

# [Synthesis of chiral drug intermediates](https://assignbuster.com/synthesis-of-chiral-drug-intermediates/)

Given the important role of phenylalanine dehydrogenase (PheDH) in the synthesis of chiral drug intermediates and detection of phenylketonuria, suggesting it is significant to obtain a PheDH with special and high activity. Here, a novel PheDH gene, pdh , encoding a Bs PDH with 61. 0% similarity to the known PheDH from Microbacterium sp ., was obtained. The Bs PDH showed the optimal activity at 60°C and pH 7. 0, and was more stable in hot environment (40-70â„ ƒ) than Nocardia sp.’s PheDH. Its activity and thermostability could be significant increased by sodium salt, showing the highest activity (138% of the activity) at 3 M NaCl, retaining nearly 100% activity at 6 M NaCl and the residual activity of Bs PDH increased from 43% to 77 % after 2 h incubation at 60â„ ƒ, compared to the absence of NaCl. These characteristics indicating Bs PDH possess better thermostability, halophilic and higher salt activation. The mechanism of the thermozyme and high salt-tolerant of Bs PDH was analyzed and verified by molecular dynamics simulation. These results provide useful information about the enzyme with high-temperature, thermostability, halophilic, higher salt activation and enantio-selectivity, and the application of molecular dynamics simulation in analyzing the mechanism of these special characteristic.

NAD(H), phenylpyruvate, L-phenylalantne and D-phenylalantne were purchased from Sigma–Aldrich Co. (Shanghai, China). All the other chemicals were analytical chromatographically pure or analytically graded and used without further purification. And they were purchased from Sinopharm Chemical ReagentCo., Ltd. (China). DNA polymerase, restriction Ex Taq TM DNA, endonucleases, T4 DNA ligase and other enzymes were purchased from TaKaRa Co., Ltd. (Dalian, China). Mutan BEST Kit, Ligation solution I, Purification Kit Ver 2. 0 and Agarose Gel DNA Fragment Recovery Kit Ver. 2. 0 were purchased from TaKaRa Co., Ltd. (Kyoto, Japan).

The marine bacterium B. halodurans (the strain number MCCC MCCC 1B00241) was obtained from the Marine Culture Collection of China (MCCC) and cultured at 30 °C in 2216 medium. Escherichia coli BL21 (DE3) and Escherichia coli DH5α ( E. coli DH5α) were used as host strains for heterologous expression and choning, respectively.

The specific primers (F-Bn-pdh: GGAATTC CATATG ATGCTAACGAAAACGCCAACTGTCAC and R-Bn-pdh: CCC AAGCTT CTATTTACGTAAGTTCCATTTCGGCC; containing Nde I and Hind III sites, underlined, respectively) were employ in amplify the pdh gene by polymerase chain reaction (PCR) under the following steps: the reaction was started at 95 °C (5 min), followed by 30 cycles: 95 °C (30 s), 55 °C (30 s), 72 °C (1min), with a final extension at 72 °C (7 min). Primerstar Max polymerase was implemented to finish this amplification and the reaction was carried out in 40 μL reaction volumes containing 1 μL of each primer, 1 μL of template DNA, 20 μL Primerstar Max and 17 μL sterile ddH 2 O. For construction a recombinant plasmid to express the Bs PDH in BL21 (DE3), the PCR product was purified and cloned into pET-28a (+) vector with Nde I and Hind III as the restriction enzyme cutting sites, generating pET-28a- pdh . After that, the recombinant plasmids were transformed into E. coli DH5α for the culture and grown at 37 °C. The establishment accuracy was confirmed by sequencing and the positive recombinant plasmids were transformed into BL21 (DE3) for further study.

The recombinant BL21 (DE3) strains were cultured in LB medium (containing 100μg/mL kanamycin) and incubated at 37°C for 12h. Subsequently, the mixture was transferred into fresh LB liquid medium (containing 100μg/mL kanamycin) and cultured at 37°C for 5 h. When OD600 reached 0. 6-0. 8, the isopropyl-β-d-thiogalactoside (IPTG) was added with a final concentration of 0. 1 mM for induce the protein expression, and the mixture was incubated at 22°C for 8 h. Then, the cells were harvested and disrupted by centrifugation at 12, 000 rpm for 20 min and resuspended in 100 mM HEPES (pH 7. 0) with High Pressure Homogenizer (Niro Soavi, Germany) and the resuspended cells were lysed by sonicating for 10 min at 4°C. After centrifugation at 12, 000 rpm for 40 min, the supernatant was applied to an AKTA Prime system equipped with a 10-mL HisTrapTMFF column (GE Healthcare, USA). Finally, the expression and purity of the enzyme was checked by 12% SDS-PAGE according to the method of Laemmli [46] and the protein concentration was calculated using Bradford Protein Assay Kit.

PheDH activity for the reductive amination was assayed at 25 °C by measuring the consumption of NADH at 340 nm (É›= 6, 220 M -1 cm -1 ) with a Hitachi U-3210 spectrophotometer in the reaction mixture (0. 5 ml) containing 100mM glycine-KCl-KOH buffer (pH 10. 4), 0. 05 mM NADH, 50 mMNH 3 ·H 2 O-NH 4 Cl buffer, 20 mM sodium phenylpyruvate, and moderate enzyme. The enzyme activity for oxidative deamination was determined at 25 °C by the reduction of NAD + (monitored at 340 nm) with D-phenylalanine or L-phenylalanine as a substrate. The reaction mixture (0. 5 ml) contained 50 mM glycine-KCl-KOH buffer (pH 10. 4), 2 mM NAD + , 20 mM D-phenylalanine or L-phenylalanine, and moderate enzyme. One unit (U) of the enzyme activity is defined as the amount of enzyme catalyzing the formation or consuming of 1μmol NADH per min in the oxidative deamination of L-phenylalanine or reductive amination of phenylpyruvate, respectively, under the standard assay conditions. Specific activity was recorded as units/mg protein.

For the part of the reductive amination, the optimal pH of Bs PDH was determined at 25°C in different buffers at pH 4-11, namely 0. 2 M acetic acid, sodium acetate buffer (containing 0. 2 NH 4 Cl, pH 4-6. 0) and 0. 2 M NH 3 ·H 2 O-NH 4 Cl buffer (6-11. 0). The optimum temperature of Bs PDH was determined by incubating the reaction mixtures at different temperatures (0-85â„ ƒ) under pH 7. 0 after pre-incubating the reaction system (without NADH) at corresponding temperatures for 20 min. The thermal stability of the enzyme was assayed under the optimal pH by pre-incubating the Bs PDH at temperatures from 37°C to 70°C for 120 min and the residual enzyme activity was measured as described above. The pH stability of the Bs PDH was determined while it was incubated at 4°C for 96 h in different buffer systems (pH 6-9. 5), and then the remaining activity was measured under the standard. The biochemical characterization of the oxidative deamination of the Bs PDH was performed with the same methods, except the buffers were 0. 2 M acetic acid-sodium acetate buffer (pH 4-6. 0), barbital sodium-hydrochloric acid buffer (6-9. 0) and 0. 05 M glycine-sodium hydroxide buffer (8. 6-11).

The effect of NaCl on the purified Bs PDH activity was determined in NH 3 ·H 2 O-NH 4 Cl buffer (pH 7. 0) or barbital sodium-hydrochloric acid buffer (7. 0) containing various concentrations of NaCl (0-6 M). The effect of NaCl on the Bs PDH thermo stability was determined by diluting the purified enzyme with 3 and 4 M NaCl for the reductive amination and oxidative deamination, respectively, and then incubating the mixture at 37, 40, 45, 50, 55, 60, 70 and 80 °C for 120 min. For each assay, a control group without NaCl was assayed under the same conditions. The residual activity of Bs PDHwas measured under standard methods conditions as described above.

The fluorescence spectra were obtained by Jasco Circular Dichroism Chiroptical Spectrometer (CD/ORD, J810, Japan). Using an excitation wavelength of 280 nm, the intrinsic fluorescence was measured. The emission spectra were recorded at the wavelengths from 180 to 280 nm. The samples were pre-incubated for 120 min at 60 °C in 0 and 3 M NaCl, respectively.

The amino acids sequence of the Bs PDH was submit to the SWISS-MODEL [1] for search template on the server. Then, the 1leh. 1. A and 3vpx. 1. A, which share the similarity of 45. 01% and 47. 01% with the Bs PDH, were obtained and used as the template for future study. The PDB file of target protein Bs PDH was constructed by manual modeling in EasyModeller4. 0 [1].

To study molecular dynamics characteristics of the halophilic Bs PDH, the molecular dynamics simulation of three groups of different salt concentrations, 0 M, 1 M, 3 M, were designed, and the atomic number of three groups of experiments were 55222, 53990 and 51530 respectively. Using the CHARM36 force field, atmospheric pressure (101. 325 kPa) as the simulation pressure, the temperature of 333 k, and in the side length for 84 Å cubic water, the three systems were molecular dynamics simulations of 15 ns by using the software NAMD [2] and visualization software VMD [3]. The methods, Langevin Piston andLangevin Thermostat were selected to control of pressure and temperature fluctuations. PME [4] was used to calculated the long-range electrostatic forces and non-bonded interactions was calculated by the potential energy truncation with a radius of 1. 35nm, for systems using periodic boundary conditions, the use SHAKE algorithm so that the water molecules remain rigid. Using periodic boundary conditions for the systems and the SHAKE algorithm makes use of keep the water molecules remain rigid. The time step was 2fs, calculation results was outputted once every 1 ps. all simulations were isothermal-isobaric ensemble under (NPT) carried out. All simulation was implements under the isothermal-isobaric ensemble (NPT).