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Medium Optimization for Improved Production of Dihydrolipohyl Dehydrogenase from *Bacillus sphaericus* PAD-91 in *Escherichia coli*

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Abstract Dihydrolipohyl dehydrogenase (DLD) is a FAD-dependent enzyme that catalyzes the reversible oxidation of dihydrolipoamide. Herein, we report medium optimization for the production of a recombinant DLD with NADH-dependent diaphorase activity from a strain of *Bacillus sphaericus* PAD-91. The DLD gene that consisted of 1413 bp was expressed in *Escherichia coli* BL21 (DE3), and its enzymatic properties were studied. The composition of production medium was optimized using one-variable-at-a-time method followed by response surface methodology (RSM). *B. sphaericus* DLD catalyzed the reduction of lipoamide by NAD^+ and exhibited diaphorase activity. The molecular weight of enzyme was about 50 kDa and determined to be a monomeric protein. Recombinant diaphorase showed its optimal activity at temperature of 30 °C and pH 8.5. K_m and V_{max} values with NADH were estimated to be 0.025 mM and 275.8 U/mL, 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 $MgSO_4$. At these concentrations, the actual diaphorase activity was calculated to be 345.0 ± 4.1 U/mL. By scaling up fermentation from flask to bioreactor, enzyme activity was increased to 486.3 ± 5.5 U/mL. Briefly, a DLD with diaphorase activity from a newly isolated *B. sphaericus* PAD-91 was characterized and the production of recombinant enzyme was optimized using RSM technique.

Keywords Dihydrolipohyl dehydrogenase (DLD) · Diaphorase · Medium optimization · Response surface methodology (RSM)

Introduction

Dihydrolipohyl dehydrogenase (DLD; EC: 1.8.1.4), also known as lipoamide dehydrogenase, is a FAD-dependent enzyme that catalyzes the reversible oxidation of dihydrolipoamide to lipoamide in the presence of NAD^+. DLD is a component of the large multienzyme complexes, pyruvate dehydrogenase and 2-oxo-acid dehydrogenase and plays a role in energy metabolism in eukaryotes [1, 2]. This flavoenzyme 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 [3, 4]. DLD enzyme is also termed diaphorase for its NAD(P)H dehydrogenase activity [5]. Diaphorases are a group of flavin-bound enzymes that catalyze the pyridine nucleotide-dependent reduction of electron-accepting molecules [6, 7]. The first such enzyme has been purified from pig heart muscle [8]. Other enzymes have been identified from various sources such as *Clostridium kluyveri*, *Bacillus stearothermophilus*, *Thermus aquaticus* and *Thermus thermophlius* [9, 10]. Diaphorases have potential application for the coupled colorimetric measurement of dehydrogenases, nitric oxide and ethanol. They have been used in biotransformation reactions [11], biosensor design [10] and in vitro diagnostic tests such as phenylketonuria (PKU) [12], maple syrup urine diseases (MSUD) and galactosemia as well [13]. Due

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to industrial applications of diaphorases, these enzymes have attracted researchers' attention for screening of novel sources and cost-benefit production.

The optimization of medium constituents is the primary step for cost-effective production of enzymes. The conventional optimization technique is one-variable-at-a-time method. Response surface methodology (RSM) is a statistical approach that has been used to optimize production medium of different enzymes [14]. Compared with classical methods, RSM can evaluate interaction from the effects of individual components and their influence on the production. This offers suitable information for executing optimization processes while simultaneously incorporating multiple responses [15]. In this communication, we report on the identification of a strain *of B. sphaericus* which produces a DLD enzyme with NADH-dependent diaphorase activity. To enhance the production of the recombinant enzyme using RSM, a central composition design method was employed for medium optimization.

Materials and Methods

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). The media, Luria–Bertani (LB), Terrific broth (TB), M9 Minimal salt (M9), SOB and SOC were prepared according to the literature [16].

Screening and Isolation of Enzyme-Producing Strains

Screening of bacteria that produced DLD with diaphorase activity was carried out in medium containing dihydrolipoamide as a selective compound [3]. Twenty-five soil samples were collected from different locations of Tehran during spring and summer seasons of 2015. The samples were placed in sterile polyethylene bags and maintained at 4 °C until processed. One gram of each soil sample was suspended in 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. Serial dilutions up to 10^{-4} were prepared. From each dilution, 0.05 mL was taken and placed on agar plates and incubated at 37 °C for 24 h [17]. The strains that grew under such conditions were selected for subsequent screening by enzyme production analysis. Production of

DLD enzyme was done in the mentioned medium at 37 °C at 180 rpm for 24 h. After the completion of growth, the fermentation broth was centrifuged at $6500 \times g$ 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 $8000 \times g$ for 20 min at 4 °C. The supernatant solution was used as crude extract for DLD activity assay [18]. DLD activity was determined by the oxidation of dihydrolipoamide in the presence of NAD^+ [18]. 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 an assay with thiazolyl blue tetrazolium bromide (MTT) as the terminal electron acceptor [19]. The standard reaction mixture contained 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. The protein concentration was determined by Bradford method with bovine serum albumin (BSA) as the standard [20]. All assay experiments were done in triplicate, and the average results were used for data analysis.

Isolate Identification

Identification of the selected 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) [21]. 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. The resultant PCR product was visualized by electrophoresis on 1% agarose gel, excised and gel-purified. 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 [22]. Phylogenic tree was constructed using the neighbor-joining algorithm using the MEGA version 5.0. The reliability of the internal branches of tree was statistically evaluated by bootstrap test with 1000 replications.

Expression and Purification of Recombinant *B. sphaericus* DLD

To amplify the putative *DLD* gene, forward and reverse primers were designed based on the consensus *DLD* sequences among *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′-CCCGTCGACTTTTACAATGTG-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 extension step at 72 °C for 7 min. The PCR product was double-digested with *Bam*HI and *Sal*I, gel-purified and then ligated into pET28a (+) expression vector. The construct bearing the desired gene (pET28aDLD) was transformed into *E. coli* strain DH5α competent cells for screening purposes. The identity of the cloned gene was also confirmed by nucleotide sequencing, and no mutation was revealed. The isolated plasmid was then 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 LB medium containing 40 μg/mL of kanamycin at 37 °C and 180 rpm. One hundred 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 OD_{600} of 0.6–0.8, DLD enzyme was expressed by the addition of 1 mM sterile isopropyl-β-ᴅ-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). The cell pellet was dissolved in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 7.4) 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 [16]. Protein bands were visualized by staining with 0.25% Coomassie Brilliant Blue R-250 in the mixture of 50% methanol and 10% acetate.

Enzyme Characterization

The total molecular mass was determined by native PAGE. The protocol for native PAGE was a modification of non-denaturing PAGE [16]. The main differences from the Laemmli protocol were the absence of SDS in electrophoresis and sample buffers, samples were not boiled and resolving gel was overlaid with purified water. The effect of temperature was analyzed by performing the diaphorase activity 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 diaphorase 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 kinetic constants were determined using GraphPad Prism version 7.00, GraphPad Software, La Jolla California USA.

Medium Optimization

The media, LB, 2× YT, M9, SOB and SOC were used for basic medium optimization. The initial pH of the medium was adjusted to 7.0 and was not controlled during the fermentation. For primary seed culture, 1.0 mL of frozen cells of recombinant *E. coli* was inoculated in 50 mL medium and grown at 37 °C and 200 rpm for 16 h. The medium (500 mL) was then inoculated with 10% (v/v) of seed culture and incubated at 37 °C and 250 rpm. After 1 h of inoculation, IPTG was added to final concentration of 1 mM and cultures were further grown at 37 °C and 250 rpm for 5 h. Cultures were harvested to activity assay of produced enzyme as above. 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). All experiments were done in triplicate, and average enzyme activity was taken as dependent variable or response. 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 [22, 23].

Batch Fermentation in 5-L Bioreactor

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

Results and Discussion

Isolation and Phylogenetic Analysis of Strain PAD-91

During the screening on selective medium containing dihydrolipoamide, 94 strains were isolated. In order to identify the enzyme-producing microorganisms, bacterial isolates were analyzed for DLD activity and NADH dehydrogenase ability. From these bacteria, only one strain, named as 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 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. 1), it was inferred that the PAD-91 strain was closely related to *B. sphaericus*. To the best of our knowledge, there is no report about production of diaphorase by *B. sphaericus*.

Construction of Expression Plasmid for Recombinant Diaphorase

The DNA fragment containing putative *DLD* gene was obtained by PCR with primers designed on the basis of conserved sequences from other *Bacillus* strains and sequenced. The 1413-bp open reading frame (ORF) had a coding capacity of 470 amino acids (Fig. 2). Expression plasmid of DLD, pET28aDLD, was constructed and transformed in *E. coli* strain BL21 (DE3).

Recombinant Enzyme Properties

The molecular mass of the recombinant diaphorase enzyme was determined to be about 50 kDa by native PAGE (Fig. 3a). 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. 3b). 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 [9]. The diaphorase reaction exhibited its maximal activity at 30 °C (Fig. 4a). 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 pH range from 3.0 to 12.0 at 30 °C. The optimum pH of the diaphorase reaction was approximately 8.5, and it showed high activity in a pH range of 7.0–9.0 (Fig. 4b). The K_m and V_{max} values for the recombinant enzyme were determined to be 0.025 mM and 275.8 U/mL, respectively. The K_m value for NADH (0.025 mM) was lower than other diaphorases such as *B. stearothermophilus* (0.07 mM) and *C. kluyveri* (0.038 mM) [9]. The calculated K_m indicated high affinity for the NADH substrate. Moreover, the turnover number of the enzyme (108.9 s^{-1}) was also higher than other previously purified diaphorases. Thus, it can be inferred that the important advantage of *B. sphaericus*

Fig. 1 Phylogenetic tree of the 16S rDNA sequences of strain PAD-91 associated with the other members of *Bacillus* species. Numbers at nodes are levels of bootstrap support (%) based on neighbor-joining (NJ) method of 1000 resampled datasets. The *scale bar* indicates 0.005 nucleotide substitution per position. Isolate is marked
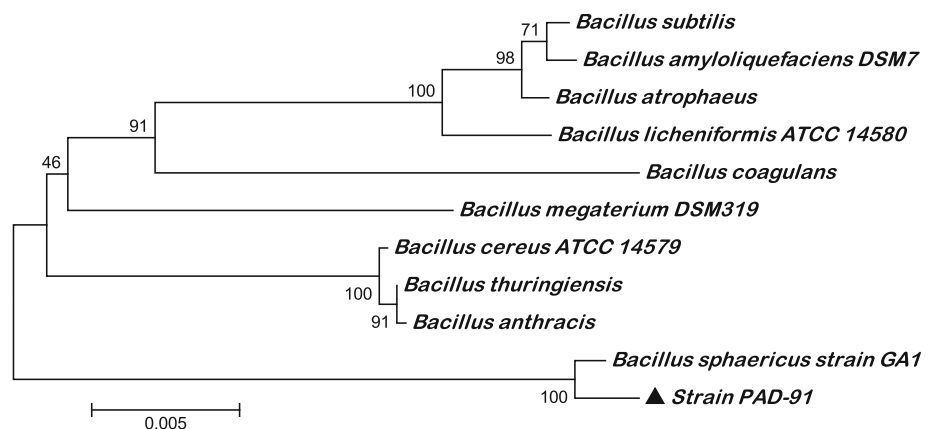
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   1 ATG GTA GTT GGA GAC TTT CCG ATT GCA TGG GGT ACA GTC GTT GTT GAT GCA GGT CGT GGT    60
  61 GGA TTC GTT GCG GGA ATT CTT GCA GAA CAA CAA GGT CTA AAG GTA GTA ATT ACT GTA AAA   120
 121 GGT ATC CTT GCT GGC GAA TGC TCA AAC GAT GGA TGT ATT CTT TCA ATA GCG AGA ATC ATT   180
 181 GCA GCT CAT CGT TTT GAG AAT GAA ATG CAT TAT GAT GAC AGG GGT ATC ACT ACA GAG TAC   240
 241 GTA ATA GTT GAC TTT ACA AGA GTT CTA GAA TGG AAA ACC GGC GTA GGT AAG ATA TTA ATT   300
 301 GGC GGT GCT GAA GGC CAT CTT ATA GGT AAC ACA GTT GTA ATC ACT CGC GCT GAA GTT TAC   360
 361 TTC GCA GAT GTT AAT ATA TTA CCC GTT ACG ACT GAA GTG GCA GCT CTA ACT TAT ATG TTT   420
 421 AAA ATT GCT GTT CCT GCA ACT GTT TCT ATA CCA ATC GAA ATT CTA GGA TTC ATA TAC TCT   480
 481 AAA CGT GTT ATC AAC TCT ACA GGC GCT TTA ATC TTA CCT GAA AGT CCT ATA AAA CTT GTT   540
 541 GTA ATC GGC GGC GCT TAC ATC GAT ATG GAA TCA GGT ACT GCA TTT GCT AAC TTC GTT ACA   600
 601 GAA GTT ATT GTA GTA GTA GCT GGC GTC GAA ATC TCA GCT GGT TTC GAA ATA GCT ATG ACC   660
 661 TCT GTT GTT ATA CGT GCT CCA CAG AAA ACA GGT AAC GTA ACT ATC CAT ATA AAA GCT ACG   720
 721 GCT AAA GGC GTT GAA GGA ACA GAA ACT GTC GTA AAA GAT AGC TTT GAA GTT ACA GGT GAA   780
 781 ATC CAA ACT GTA GAA GTA GAT TAC GTA GGA GTA ACT GTA GGT CGT CGT CCA AAC ATT CAA   840
 841 GAA ATC GGT CTT GAG CAA GTT GGA GGT AAA ATG ACT GAC CGC GGC CTC ATC GAA TTC GAT   900
 901 GAG CAA TGT GGT ACA AAC GTA TCA AAC ATC AAT GCA ATC GCT GAT ATC TTT CCT GCA CCA   960
 961 CCA TTA GCT CAC AAA GCT ACT TAC GAA GGT TAA GTA GCT CTA GAA GCA CTT AGT GTC CAT 1,020
1,021 GCA TCA GCT ATC GAT GAC ATC GGA TTT CCT GCA GTA AGC TTC ACT CAT CCA GAA TTA TCA 1,080
1,081 TCT GTT GGT TAC AGT AAG AAA CAA GCT TCA GAA GCT GGA CTG ACT GTA ACT TTA TCT AAG 1,140
1,141 TTC CCA TTC GCT GCT AAC GGT AGT GCA TTA TCA TTA TAC AGC ACT TAC GGT TTC TTA GGA 1,200
1,201 CTT GTA ACA CAT AAA GAA GAT GGT CAT CTT GTA GGT GCT CTA GTT GCA GGT GTA GGC GCT 1,260
1,261 TCT GAT ATT AAT TCT GAG ATT GCT TTA GCT ATC GTA GCT GGA ATG AGA GCA GAA GTT ATC 1,320
1,321 GCT CAA ACA ATC CAC GAT CAC CCA ACA CAA GGT GAA ATC AGA ATG GAA GCA GCT GTA GTT 1,380
1,381 GCT CTT CGA ATG CCA ATT TAC ATT GTA AAA TAA . . . . . . . . . . . . . . . . . . 1,413
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Fig. 2 Nucleotide sequence of DLD from *B. sphaericus* PAD-91. The numbers on the *left* and *right* are nucleotide accounts
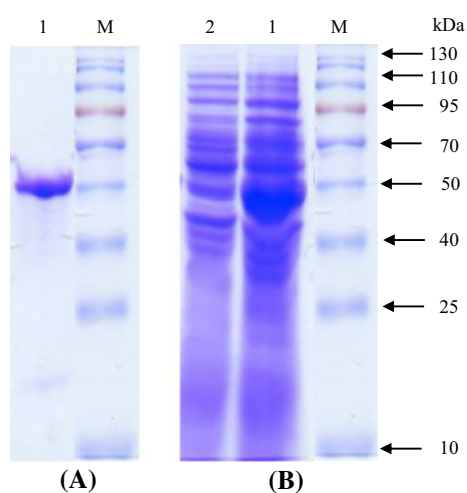
Fig. 3 Native PAGE (**a**) and SDS-PAGE (**b**) analysis of recombinant enzyme. **a** *Lane M* standard protein molecular weight marker; *lane 1* purified enzyme. **b** *Lane M* standard protein molecular weight marker; *lane 1* whole cell lysate from a sample after medium optimization; *lane 2* whole cell lysate from a sample before medium optimization (basal medium). The quantity of whole cell lysates and purified enzyme applied to SDS-PAGE and native PAGE gels were 40 and 10 μg, respectively

diaphorase over previously studied enzymes was its kinetic features. Suitable kinetics parameters offer promise for our identified diaphorase to be a good candidate for biotechnological applications.
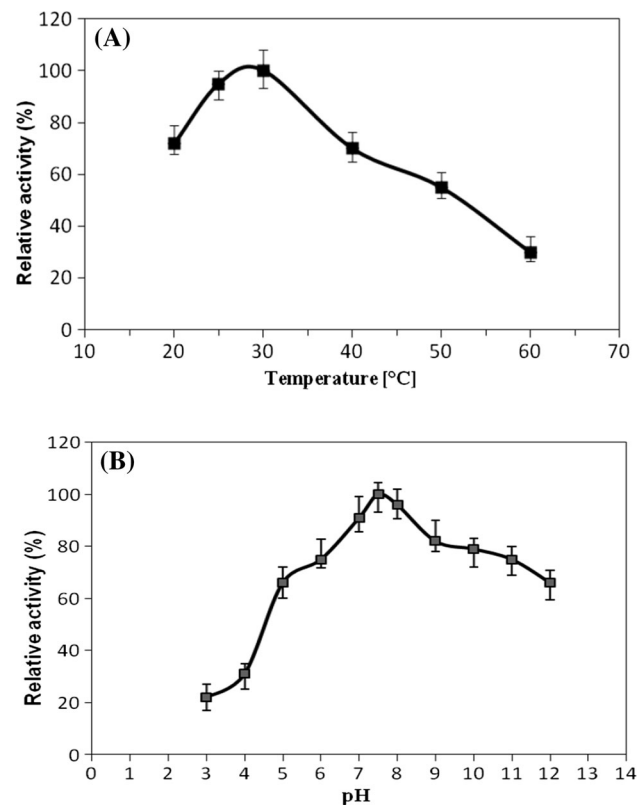
Fig. 4 Influence of temperature (**a**) and pH (**b**) on the activity of diaphorase reaction. Each value represents mean ± SD (n = 3)
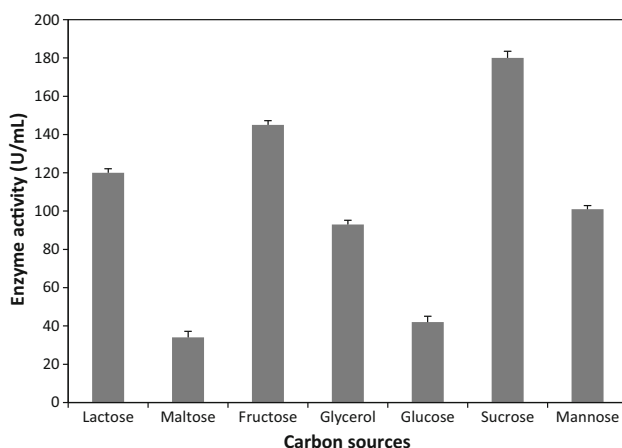
Effect of Basal Medium on Enzyme Production

In order to determine the optimal medium for recombinant strain *E. coli* BL21 (DE3) to grow and produce enzyme, the influence of liquid media of LB, 2× YT, SOB was investigated. The results indicated that 2× YT medium was the most favorable for cell growth. Under this condition, enzyme activity of 180 U/mL was achieved.

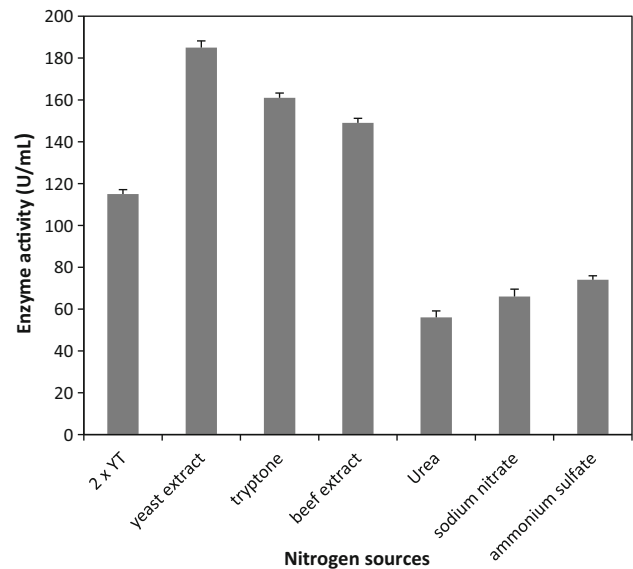## Effect of Carbon Source on Enzyme Production

The influence of carbon source in 2× YT medium was studied by adding of maltose, glucose, lactose, sucrose, mannose and fructose at concentration of 10 g/L. As shown in Fig. 5, 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 [24, 25]. This phenomenon is in accordance with the suppressive role of this parameter, which is attributed to its catabolic repression influence [26]. In contrast, there are reports that glucose improves enzyme production [27].

Effect of Nitrogen Source on Enzyme Production

The nitrogen sources, yeast extract and tryptone in 2× TY medium were replaced by tryptone, yeast extract, beef extract, ammonium sulfate, ammonium nitrate and urea at concentration of 20 g/L. When yeast extract was used (Fig. 6), 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 [28]. Similar observations have been reported in production of other enzymes such as

Fig. 6 Influence of nitrogen sources on diaphorase activity. Each value represents mean ± SD (n = 3)

validoxylamin glycosyltransferase [29], inulinase and invertase [30].

Effect of Inorganic Salts on Enzyme Production

The influence of inorganic salts in 2× YT 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. 7). 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

Fig. 5 Influence of carbon sources on diaphorase activity. Each value represents mean ± SD (n = 3)
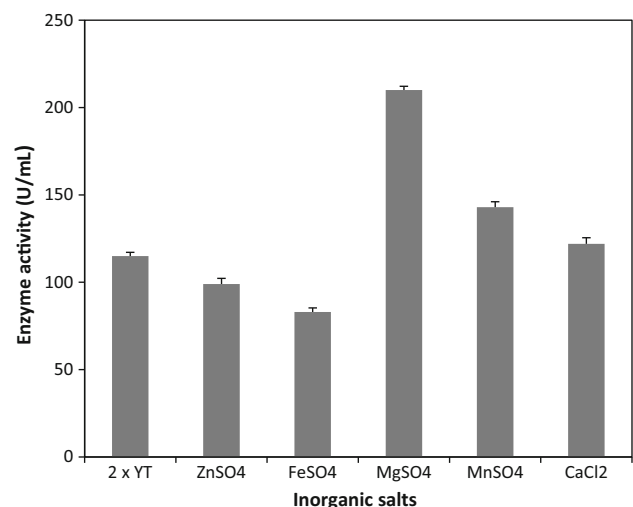
Fig. 7 Influence of inorganic salts on diaphorase activity. Each value represents mean ± SD (n = 3)

involved in DNA processing [15, 31]. However, in some cases Mg^{2+} has exhibited negative effects on the production of enzymes [32, 33].

Optimization of Enzyme Production

The most effective factors including sucrose, yeast extract, NaCl and $MgSO_4$ were selected based on the results of single-factor research. A four-factors and three-level experiment was designed. The coding levels of factors and experimental results are shown in Table 1. The property of polynomial model equation was expressed by R^2, and its statistical significance was determined by F test. The analysis of variance results are shown in Table 2. The ANOVA for response surface quadratic regression model

showed that the model was highly significant (p value of 0.0005) 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. An adequate precision measures the signal-to-noise ratio and a ratio greater than 4 is desirable. 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 (CV %) (6.77) indicated a high degree of precision and reliability of the experimental values. As found from Table 2, sucrose and yeast extract had significant effect on enzyme activity with p value <0.05, while NaCl and $MgSO_4$ had insignificant effect.

Table 1 Diaphorase activity in different compositions of culture media using a Box–Behnken design

Run	Sucrose (g/L)		Yeast extract (g/L)		NaCl (g/L)		MgSO4 (g/L)		Enzyme activity (U/mL)	
	Code X1	X1	Code X1	X2	Code X1	X2	Code X1	X2	Experimental	Predicted
1	1	10	0	20	−1	2.5	0	0.25	217	220.09
2	0	7.5	−1	15	−1	2.5	0	0.25	275	243.59
3	1	10	1	25	0	5	0	0.25	345	321.76
4	0	7.5	−1	15	0	5	−1	0.1	229	239.76
5	0	7.5	0	20	1	7.5	1	0.4	240	247.51
6	0	7.5	1	25	0	5	−1	0.1	257	261.84
7	0	7.5	0	20	−1	2.5	1	0.4	267	266.01
8	1	10	−1	15	0	5	0	0.25	253	249.34
9	0	7.5	−1	15	0	5	1	0.4	226	215.92
10	−1	5	0	20	−1	2.5	0	0.25	286	293.42
11	1	10	0	20	0	5	−1	0.1	260	243.92
12	0	7.5	0	20	0	5	0	0.25	270	271.42
13	1	10	0	20	1	7.5	0	0.25	213	222.67
14	0	7.5	0	20	1	7.5	−1	0.1	262	290.84
15	0	7.5	0	25	1	7.5	0	0.25	234	241.01
16	0	7.5	0	15	1	7.5	0	0.25	244	270.17
17	0	7.5	1	25	0	5	1	0.4	241	228.76
18	0	7.5	0	20	0	5	0	0.25	280	284.76
19	−1	5	−1	15	0	5	0	0.25	238	231.09
20	−1	5	0	20	0	5	−1	0.1	270	280.09
21	−1	5	1	25	0	5	0	0.25	220	218.09
22	0	7.5	0	20	−1	2.5	−1	0.1	320	291.26
23	0	7.5	0	20	0	5	0	0.25	233	245.59
24	−1	5	0	20	1	7.5	0	0.25	284	269.76
25	0	7.5	0	20	0	5	0	0.25	253	256.17
26	0	7.5	1	25	−1	2.5	0	0.25	253	256.17
27	0	7.5	0	20	0	5	0	0.25	253	256.17
28	1	10	0	20	0	5	1	0.4	253	256.17
29	−1	5	0	20	0	5	1	0.4	253	256.17

The relation between factors and enzyme activity was indicated by the surface plots (Fig. 8a–f). The response surface curve in Fig. 8a represents the interaction between yeast extract and sucrose, which showed that the maximum diaphorase activity was achieved with high levels of these variables. Figure 8b depicts the interaction between $MgSO_4$ and sucrose. The results showed that enzyme activity was mostly affected by sucrose than $MgSO_4$. The response surface curve for the interaction of yeast extract and $MgSO_4$ is represented in Fig. 8c. The shape of response showed that diaphorase activity was mostly affected by yeast extract levels than $MgSO_4$. The same trend was observed for the interaction of sucrose and NaCl (Fig. 8d) as well as yeast extract and NaCl (Fig. 8e) where the maximum response was mostly influenced with sucrose and yeast extract. Figure 8f shows the interaction of NaCl and $MgSO_4$. As found, there was a less significant interaction between NaCl and $MgSO_4$ and the optimum response was obtained with the intermediate levels of these two factors. There was no regular trend between concentration of these factors and enzyme activity. By analyzing the response surface plots, the optimum levels for sucrose, yeast extract, NaCl and $MgSO_4$ were determined to be 10, 25, 5 and 0.25 g/L, respectively.

Experimental Validation of Optimized Medium

After repeated experiments employing the optimized medium, the average actual enzyme activity was 345.0 ± 4.1 U/mL, indicating a good agreement between the observed and predicted value. As shown in Fig. 3, the

Fig. 8 Response surface plot for the diaphorase activity as a function ▶ of yeast extract and sucrose (**a**), $MgSO_4$ and sucrose (**b**), $MgSO_4$ and yeast extract (**c**), NaCl and sucrose (**d**), NaCl and yeast extract (**e**) and $MgSO_4$ and NaCl (**f**)

production level of target enzyme was higher compared with basal medium. Overall, these results confirmed the validity and precision of the model. The obtained activity for recombinant *B. sphaericus* diaphorase was significantly higher than reported ones in other literatures [9]. Therefore, produced enzyme has the potential to be used as a component of diagnostic tests such as phenylketonuria, maple syrup urine diseases and galactosemia [12].
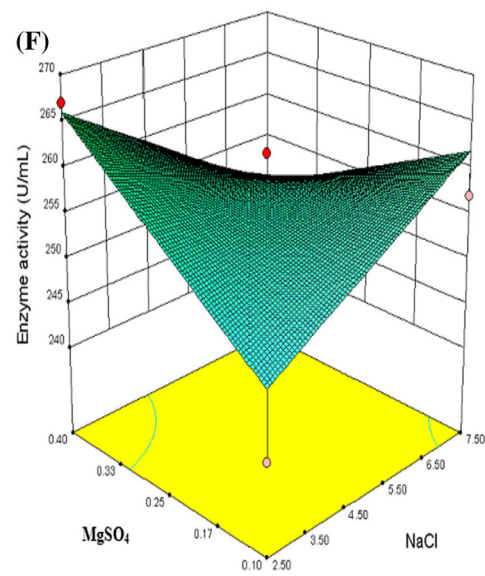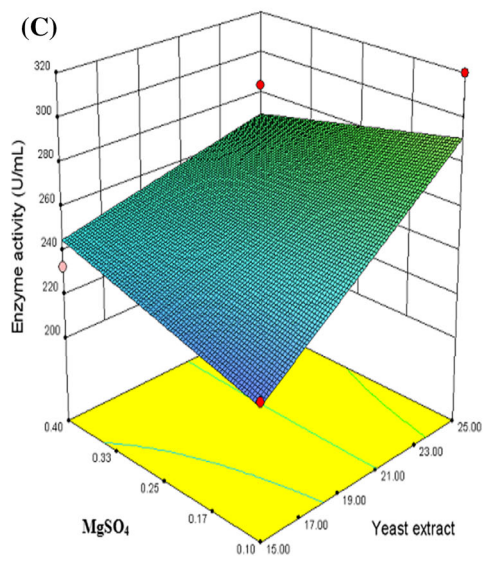
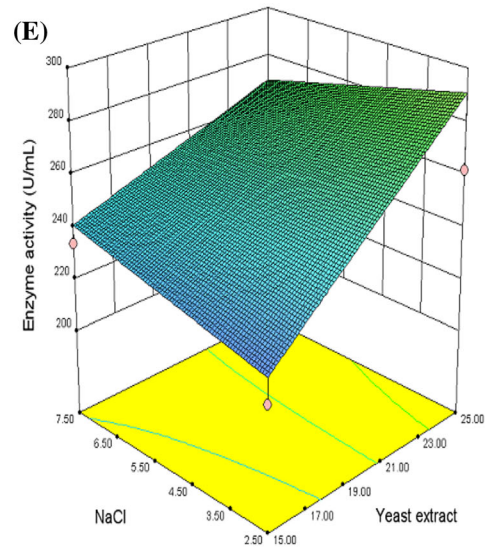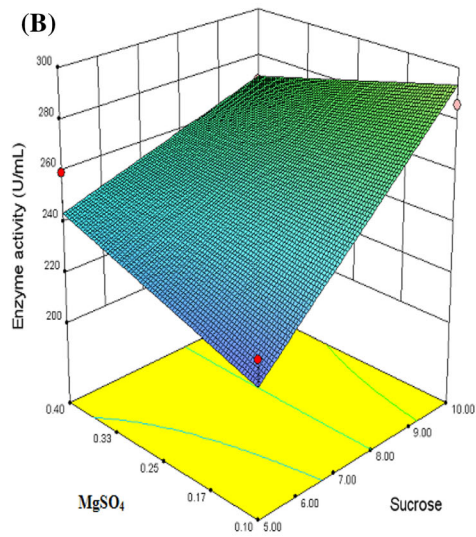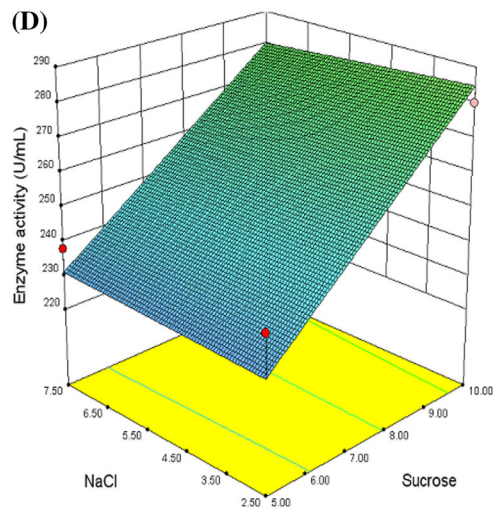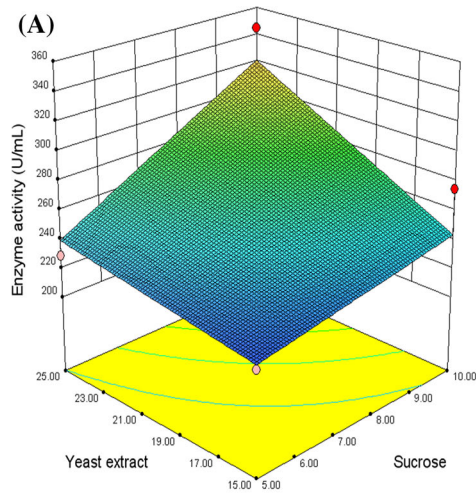## Scale-up Production of Recombinant Diaphorase

Based on the shake flask results, target enzyme production in a batch fermenter was studied to obtain higher enzyme activity. The experiments with the initial pH of the production medium showed that amylase production was optimized at pH 7.0 (Fig. 9a). The effects of inoculum size (v/v) and agitation on diaphorase production were investigated. Maximum production was obtained with 10% inoculum. Further increase in inoculum size causes the reduction in diaphorase production (Fig. 9b). The decrease in enzyme production with the increase in the percentage of inoculum might be due to the initial rapid growth of bacterium and lack of sufficient nutrient available in the medium for higher volume of bacteria. Optimum diaphorase production was obtained at 90 rpm (Fig. 9c). Further increase in agitation that decreased diaphorase

Table 2 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

(A)

(D)

(B)

(E)

(C)

(F)
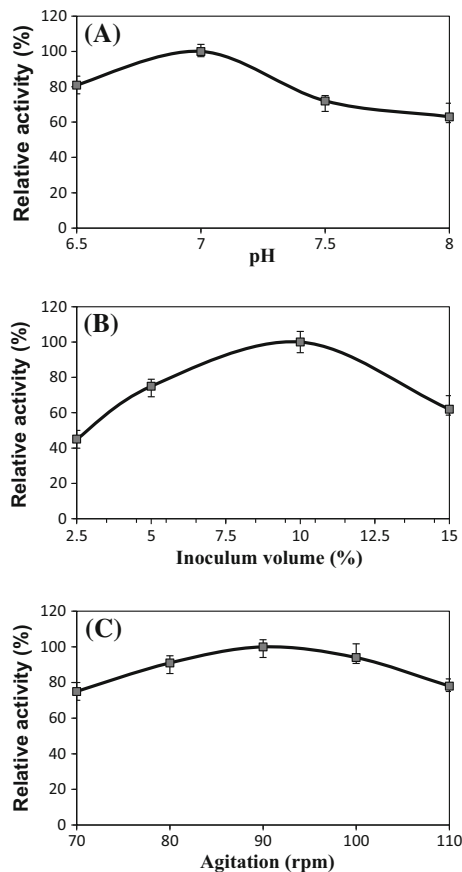
Fig. 9 Effects of initial pH (**a**), inoculum volume (**b**) and agitation (**c**) on recombinant diaphorase production in bioreactor
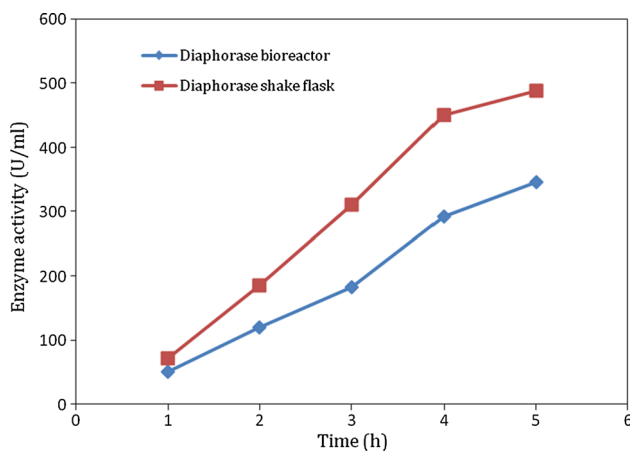
Fig. 10 Comparison of diaphorase production in shake flask and fermenter under optimized condition

production might be due to cell lysis at higher agitation. The activity of recombinant enzyme under optimized conditions in bioreactor was higher (486.3 ± 5.5 U/mL) than that obtained in parallel sake flask culture (345.0 ± 4.1 U/mL) (Fig. 10).

Conclusion

In this article, we presented recombinant expression, characterization and medium optimization of a new DLD enzyme with diaphorase activity from a newly identified strain of *B. sphaericus*, named PAD-91. The *DLD* gene consisted of 1413 bp encoding a protein with MW of 50 kDa. Recombinant diaphorase produced in *E. coli* BL21(DE3) exhibited its optimal activity at temperature of 30 °C and pH 8.5. K_m and V_{max} values with NADH were determined to be 0.025 mM and 275.8 U/mL, respectively. The most appropriate medium for the production of recombinant enzyme was composed of sucrose 10 g/L, yeast extract 25 g/L, NaCl 5 g/L and $MgSO_4$ 0.25 g/L. At optimized condition, the actual recombinant diaphorase activity was calculated to be 345.0 ± 4.1 U/mL. Fermentation in 5-L bioreactor resulted in a further enhancement of the enzyme activity to 486.3 ± 5.5 U/mL. Collectively, optimization of culture medium and growth conditions reduced the cost of production and improved the feasibility of commercial production of the target enzyme. Our results make it valuable to do further investigations on diaphorase from *B. sphaericus* PAD-91.

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.

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

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