Protein Expression and Purification 146 (2018) 1–7

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Enzymatic characterization of a NADH-dependent diaphorase from *Lysinibacillus* sp. strain PAD-91

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ARTICLE INFO

Keywords:
Lysinibacillus sp.
Characterization
Diaphorase
Medium optimization

ABSTRACT

Diaphorases are flavin-containing enzymes with potential applications in biotransfomation reactions, biosensor design and in vitro diagnostic tests. In this paper, we present recombinant expression, characterization and medium optimization of a lipoamide dehydrogenase (DLD) with NADH-dependent diaphorase activity from a *Lysinibacillus* sp. strain. DLD encoding sequence showed an open reading frame of 1413-bp encoding a 470 amino acid chain. *Lysinibacillus* sp. DLD catalyzed the NADH-dependent reduction of electron acceptors and exhibited diaphorase activity. The molecular mass of the isolated enzyme was found to be about 50 kDa, and determined to be a monomeric protein. The optimum pH and temperature for the catalytic activity of the enzyme was about pH 7.5 and 30 °C. The K_m and V_{max} values were estimated to be 0.025 mM and 1.33 μmol/min, respectively. Recombinant enzyme was optimally produced in fermentation medium containing 10 g/L sucrose, 25 g/L yeast extract, 5 g/L NaCl and 0.25 g/L $MgSO_4$. By Scaling up fermentation from flask to bioreactor, enzyme activity was increased to 487.5 U/ml. This study provides data on the identification, characterization and medium optimization of a NADH-dependent diaphorase from a newly isolated *Lysinibacillus* sp. PAD-91.

1. Introduction

Diagnostic enzymes are used directly or as a component of an assay system for the determination of many different substances [1]. 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 phenylketonuria [2], maple syrup urine diseases (MSUD) and galactosemia. Diaphorases belong to the family of oxidoreuductases that act on NADH or NADPH with hydrogen acceptors [3]. Lipoamide dehydrogenase (DLD; EC: 1.8.1.4), is a FAD-dependent enzyme that catalyses the reversible oxidation of dihydrolipoamide to lipoamide in the presence of NAD^+ [4,5]. This flavoenzyme contains a reactive disulfide bridge and a FAD cofactor per subunit. It has been extensively characterized from a variety of organisms belonging to prokaryotes, eukaryotes and archaeobacteria [6,7]. DLD enzyme is also termed diaphorase for its NAD(P)H dehydrogenase activity [8,9]. The first diaphorase enzyme has been purified from pig heart muscle [10,11]. Other enzymes have been identified from various sources such as *Clostridium kluyveri*, *Bacillus stearothermophilus*, *Thermus aquaticus* and *Thermus thermophlius* [12,13]. Diaphorases have potential application for the coupled colorimetric measurement of dehydrogenases, nitric oxide and ethanol. They have been used in biotransfomation reactions [14], biosensor design [13] and neonatal screening tests such as PKU, MSUD and galactosemia. Due to industrial applications of diaphorases, these enzymes have attracted researcher's attentions for screening of novel sources and cost-benefit production. In this article, we report a strain of *Lysinibacillus* sp. which produced a DLD with NADH-dependent diaphorase activity. To enhance the production of recombinant enzyme, central composition design method was employed for medium optimization.

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https://doi.org/10.1016/j.pep.2018.01.005
Received 7 December 2016; Received in revised form 20 November 2017; Accepted 17 January 2018

2. Materials and methods

2.1. Chemicals and media

Reagents for cultivation were purchased from Merck (Darmstadt, Germany). Lipoamide and NAD^+ were obtained from Sigma (St. Louis, MO, USA) and NADH was from Roche (Germany). *Escherichia coli* BL21 (DE3) and pET-28b (+) were purchased from Novagen (Philadelphia, PA).

2.2. Isolation and screening of strains producing enzyme

One gram of each soil sample was suspended in a selective liquid medium that contained (per liter): 0.5% dihydrolipoamide, 1 g NaCl, 2 g K_2HPO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 5 g yeast extract, 5 g polypeptone in 1 L of tap water, pH 7.0, then incubated with shaking at 37 °C at 180 rpm for 24 h [6]. From each dilution, 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 subsequent screening by 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 lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 7.4) and disrupted by ultrasonic oscillator for 10 min. Cells and insoluble materials were removed by centrifugation at 10000 rpm for 20 min at 4 °C. The supernatant solution was used as crude extract for DLD activity assay. DLD activity was determined by the oxidation of dihydrolipoamide in the presence of NAD^+ [15]. 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 composited 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 [3].

2.3. 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) [16]. 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 [17]. Phylogenic tree was constructed using the neighbor-joining algorithm using the MEGA version 5.0.

2.4. 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′- CCCGTCGACTTATTTTACAATGTG-'3), which contained the restriction sites for *Bam*HI and *Sal*I, respectively (underlined). The construct bearing the desired gene (pET28aDLD) was transformed into *E. coli* strain BL-21 (DE3) competent cells. *E. coli* strain BL21 (DE3) cells bearing pET28aDLD were cultivated overnight in 100 ml of LB medium containing 40 µg/ml of kanamycin at 37 °C and 180 rpm. 100 ml of pre-cultured 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 OD_{600} of 0.6–0.8, DLD enzyme was expressed by the addition of 1 mM sterile isopropyl-β-D-thiogalactopyranoside (IPTG). After 5 h induction at 37 °C, cells were harvested, washed twice with 0.9% NaCl solution and stored at −20 °C. Expression of recombinant DLD in different conditions was evaluated by activity assay and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After centrifugation at 10000 rpm for 20 min at 4 °C, the cell pellet was 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). SDS-PAGE was performed using discontinuous gels (10 cm × 10 cm) with a 12% separating gel and a 6% stacking gel [18]. Protein bands were visualized by staining with 0.25% Coomassie brilliant Blue R-250 in the mixture of 50% methanol and 10% acetate.

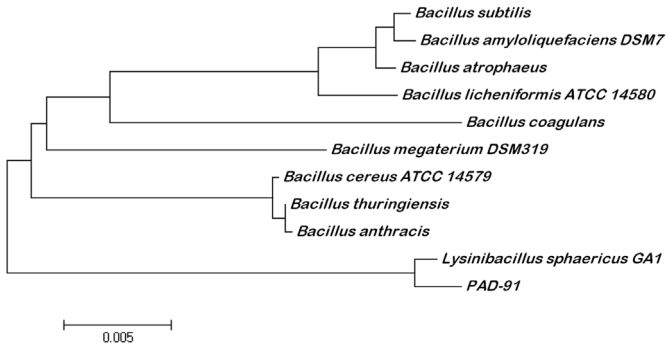

Fig. 1. Phylogenetic tree of the 16S rDNA sequences of strain PAD-91 associated with the other members of *Bacillus* species.

2.5. Enzyme characterization

The effect of temperature was analyzed by performing the enzyme assay at various temperatures (30-60 °C). Reaction mixture was pre-incubated at the desired temperatures for 4 min. The effect of pH on the enzymatic reaction of DLD was assessed 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 experiments of enzyme kinetic were determined by varying concentrations of NADH. Kinetic constants (K_m and V_{max}) were calculated using GraphPad Prism version 7.00, GraphPad Software, La Jolla California USA.

2.6. Bioinformatics analysis

BLAST through NCBI was used to identify homologous structures of DLD, with default settings against the database of protein sequences in the protein data bank (PDB). Multiple alignment of amino acid sequences was performed with DNASIS MAX software and displayed with ESPript 3.0 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi).

2.7. Medium optimization

The media, LB, 2x TY, M9, SOB and SOC were used for basic

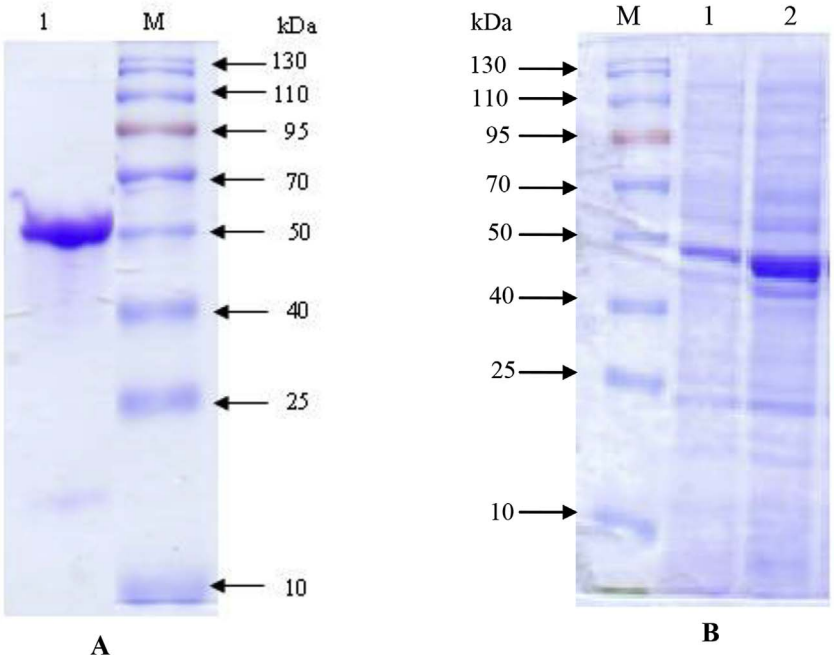

Fig. 2. Native SDS-PAGE analysis of recombinant enzyme. (A) Purification of recombinant diaphorase. Lane M: standard protein molecular weight marker; Lane 1: purified enzyme. (B) Expression analysis of recombinant enzyme in basal and optimized medium. Lane 1: whole cell lysate from a sample before medium optimization (basal medium). Lane 2: whole cell lysate from a sample after medium optimization.

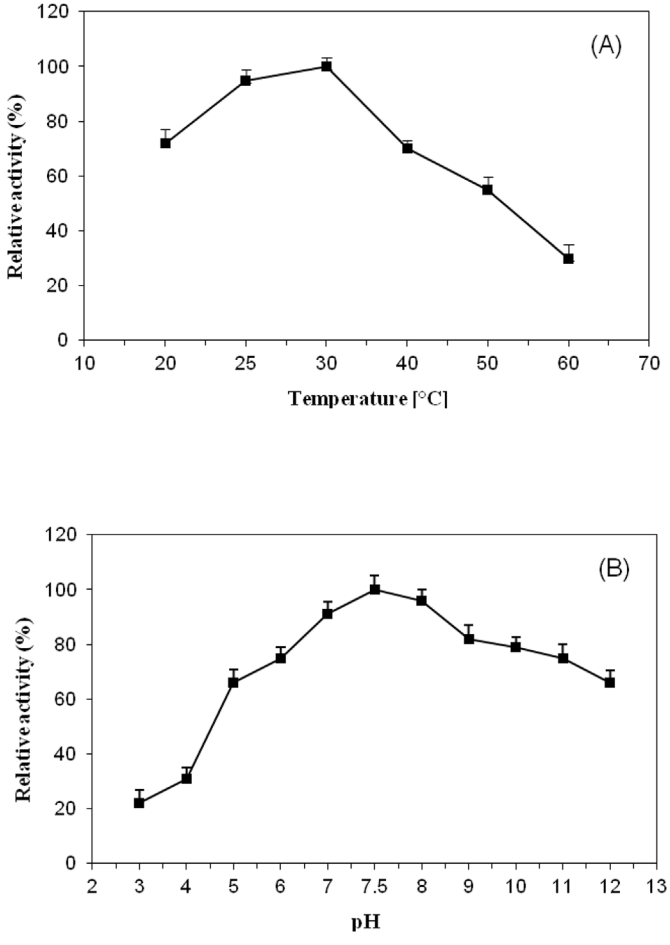

Fig. 3. Influence of temperature (A) and pH (B) on the activity of diaphorase enzyme. Each value represents mean ± SD (n = 3).

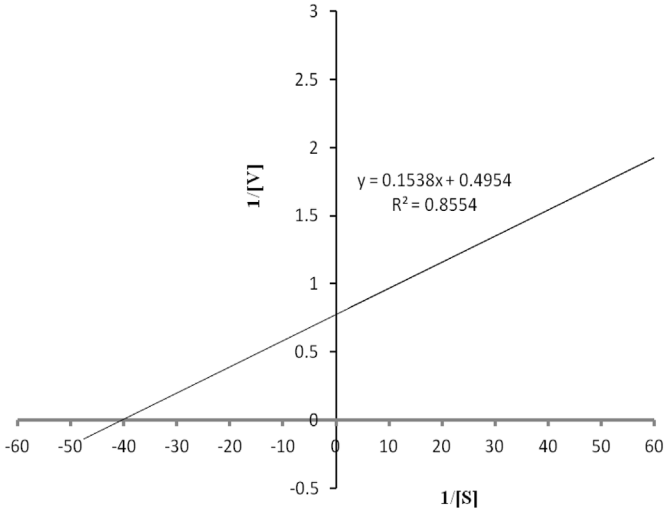

Fig. 4. Lineweaver-Burk plot for recombinant diaphorase from *Lysinibacillus* sp.

medium optimization. A central composite face-centered design (CCFD) was used to optimize medium composition of recombinant enzyme using Design-Expert software (version 8.0.4, State-Ease, Inc., USA). All variables were studied at two levels (+1, −1). Analysis of variance (ANOVA) was used for the determination of significant variables. The statistical significance of the proposed model was evaluated by Fisher's statistical test (*F*-test). The regression equations were also summated to the *F*-test to determine the coefficient of determination (R^2). The fitted polynomial equation was expressed as three-dimensional surface plots to visualize the relationship between the responses (dependent variables) and the experimental levels of each factor (independent variables). The optimized parameters were tested experimentally to confirm the model validity [19]. To further optimize the production of recombinant diaphorase, additional cultural conditions were studied. Working volume was 3.0 L in 5-L stirred tank bioreactor (BioFlo 3000, New Brunswick Scientific, Edison, New JersEy, USA). . The pH was controlled at 7.0 ± 0.2 by addition of 25% (v/v) ammonia and 20% (v/v) HCl. Pre-inoculum was prepared by transferring freshly grown cells into 500 ml flask containing 300 ml medium and incubating at 37 °C and 250 rpm for 12 h. The initial batch culture was started by addition of 10% (v/v) of primary seed culture into the bioreactor. After 1 h of cultivation in bioreactor (OD_{600} = 0.6–0.8), IPTG was added to a

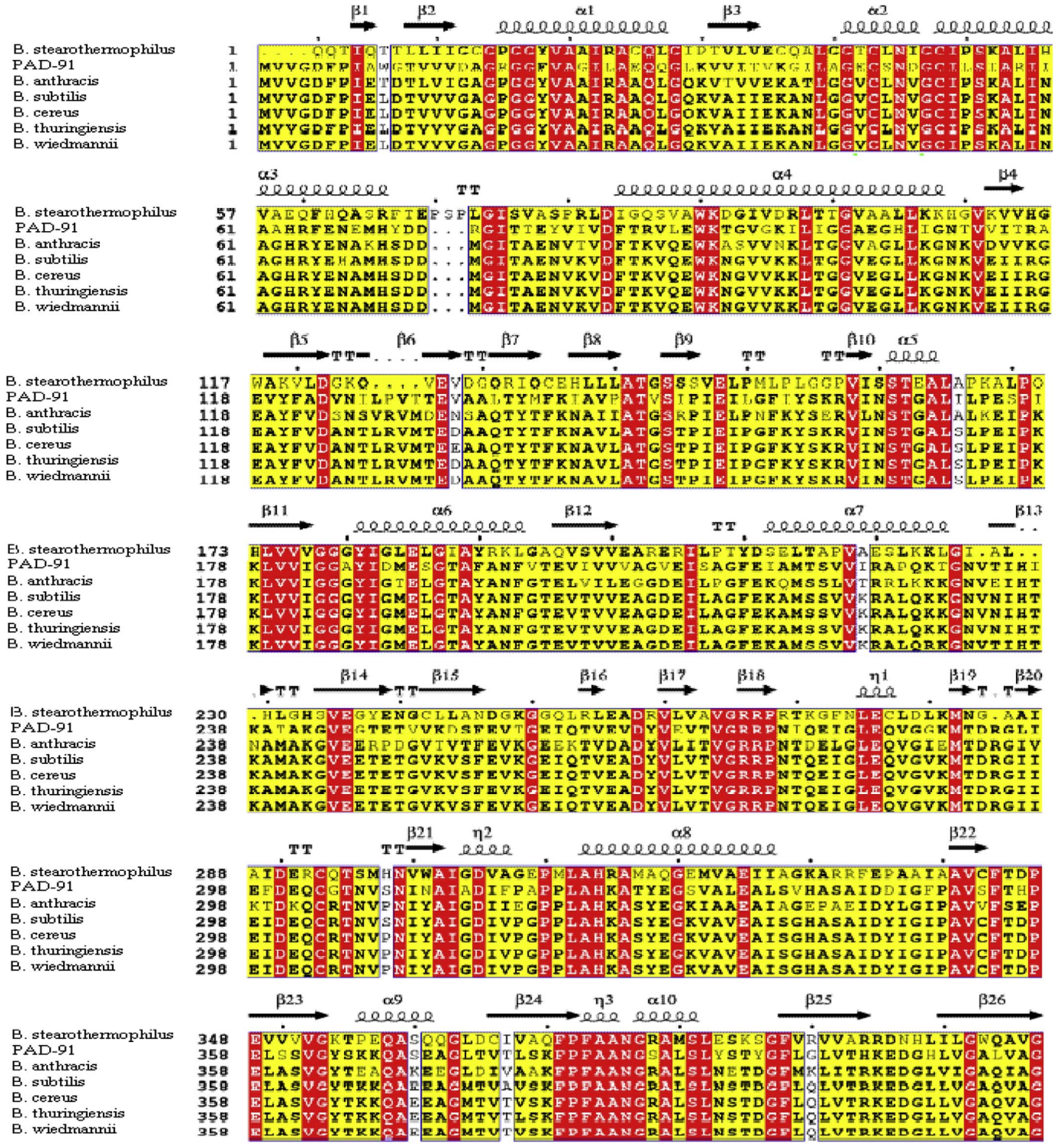

Fig. 5. Multiple alignment of DLD amino acid sequences from different *Bacillus* strains. The alignment was done by using the DNASIS software and displayed with ESPript 3.0 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi). Highly identical residues are depicted by red color and the less strongly conserved residues are colored by yellow font. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

final concentration of 1 mM to induce expression and culture was further grown at 37 °C and 400 rpm for 4 h. Culture was sampled at regular intervals for enzyme activity assay.

2.8. Nucleotide sequence accession number

The nucleotide sequence of PAD-91 DLD has been deposited in GenBank database under the accession number; MF496040.

3. Results and discussion

3.1. Isolation of strain PAD-91

Bacterial isolates were analyzed for 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

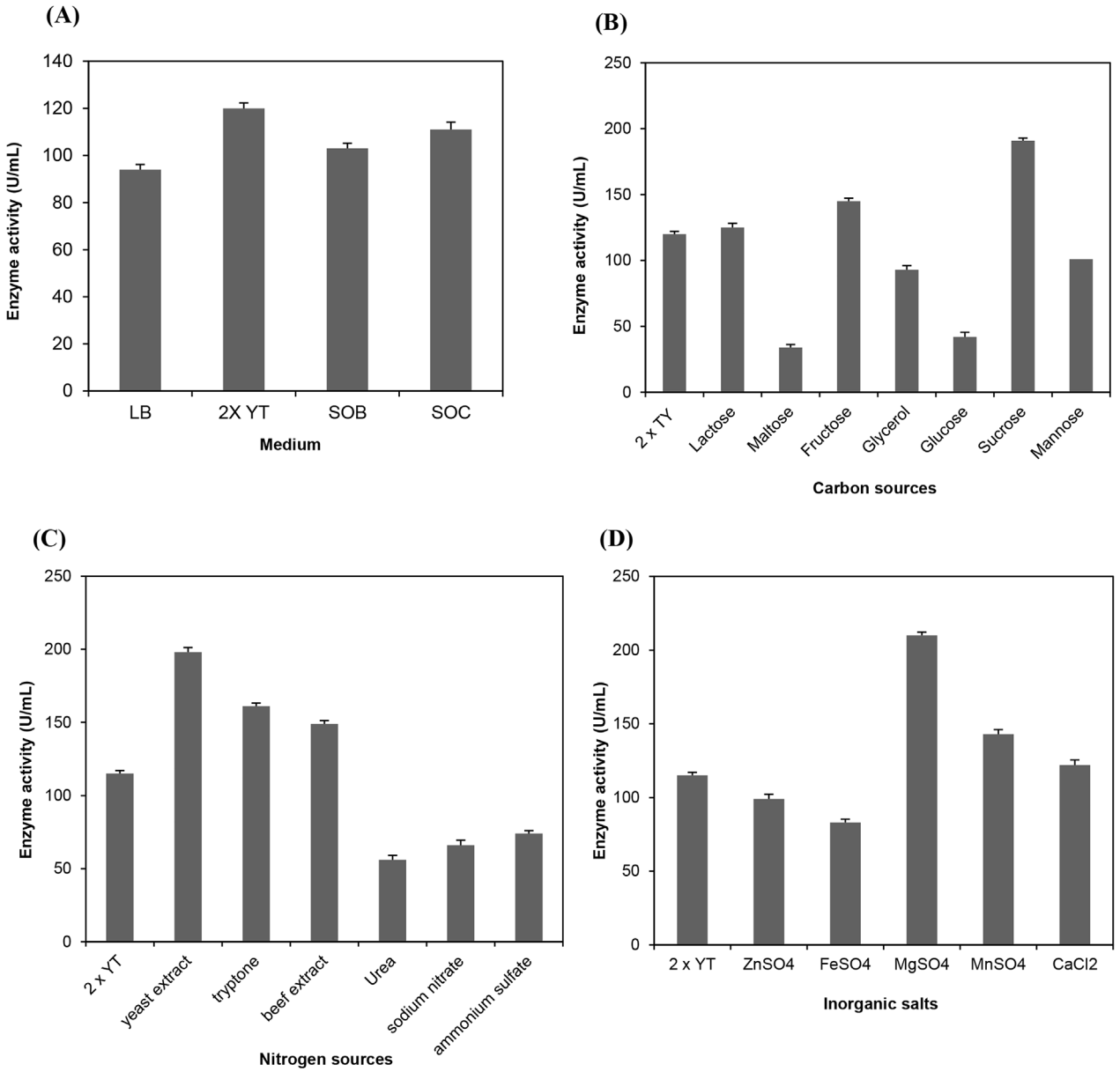

Fig. 6. (A) Influence of basal medium on diaphorase activity. (B) Influence of carbon sources on diaphorase activity. (C) Influence of nitrogen sources on diaphorase activity. (D) Influence of inorganic salts on diaphorase activity. Each value represents mean ± standard deviation (n = 3).

Table 1
Results of ANOVA for response surface quadratic model.

Source	SS	DF	MS	F-value	*p*-value	
Model	18104.17	10	1810.42	6.02	0.0005	significant
A-Sucrose	8268.75	1	8268.75	27.51	< 0.0001	
B-Yeast extract	7105.33	1	7105.33	23.64	0.0001	
C-NaCl	4.08	1	4.08	0.014	0.9085	
D-$MgSO_4$	27	1	27	0.09	0.7678	
AB	841	1	841	2.8	0.1117	
AC	12.25	1	12.25	0.041	0.8423	
AD	625	1	625	2.08	0.1665	
BC	380.25	1	380.25	1.27	0.2755	
BD	600.25	1	600.25	2	0.1747	
CD	240.25	1	240.25	0.8	0.3831	
ABC	0	0				
Residual	5409.97	18	300.55	15.93	0.0043	
Lack of Fit	5409.97	14	386.43			
Pure Error	0	4				
Core Total	23514.14	28	112.83	5.11	0.0089	

DF: degree of freedom, SS: sum of squares, MS: mean squares.

database. The 16S rRNA nucleotide was analyzed with the BLAST program which showed 97% homology with *L. sphaericus*. According to the created phylogenetic tree (Fig. 1), it was inferred that the PAD-91 strain belonged to the genus of *Lysinibacillus*.

3.2. Enzyme properties

The DNA fragment containing *DLD* gene was obtained from the PAD-91 strain by PCR with primers designed on the basis of conserved sequences from other *Bacillus* strains. The 1413-bp open reading frame (ORF) had a coding capacity of 470 amino acids. The molecular mass of diaphorase enzyme was determined to be about 50 kDa (Fig. 2). The subunit structure was examined by SDS-PAGE. SDS-PAGE gel analysis showed a single band indicating that the enzyme consists of one subunit (Fig. 2). 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 [12]. The diaphorase reaction exhibited its maximal activity at 30 °C (Fig. 3a). As seen, a sharp decrease in enzyme activity was observed above 30 °C and was completely

inactivated at 60 °C. 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 7.5 and it showed high activity in a pH range of 7.0–9.0 (Fig. 3b). The K_m and V_{max} values for the recombinant enzyme were determined to be 0.025 mM and 1.33 μmol/min, respectively (Fig. 4). The K_m value for NADH (0.25 mM) was lower than other previously reported diaphorases such as *B. stearothermophilus* (0.7 mM) and *C. kluyveri* (0.38 mM) [12]. Thus, it can be said that the important advantage of *Lysinibacillus* sp. diaphorase over previously studied enzymes was its kinetic features. Suitable kinetics parameters offered that our identified diaphorase to be a good candidate for biotechnological applications.

3.3. In silico analysis

The multiple alignment of *Lysinibacillus* sp. PAD-91 DLD amino acid sequence with other DLDs was done (Fig. 5). The DLD protein (470 amino acids) of *Lysinibacillus* sp. showed 68.30%, 54.89%, 68.30%, 68.51% and 68.09% homology with the DLD of. *B. anthracis*, *B. subtilis*, *B. cereus*, *B. thuringiensis* and *B. wiedmannii* strains, respectively.

3.4. Selection of the best parameters for enzyme production by one-variable-at-a-time approach

The results indicated that 2 x TY medium was the most favorable for cell growth (Fig. 6a). Under this condition, enzyme activity of 120 U/ml was achieved. The influence of carbon source in 2 x TY medium was studied by adding of maltose, glucose, lactose, sucrose, mannose, and fructose at concentration of 10 g/L. As shown in Fig. 6b, enzyme activity was the highest when sucrose was employed as carbon source. Low enzyme activity was seen in culture media containing glucose and maltose. The negative effect of glucose has been reported for medium optimization of other enzymes such as lipase [19,20]. This phenomenon is in accordance with the suppressive role of this parameter, which is attributed to its catabolic repression influence [21]. In contrast there are reports that glucose improves enzyme production [22]. The nitrogen sources, yeast extract and tryptone in 2 × YT medium were replaced by tryptone, yeast extract, beef extract, ammonium sulfate, sodium nitrate, and urea at concentration of 20 g/L. When yeast extract was used (Fig. 6c), enzyme activity was the highest. So the yeast extract was found as the best nitrogen source. Yeast extract has been used in most fermentation studies as a supplier of vitamins and growth factor [23]. Similar observations have been reported in production of other enzymes such as validoxylamin glycosyltransferase [24], inulinase and invertase [25]. The influence of inorganic salts in 2 x TY medium was studied by adding of $ZnSO_4$, $FeSO_4$, $MgSO_4$, $MnSO_4$ and $CaCl_2$ at concentration of 0.2 g/L. The results showed that $MgSO_4$ was an important additive (Fig. 6d). In contrast, other inorganic salts had negative effects on enzyme production. The reason of promoting function of Mg^{2+} may be that, Mg^{2+} is highly necessary to maintain genomic stability and is an essential cofactor in almost enzymatic systems involved in DNA processing [26,27]. However, in some cases Mg^{2+} has exhibited negative effects on the production of enzymes [28,29].

3.5. Production optimization using RSM

A four factors and three levels experiment was designed. The property of polynomial model equation was expressed by R2 and its statistical significance was determined by *F* test. The analysis of variance results were shown in Table 1. The ANOVA for response surface quadratic regression model showed that the model was highly significant (*p* value of .0005) with an F-value of 6.02. In addition, the model R^2 was 0.97, indicating that the fitting degree of equation was good, and there was a high degree of correlation between the predicted and measured values. Thus it could be applied to predict theoretical enzyme activity. The adequate precision value of 9.865 indicated an adequate signal and suggested that the model could be used to navigate the design space. A low value of coefficient of variance (C.V. %) (6.77) indicated a high degree of precision and reliability of the experimental values. As found from Table 1, sucrose and yeast extract had significant effect on enzyme activity with p value < .05 while NaCl and $MgSO_4$ had insignificant effect. By analyzing or data, the optimum levels for sucrose, yeast extract, NaCl, and $MgSO_4$ were determined to be 10 g/L, 25 g/L, 5 g/L, and 0.25 g/L, respectively. After repeated experiments employing the optimized medium, the average actual enzyme activity was 345.0 U/mL, indication a good agreement between the observed and predicted value. The obtained activity for recombinant *Lysinibacillus* sp. diaphorase was significantly higher than reported in other literature [12]. Based on the shake flask results, target enzyme production in a batch fermenter was studied to obtain higher enzyme activity. The optimal values of agitation speed, aeration rate, and initial pH were 86 rpm, 2.1 vvm and 7.0, respectively. The activity of recombinant enzyme was higher (487.5 U/ml) than that obtained in parallel shake flask culture (345.0 U/ml).

4. Conclusion

We presented recombinant expression, characterization and medium optimization of a DLD enzyme with diaphorase activity from a strain of *Lysinibacillus* sp. 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 7.5. K_m and V_{max} values with NADH were determined to be 0.025 mM and 1.33 μmol/min, respectively. The most appropriate medium for the production of recombinant enzyme was composed of sucrose10 g/L, yeast extract 25 g/L, NaCl 5 g/L, and $MgSO_4$ 0.25 g/L. At optimized condition, the actual DLD activity was calculated to be 345.0 U/mL. Fermentation in 5-L bioreactor resulted in a further enhancement of the enzyme activity to 487.5 U/ml.

Acknowledgments

This research project was financially supported by Biochemistry & Metabolic Disorders Research Center, Department of Research & Technology, Golestan University of Medical Sciences, Gorgan, Iran and the office of vice chancellor for research and technology from the Islamic Azad University, Bandar Jask Branch.

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