, $n = 3$

Table 2 Kinetic parameters of *B. sphaericus* diaphorase

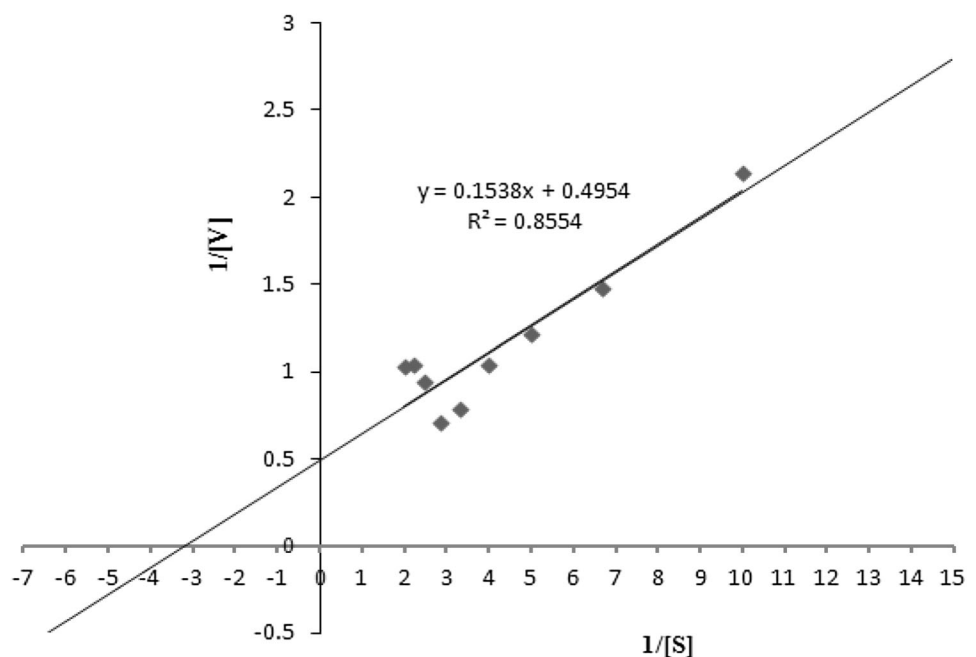
Substrate	Specific activity (U/mg)	K_m (mM)	V_{max} (U/ml)	K_{cat} (s ⁻¹)	K_{cat}/K_m (mM ⁻¹ s ⁻¹)
NADH	250.72	0.33	200.0	108.9	330.0

in Table 1, none of them enhanced or inhibited the enzyme activity. This was similar to the results achieved by other diaphorases (Dietrichs et al. 1990; Argyrou et al. 2003). Kinetic constants of the recombinant purified enzyme for

NADH substrate were determined. The results of kinetic parameters are depicted in Table 2. K_m and V_{max} values for NADH were estimated to be 0.33 mM and 200.0 U/ml, respectively (Fig. 8). The K_m value for NADH (0.33 mM) was lower than the other previously reported diaphorases such as *B. stearothersophilus* (0.7 mM) and *C. kluyveri* (0.38 mM) (Cakraborty et al. 2008). The calculated K_m of the recombinant enzyme indicated high affinity for the NADH substrate. Moreover, the turnover number of the enzyme (108.9 s⁻¹) was also higher than the other previously purified diaphorases. These kinetic features are very useful for the enzymological applications of *B. sphaericus* diaphorase.

The potential of *B. sphaericus* diaphorase as a diagnostic tool

Diaphorase enzymes are important analytical tools in clinical chemistry. They have the potential to be used as a component of in vitro diagnostic tests, e.g., diagnostic assays for PKU (Dilipkumar et al. 2013), MSUD and galactosemia (Shahbazmohammadi and Omidinia 2011). A diagnostic test in which the potential of desired enzyme was examined can be exemplified in the determination of galactose. As seen in Fig. 9, the regression equation for the two distinct reactions in the presence and without diaphorase enzyme was achieved as $Y = 0.0126x + 0.0296$ and, $Y = 0.0089x + 0.0331$, respectively. The correlation of coefficient (R^2) for the reaction with diaphorase was 0.997 in comparison with without diaphorase (0.912). The short analysis time was another advantage of using

Fig. 8 Lineweaver–Burk plot for recombinant *B. sphaericus* diaphorase

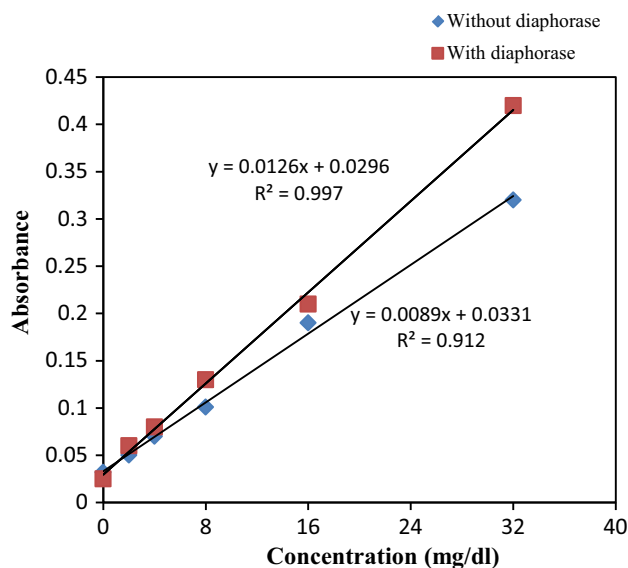


Fig. 9 Calibration curve for comparison of galactose concentration measurement with and without diaphorase. Experiments for each concentration were performed in triplicate run

diaphorase enzyme. Time required for assay in the presence diaphorase was 20 min which was favorably comparable than independent-diaphorase method (40 min). Resuming, the obtained results by diaphorase was that regression parameters of test was improved and assay time was decreased. These effects seemed to be related to the ability of target enzyme in electron transfer from NADH to electron acceptor (INT) as well as regeneration of NADH (Fig. 1) which leads to enhance of reaction velocity and better linearity (Gunther and Simon 1987). Similar results have also been found by *C. kluyveri* diaphorase (Cakraborty et al. 2008). Further examples where diaphorase can be applied in the measurement of metabolites are in the determination of phenylalanine and leucine (Kianmehr et al. 2016). Hence, given the above results, it can be concluded that *B. sphaericus* enzyme had potential to be a candidate for application in medicine as a diagnostic enzyme.

Conclusion

This paper presents recombinant expression, characterization and application of a DLD enzyme with diaphorase activity from a strain of *B. sphaericus* PAD-91. The *DLD* gene consisted of 1413 bp encoding a protein with MW of 50 kDa. Diaphorase exhibited its optimal activity at temperature of 30 °C and pH 8.5. The enzyme was compatible with different metal ions and inhibitors. K_m and V_{max} values of *B. sphaericus* enzyme with NADH were determined to be 0.33 mM and 200.0 U/ml, respectively. Collectively,

the suitable kinetic features of *B. sphaericus* diaphorase can make this biocatalyst useful for application as a diagnostic enzyme.

Acknowledgements This Project has been financially supported by the office of vice chancellor for research and technology from the Islamic Azad University, Bandar Abbas Branch.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This article does not contain any studies with human participants performed by any of the authors.

Statement on the welfare of animals All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

References

- Adams MA, Jia Z (2006) Modulator of drug activity B from *Escherichia coli*: crystal structure of a prokaryotic homologue of DT-diaphorase. *J Mol Biol* 359:455–465
- Antiochia R, Cass AEG, Palleschi G (1997) Purification and sensor applications of an oxygen insensitive thermophilic diaphorase. *Anal Chem Acta* 345:17–28
- Argyrou A, Sun G, Palfey BA, Blanchard JS (2003) Catalysis of diaphorase reactions by *Mycobacterium tuberculosis* lipoamide dehydrogenase occurs at the EH4 level. *Biochem* 42:2218–2228
- Benen JAE, Van Berkel WJH, Van Dongen WMAM, Muller F, Kok AD (1989) Molecular cloning and sequence determination of the *Ipd* gene encoding lipoamide dehydrogenase from *Pseudomonas fluorescens*. *J Gen Microb* 135:1787–1797
- Bhushan B, Halasz A, Spain JC, Hawari J (2002) Diaphorase catalyzed biotransformation of RDX via N-denitration mechanism. *Biochem Biophys Res Commun* 296:779–784
- Boething RS, Weaver T (1979) A new assay for diaphorase activity in reagent formulations, based on the reduction of thiazolyl blue. *Clin Chem* 25(12):2040–2042
- Cakraborty S, Sakka M, Kimura T, Sakka K (2008) Characterization of a dihydrolipoamide dehydrogenase having diaphorase activity of *Clostridium kluyveri*. *Biosci Biotech Biochem* 72(4):982–988
- Dietrichs D, Meyer M, Schmidt B, Andreesen JR (1990) Purification of NADPH-dependent electron transferring flavoproteins and N-terminal protein sequence data of the dihydrolipoamide dehydrogenase from anaerobic, glycine-utilizing bacteria. *J Bacteriol* 172:2088–2095
- Dilipkumar M, Rajasimman M, Rajamohan N (2013) Enhanced inulinase production by *Streptomyces* sp. in solid state fermentation through statistical designs. *3 Biotech* 3:509–515
- Gunther H, Simon H (1987) The use of pig heart dihydrolipoamide dehydrogenase (diaphorase) for the regeneration of NADH or NAD. *Appl Microbiol Biotechnol* 26(1):9–12
- Kaplan F, Setlow P, Kaplan NO (1969) Purification and properties of a DPNH-TPNH diaphorase from *Clostridium kluyveri*. *Arch Biochem Biophys* 132:91–98
- Ki J-S, Zhang W, Qian P-Y (2009) Discovery of marine *Bacillus* species by 16S rRNA and rpoB comparisons and their usefulness for species identification. *J Microbiol Methods* 77:48–57
- Kianmehr A, Mahrooz A, Ansari J, Oladnabi M, Shahbazmohammadi H (2016) The rapid and sensitive quantitative determination of

- galactose by combined enzymatic and colorimetric method: application in neonatal screening. *Appl Biochem Biotechnol* 179:283–293
- Madiagan RA, Mayhew SG (1993) Preparation of the apoenzyme of the FMN-dependent *Clostridium Kluyveri* diaphorase by extraction with apofalvodoxin. *Biochem Soci Transform* 22:578–585
- Mashayekhi Mazar F, Shahbazmohammadi H, Ebrahimi-Rad M, Gregorian A, Omidinia E (2012) Isolation, purification and characterization of a thermophilic alkaline protease from *Bacillus subtilis* BP-36. *J Sci* 23:7–13
- Rlyachko NL, Shchedrina VA, Efimov AV, Kazakov SV, Gazaryna IG, Kristal BS, Brown AM (2006) pH-dependent substrate preference of pig heart lipoamide dehydrogenase varies with oligomeric state. *J Biol Chem* 280(16):16106–16114
- Sambrook J, Fritsch EF, Maniatis T (1994) *Molecular cloning: a laboratory manual*, 2nd edn. Cold Spring Harbor Laboratory press, Cold Spring Harbor, pp 1847–1857
- Serrano A (1992) Purification, characterization and function of dihydrolipoamide dehydrogenase from cyanobacterium *Anabaena* sp. strain P.C.C.7119. *Biochem* 288:823–830
- Shahbazmohammadi H, Omidinia E (2011) New enzymatic colorimetric method for the quantitative determination of phenylalanine in dry-blood spots. *J Sci* 22:15–20
- Shahbazmohammadi H, Omidinia E, Sahebghadam Lotfi A, Saghiri R (2007) Preliminary report of NAD⁺-dependent amino acid dehydrogenase producing bacteria isolated from soil. *Iran Biomed J* 11(2):131–135
- Shahbazmohammadi H, Mostafavi SS, Soleimani S, Bozorgian S, Pooraskari M, Kianmehr A (2015) Response surface methodology to optimize partition and purification of two recombinant oxidoreductase enzymes, glucose dehydrogenase and D-galactose dehydrogenase in aqueous two-phase systems. *Protein Expr Purif* 108:41–47
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28:2731–2739
- Tedeschi G, Chen S, Massey V (1995) Active site studies of DT-diaphorase employing artificial flavins. *J Biol Chem* 270:2512–2516
- Vaubel RA, Rustin P, Isaya G (2011) Mutation in the dimer interface of dihydrolipoamide dehydrogenase promote site-specific oxidative damages in yeast and human cells. *J Biol Chem* 286(46):40232–40245
- Xia L, Björnstedt M, Nordman T, Eriksson LC, Olsson JM (2001) Reduction of ubiquinone by lipoamide dehydrogenase. An antioxidant regenerating pathway. *Eur J Biochem* 268(5):1486–1490
- Yan L-J, Thangthaeng N, Sumien N, Forster MJ (2013) Serum dihydrolipoamide dehydrogenase is a labile enzyme. *J Biochem Pharmacol Res* 1(1):30–42