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Isolation, purification and characterization of a salt-active and organic-solvent-thermostable phenylalanine dehydrogenase from *Bacillus nanhaiensis* DSF-15A2

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ABSTRACT

Background: As phenylalanine dehydrogenase (EC 1.4.1.20, PheDH) activity and stability was still diminished in organic solvent and biocatalytic reaction in non-aqueous media has presented as a great alternative to chemical synthesis and biosynthesis, suggesting that it is crucial to obtain a PheDH with high organic solvent-tolerant.
Results: It is the first case when thermo-solvent tolerant PheDH producing bacterium was isolated from a marine sample. The *Bn*PheDH' stability could be significantly improved by 1 M NaCl, which was analyzed by molecular dynamics simulation, and it showed better stability in methanol, ethanol, ethylene glycol, ethylene glycol monoethyl ether and acetone. The k_{cat}/Km in 30% (v/v) methanol and ethylene glycol monomethylether was 1.15- and 1.35-fold than that of in the aqueous phase, indicating that the catalytic efficiency was significantly improved by the methanol and ethylene glycol monomethylether.
Conclusions: The results indicated that the affinity of enzyme, substrate and co-factor and the catalytic efficiency can be significantly improved by organic solvents, suggesting that the *Bn*PheDH had great application value in organic phase or aqueous-organic phase catalytic reaction, and it would be used as an robust biocatalyst to synthesize synthons in the food and pharmaceutical industries.

1. Introduction

Enzymatic synthesis reaction in non-aqueous media has presented as an attractive alternative to biosynthesis and chemical synthesis. Numerous methods have been exploited for carrying out biosynthesis reactions in organic media containing multiphase reverse micelles media, aqueous-organic and monophasic media [1–5]. Biocatalysts are useful for biosynthesis and organic synthesis [6] in consideration of their better stereo-selectivity, high product yield and high catalytic efficiency. Compared to chemical catalysts, biocatalysts are also ecologically friendly. However, biocatalysts ideally need high stability and activity in circumstances containing organic solvents for industrial production. To date, majority of synthetic uses of enzymes, including those in aqueous-organic and non-aqueous solvents, have included hydrolases like lipases. The influences of organic media are extra straightforward in these cases, especially for the stereoselectivity [7,8]. It is well known that organic solvents can be used to modulate the stereoselectivity as it can change the enzymatic selectivity of hydrolases [9]. Several cases of solvent-controlled stereoselectivity of the lipases have been reported [10]. Furthermore, lipases showed an increased thermostability, the probability of using highness hydrophobic substrates in aqueous phases which are insoluble, and an improved control of unwanted side-reactions in the presence of organic solvents.

Oxidoreductases have a great advantage up lipases in the manufacture of enantiomerically pure compounds as they can permit the substrate conversion over 100%. In this connection lots of alcohol dehydrogenases display a broad substrate range and high stereoselectivity [11], while dehydrogenases have been used fewer often in organic solvents [12] as the prime drawback being their poor stability in non-aqueous solvents and the need for a cofactor

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which is unstable and sparingly soluble in some organic solvents [13]. Even the interesting contributions have appeared on alcohol dehydrogenases [14], PheDH with high stability and activity in organic-aqueous solvent have few reported.

PheDH is a NAD^+-dependent oxidation-reduction enzyme that catalyzes reversible deamination of phenylalanine and a few of its analogues to the homologous keto acids. PheDHs have been broadly used as biocatalysts to synthesize synthons for the food and pharmaceutical industries e.g. production of L-phenylalanine (L-phe) for aspartame (sweetener) production and enantiomerically pure non-natural amino acids for drug precursors [15,16], and also as diagnostic kits for phenylketonuria (PKU) [17,18]. Organic solvents own the following advantages: (i) favourable shifts of reaction equilibrium, (ii) greatly increased solubility of hydrophobic substrates, (iii) suppression of water-dependent side reactions, (iv) catalysis of reactions impossible in water and (v) resistance to microbial contamination [19,20]. Bear these conditions limit the application of enzymes in industrial production. Hence, the hunt for robust enzymes e.g. PheDH with good stability and high activity in conditions containing organic solvents is of grand practical importance.

Marine organisms are significantly different from terrestrial microorganisms for their special and various living environments and are still an unexploited resource with potential profits for industrial application [21,22]. Considering these reasons, a marine microorganism *Bacillus nanhaiensis* DSF-15A2 with high PheDH activity, was isolated. A gene encoding a novel *Bn*PheDH expected to be organic solvents tolerant and salt-adaptative was excavated and expressed in *Escherichia coli* BL21 (DE3). The biochemical properties of the *Bn*PheDH were studied and its stability towards aqueous phase and organic solvents were also determined.

2. Materials and methods

2.1. Materials

The marine bacterium *Bacillus nanhaiensis* DSF-15A2 (the strain number CGMCC 8969) was preserved in the China General Microbiological Culture Collection Center (CGMCC) and cultured at 28 °C when it was used. Enzymes such as Taq DNA polymerases, PrimeSTAR Max, DNA polymerase, restriction endonucleases, T4 DNA ligase were purchased from Takara (Dalian, China). L-phe, NADH, NAD^+ and other compounds were obtained from Sigma (St. Louis, MO, USA). Buffers and chemicals were of biotechnology grade or HPLC grade. DNA purification Kit and Protein Quantification Kit were purchased from Pharmacia Co. (Sweden) or Axygen Co. (USA). All oligonucleotide primers (Table 1) and fragments were synthesized and sequence by Sangon Biotech Co. (Shanghai, China). *Escherichia coli* BL21 (DE3) (Novagen, USA) and *Escherichia coli* DH5a (Dalian, China) were used as the bacterial hosts for heterologous expression and plasmid amplification, respectively. Luria–Bertani (LB) medium containing kanamycin (50 μg/mL) was used to culture *Escherichia coli* DH5a and *Escherichia coli* BL21 (DE3), and marine agar 2216 medium was used to incubate the marine bacteria.

2.2. Isolation of bacterial strains

To isolate microorganisms with high PheDH activity, the bacteria samples, which were supplied by Marine Culture Collection of China (MCCC, China), were cultured in marine agar 2216 medium at 30 °C for 3–4 days, until the optical density at 600 nm (OD 600) of 0.8–1.0. Then, cells were harvested for enzyme assay and then washed with PBS buffer (0.8% NaCl, 0.02% KCl, 0.142% Na_2HPO_4, 0.027% KH_2PO_4; pH 7.2) by centrifugation at 6000*g* for 10 min. Such treated cells were suspended in PBS (pH 7.0), disrupted by an Ultrasonic Cell Disruption System and the crude enzyme was obtained following by centrifuged 30 min at 4 °C, 8000*g*. Based on the PheDH activity, the marine bacterium, *Bacillus nanhaiensis* DSF-15A2 (CGMCC No. 8969) showed the highest PheDH activity was selected and used in this work.

2.3. Cloning of the Bn*PheDH gene*

We cannnot directly clone the *pdh* gene from *Bacillus nanhaiensis* DSF-15A2 as there is no reported about its genome information. Using NCBI genome database, 10 PheDH genes information (ACF96938.1 and BAA08816.1 of similarity for 100%; EWG09095.1 and EFV75869.1 of similarity for 99%; EDL64419.1 and EAR66050.1 of similarity for 95%; CEG27346.1; EIT85807.1 and EZP75760.1 of similarity for 95%; AAA22646.1) which come from *Bacillus* genus, was found. Then, 6 group primers (Table 1) were designed according to the homology comparison.

Degenerate primers (F1-6/R1-6; containing *Nde*I and *Xho*I sites, red, respectively) were applied to amplify the *pdh* gene. PCR was executed using PrimSTAR Max as follows: the reaction was started at 94 °C (10 min), followed by 35 cycles: 94 °C (15 s), 54 °C (15 s), 72 °C (45s), with a final extension at 72 °C (10 min). The reaction was carried out in 20 μL reaction volumes containing 2 μL of each primer, 1 μL of template DNA, 1.0 mM of dNTP, 10 μL PrimSTAR Max. Recombinant plasmids were constructed to express the PheDH in a host of *Escherichia coli*. The PCR product was purified and cloned into pET-28a(+) vector at *Nde*I and *Xho*I sites. After that, the recombinant plasmids were transformed into *Escherichia coli* DH5a and the cell was grown at 37 °C. After sequence, the right recombinant plasmids were transformed into *Escherichia coli* BL21 (DE3) for PheDH expression.

2.4. Expression and purification of recombinant enzyme

The positive plasmid pET-28a-*pdh* was transformed into *Escherichia coli* BL21 (DE3) to express the *Bn*PheDH. The cells were grown in LB medium with kanamycin (50 μg/mL) at 37 °C, subsequently the mixture was transferred to new LB medium (1–3:100 dilution) with kanamycin (50 μg/mL) and cultured at 37 °C for 2–4 h until the OD 600 of 0.5–0.7. After that, a final concentration of 0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) was added, followed by cultivation for 12 h at 18 °C and 200 rpm. Then, cells were harvested and washed with PBS buffer twice by centrifugation at 6000*g* for 10 min, suspended in PBS (pH 7.2) and disrupted using an Ultrasonic Cell Disruption System and the crude enzyme was obtained by centrifuged 30 min at 4 °C, 8000 g. Then His-tagged enzyme was purified by employing Ni-IDA column.

2.5. Enzyme assay

PheDH activity was measured at 25 °C spectrophotometrically by detecting the change in concentration of NADH at 340 nm [23,24]. One unit (U) of enzyme activity was defined as the oxidation of 1 μmol of NAD^+ to NADH per-minute. The assay buffer contained 20 μmol glycine-NaOH, 5.0 μmol NAD^+, the debita spissitudine amino acid substrate, PheDH and organic solvent in a total volume of 500 μL. Kinetic parameters for each substrate were determined at a fixed concentration of 200 mM amino acid substrate or 5.0 mM NAD^+. Specific activity of the *Bn*PheDH was expressed as U/mg protein. The method of Bradford was used to determine the protein concentrations.

2.6. Biochemical characterization of the enzyme

The optimal pH of *Bn*PheDH was measured at 25 °C by introducing the purification *Bn*PheDH into three different buffers: PBS