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RESEARCH

Efficient bioremediation of multiple steroid hormones by halotolerant 17β-hydroxysteroid dehydrogenase derived from moderately halophilic *Pontibacillus chungwhensis* HN14

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Abstract

Steroid hormones exhibit potent endocrine disrupting activity and have been shown to disrupt the equilibrium of aquatic ecosystems and pose a threat to public health through their persistent and carcinogenic effects. *Pontibacillus chungwhensis* HN14, a moderately halophilic bacterium with the capacity to effectively degrade various polycyclic aromatic hydrocarbons and other organic pollutants, was previously isolated. Additionally, the strain HN14 showed strong environmental adaptability under various environmental stress conditions. In this study, the steroid degradation by strain HN14 was studied for the first time. We demonstrated that strain HN14 could degrade estradiol (E2) to maintain the growth of the strain and could convert E2 to estrone. Additionally, the efficient substrate degradation efficiency of *P. chungwhensis* HN14 under high salinity and high substrate concentration conditions was demonstrated. Furthermore, a 17β-hydroxysteroid dehydrogenase, 17β-HSD(HN14), was identified in strain HN14. Comparative analysis reveals that 17β-HSD(HN14) shares approximately 38% sequence identity with 17β-HSDx from *Rhodococcus* sp. P14. In addition, 100 μg of purified 17β-HSD(HN14) could effectively convert about 40% of 0.25 mM of E2 within 1 h period, with an enzyme activity of 17.5 U/mg, and catalyze the dehydrogenation of E2 and testosterone at the C-17 position. The characterization of purified enzyme properties reveals that 17β-HSD(HN14) exhibits exceptional structural robustness and enzymatic efficacy even under high salinity conditions of up to 20%. Overall, this study enhances our comprehension of steroid biodegradation in strain HN14 and contributes novel ideas and theoretical underpinnings for advancing bioremediation technologies targeting steroid pollution in high-saline environments.

Keywords 17β-HSD · Steroids · *Pontibacillus* · Biodegradation · High salinity

Introduction

Endocrine disrupting compounds (EDCs) exert potent endocrine-disrupting effects (Narindri Rara Winayu et al. 2024). Steroid hormones, as the typical type of EDCs, pose a severe threat to humanity and the ecological environments due to their carcinogenic effects. Previous studies have shown that steroid hormones, including estrone (E1), estradiol (E2), estriol (E3), and testosterone (T) detected in the environment are mostly discharged from wastewater of municipal sewage treatment plants, hospitals, animal husbandry, and aquaculture industry (Tian et al. 2022; Zhang et al. 2016, 2024). More seriously, steroid hormones can accumulate in marine sediments, and soils (Ye et al. 2019). In comparison with other natural estrogens, E2 has the highest estrogenic activity and biological toxicity and is more likely to cause

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breast cancer in women (Samavat and Kurzer 2015). Furthermore, low concentrations of E2 can induce the development of female characteristics in male fish suggesting that E2 can cause endocrine disorders in animals (Song et al. 2018). Therefore, it is imperative to reduce or eliminate the toxicity of E2 and its metabolic intermediates.

Microbially mediated bioremediation represents an efficient and environmentally friendly approach for eliminating E2 from the environment. Numerous studies have indicated that bacteria play essential roles in E2 biodegradation (Silva et al. 2012; Pratush et al. 2020). Bacteria including *Bacillus*, *Rhodococcus*, *Pseudomonas*, *Buttiauxella*, *Virgibacillus*, and *Microbacterium* exhibit remarkable efficiency in degrading steroids as natural degraders in the environment (Zhang et al. 2011; Fernández et al. 2017; Ye et al. 2019; Miao et al. 2023). The microbial degradation of E2 involves a series of enzymatic catalytic reactions, which typically include dehydrogenation, hydroxylation, isomerization, methoxylation, acylation, and hydrolysis. In addition, these reactions are crucial steps in breaking down E2 into simpler and less harmful compounds (Talalay and Wang 1955; Ye et al. 2017; Van der Geize et al. 2008). These functional enzymes include dehydrogenase, cytochrome P450 monooxygenase, ring-cleavage dioxygenase, decarboxylase, hydroxylase, isomerase, aldolase, and demethylase (Ye et al. 2019; Genti-Raimondi et al. 1991; Yang et al. 2024; Kristan and Rižner 2012). Prior studies have identified that bacteria can catalyze the dehydrogenation of E2 at C-17 and convert E2 into E1 by using dehydrogenase, including OecA and 17β-HSD (Wu et al. 2019; Ye et al. 2019). This biotransformation process reduces the toxicity and harm of E2 and reduces its damage to microorganisms themselves (Chen et al. 2018). In addition, hydroxylation by monooxygenase enzymes such as OecB or other monooxygenases at the C4 position of E1 can lead to the cleavage of the A ring structure of the molecule (Chen et al. 2017; Miao et al. 2023; Tian et al. 2022; Peng et al. 2023). Similarly, oxidation of B and D ring allow E1 to be converted into different ring cleavage products (Li et al. 2012; Horinouchi et al. 2019; Ye et al. 2022). These ring cleavage products are further degraded and finally enter the TCA cycle.

The genus *Pontibacillus* is a typical moderately halophilic bacterium and exhibits strong environmental adaptability, including high salt and heavy metal tolerance and organic pollutant degradation (Yang et al. 2024). In previous studies, *Pontibacillus chungwhensis* HN14 was isolated from mangrove surface sediments and identified to have the ability to degrade various polycyclic aromatic hydrocarbons (PAHs) (Qian et al. 2022, 2024). However, limited information about the degradation characteristics and mechanism of steroids in *Pontibacillus* has been reported.

This study evaluated the degradation ability of E2 in strain *P. chungwhensis* HN14 under different stress conditions by substrate degradation assay. Additionally, a 17β-hydroxysteroid dehydrogenase from strain HN14, 17β-HSD(HN14), was identified and characterized. 17β-HSD(HN14) has low amino acid similarity to the reported 17β-HSDs and exhibits excellent structural stability and catalytic activity under high salinity conditions. The degradation products were identified by liquid chromatography-tandem mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS). The in-depth study suggested that strain HN14 has more advantages of degrading steroids in harsh environments, and dehydrogenase 17β-HSD(HN14) may be useful in bioremediation of hypersaline environments contaminated with steroids.

Materials and methods

Strain and chemical preparation

P. chungwhensis HN14 (GDMCC 62925) used in this study was isolated from mangrove surface sediments, Hainan, China. Table S1 has listed the other plasmids and strains used in this study. Sigma Aldrich Co. (St. Louis, MO, USA) provided E2, E1, E3, 17α-ethynylestradiol (EE2), T, and androst-4-ene-3,17-dione (AD) (> 98% purity). All chemicals were purchased from standard manufacturers and were of chromatographic grade. The Bacteria Genomic DNA Quick Extraction Kit, High Pure Maxi Plasmid Extraction Kit, EasyTaq® DNA polymerase, and dNTPs were purchased from TIANGEN BIOTECH Co., LTD. (Beijing, China). The restriction enzymes *Eco*R I and *Pst* I and their corresponding buffers, and DNA polymerase were purchased from Takara (Dalian, China).

5 g of tryptone, 1 g of yeast extract, 0.074 g of K_2HPO_4, and 0.002 g of $Fe_2(SO_4)_3$ were dissolved in 1 L filtered seawater to obtain the 2216E medium. The minimal salt medium (MSM, L^{-1}) consisted of 1 g $(NH_4)_2SO_4$, 0.8 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g $MgSO_4$, 10 g NaCl, 0.005 g $FeCl_3$, and 0.001 g $(NH_4)_2MoO_4$. The Luria-Bertani broth (LB, L^{-1}) comprises 10 g tryptone, 5 g yeast extract, and 10 g NaCl. The M9 liquid medium (M9, L^{-1}) contained 1 g NH_4Cl, 6 g Na_2HPO_4, 2.5 g NaCl, 3 g KH_2PO_4, 1 mL $MgSO_4$ solution (1 mol/L), 5 mL glucose solution (20% (W/V)), and 100 µL $CaCl_2$ solution (1 mol/L).

E2 degradation characteristics in *P. chungwhensis* HN14

The strain HN14 was cultured to the logarithmic growth period in 2216E medium. The bacteria were collected

by centrifugation at 5000 ×g, 4 °C for 15 min, and were washed with MSM medium three times. Then the cells were resuspended in 4 mL of MSM liquid medium (Prakash et al. 2023). The suspensions were added to MSM supplemented with 20 mg/L E2. The initial concentration of strain HN14 was adjusted to OD_{600nm} of 0.2. The bacterial growth was monitored by measuring the value of OD_{600nm}. In addition, for the degradable substrate concentration, 20, 50, 100, and 200 mg/L E2 were regarded as the initial substrate concentrations. Salinity gradients ranging from 1 to 25% (mass/volume) NaCl concentration (1%, 3%, 5%, 7%, 10%, 15%, 20%, and 25%) were employed for the degradation assay. The samples of different treatments were placed in the instrument SHAKER INCUBATOR (TS-200B) for culture in the dark. The culture temperature was set at 30℃, and the rotation speed was adjusted to 150 rpm. Non-inoculated MSM medium with matching E2 concentration and salinity levels was set as the control experiment. Cultures were incubated for between 1 and 9 d. All treatments were replicated three times. Following induction, the remaining concentration of E2 was analyzed using High-performance liquid chromatography (HPLC). The calculation method for degradation efficiency was consistent with previous study (Yang et al. 2024). The metabolic intermediates formed during substrate degradation were identified by GC-MS (Agilent Technologies/Hewlett Packard) and LC-MS (Thermo UltiMate 3000/TSQ ENDURA). The sample preparation methods for GC-MS and LC-MS were consistent with 2.6. The detected results of GC-MS were analyzed according to the mass spectrometry ion peaks (fragment ions and parent ions) and compared with the standards in the mass spectrometry database of NIST14. Details of GC-MS and LC-MS were consistent with 2.3. The detected results of LC-MS were analyzed according to the LC-MS profile of E2 degradation intermediates (Tian et al. 2022).

Data analysis

For characterizing the degradation rate of E2 or T, EE2 was included as an internal reference in 50 mL cultures. These cultures were subsequently extracted using equal volumes (50 mL) of ethyl acetate. Subsequently, 1 mL of the organic extract was injected into a chromatographic bottle. After the ethyl acetate was completely volatilized, it was redissolved with 1 mL acetonitrile: water = 1: 1 solution and used for HPLC detection. HPLC was performed using the Agilent 1260 Infinity with WondaSilTM C18 columns (4.6 m, 250 mm diameter, 5 µm film thickness). The specific experimental parameters are 50% acetonitrile, 50% water, 1 mL/min, 35 °C, pressure of 107 Bar, and injection volume of 20 µL. The wavelength of 205 nm was set to detect E2, E3, and EE2 (EE2 as an internal reference) (Zhang et al. 2018). The wavelength of 245 nm was set to detect T. The data were used to calculate the degradation efficiency of E2 by *P. chungwhensis* HN14.

The degradation rate formula η was defined as

$$Cs = Cdn * Csd/Csn;\ \eta = (Cdd - Cs)/Cdd \times 100\%$$

Where Cs was the actual peak area of E2 or T in the experimental group; Cdn was the peak area of internal reference EE2 in the control group; Csd was the peak area of E2 or T in the experimental group; Csn was the peak area of EE2 in the experimental group; Cdd was the peak area of E2 or T in the control group.

For GC-MS analysis, the detection program was adjusted according to modifications described in a previous study (Zhang et al. 2024). The initial column temperature was adjusted to 50 °C and held for 3 min. Subsequently, the column temperature was increased at a rate of 10 °C/min to 280 °C and held for 9 min. In full-scan mode over 5.5 to 33 min, the mass spectrometer was operated in electron impact mode at 70 eV from 50 to 500 m/z. The injection mode was non-split mode. Detector and injector temperatures were set to 200°Cand 280 °C, respectively (Chen et al. 2018).

For LC-MS, the column was Hypersil GOLD™ Dim. The mobile phase was acetonitrile: water (v/v) = 1: 1. The flow rate was set to 0.2 mL/min, and the injection volume was 10 µL. The column compartment temperature was maintained at 30℃. For ion source properties, the ion source type was electrospray ionization (H-ESI), the scanning mode was positive and negative ion common scanning mode with a capillary voltage of 3500 V and 3000 V, respectively, the sheath gas was 45 Arb, the aux gas was 15 Arb, the ion transfer tube temperature was 300℃, and the vaporizer temperature was 220℃. The ESI scan range was 50 to 600 m/z (Tian et al. 2022).

Bioinformatics analyses

The genome information for *P. chungwhensis* HN14 can be found in GenBank under the accession number CP126449. The protein sequence of 17β-HSD(HN14) has been submitted to NCBI under accession number WIF96803.1. The target sequence was retrieved using the alignment tool NCBI-Blast-2.12.0+ (Camacho et al. 2009). The query sequence used for the Blast search is the protein-coding sequence from 17β-HSDx of strain *Rhodococcus* sp. P14 (ASC49558.1) (Ye et al. 2019). Multiple sequence alignments were performed by DANMAN, and all parameters were set to default values (Ye et al. 2017). Additionally, the protein sequences of 17β-HSDs with the same dehydrogenation function and from different hosts were downloaded

from the NCBI database and regarded as the reference sequence needed to construct the phylogenetic tree. Subsequently, an initial Neighbor-Joining phylogenetic tree was constructed using MEGA7 (Kumar et al. 2016). The obtained phylogenetic trees were enhanced using Tree Visualization by One Table (tvBOT) (Xie et al. 2023). AlphaFold2 was applied to predict the three-dimensional structure of protein 17β-HSD(HN14) (Jumper et al. 2021). Subsequently, the molecular structure of E2 was obtained from the PubChem compound database (Kim et al. 2023). Autodock Vina 1.2.0 was employed for molecular docking analysis (Eberhardt et al. 2021). Briefly, the molecular docking box was automatically generated by the software Autodock Vina 1.2.0. Subsequently, the size of the molecular docking box was adjusted to completely cover the catalytic domain of protein 17β-HSD(HN14) with the substrate E2. After conducting docking simulations, the optimal docking model was chosen based on the binding energy between the substrate and the protein for further analysis (Ye et al. 2017, 2019).

Heterologous expression and purification of 17β-HSD(HN14)

The gene *17β-HSD(HN14)* was amplified by polymerase chain reaction with primers hsd-F and hsd-R carrying restriction endonuclease sites (Table S2). The experimental conditions of PCR were provided in the Experiment S1. Then, the restriction enzymes *Eco*R I and *Pst* I were used to excise the gene *17β-HSD(HN14)* and recombinant plasmid pETDuet-1. Subsequently, the recombinant plasmid pETDuet-*17β-HSD(HN14)* was constructed through ligation of target gene fragments by T4 ligase. The expression plasmid pETDuet-*17β-HSD(HN14)* was transferred into strain *E. coli* BL21(DE3) to obtain the genetically engineered strain for heterologous expression of protein 17β-HSD(HN14).

The strain *E. coli* BL21(DE3)-pETDuet-*17β-HSD(HN14)* and strain *E. coli* BL21(DE3) carrying original plasmid pETDuet-1 were incubated in LB medium with ampicillin until the value of OD_{600nm} reached 0.6–0.8, respectively. Then, 0.2 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the flask and induced at 16°C for 18 h. The cultured cells were harvested at 5000 g, 4°C for 15 min and resuspended using 20 mM Tris-HCl buffer. The collected cells were disrupted using an ultrasonic disruptor (15% power, 10 min) and centrifuged at 5000 ×g, 4°C for 15 min (Ye et al. 2019). Subsequently, the supernatant obtained was labeled as cell crude lysate, while the precipitation solution dissolved in 20 mM Tris-HCl was termed cell debris. Then, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to detect 17β-HSD(HN14) expression. Furthermore, Western blotting analysis assay was carried out with recombinant protein to verify the existence of the target protein as described previously (Ye et al. 2017). The recombinant protein was adsorbed by Ni-NTA affinity chromatography. The purified protein 17β-HSD(HN14) was eluted and obtained with various concentrations of imidazole buffer (20 mM, 50 mM, 100 mM, 200 mM, 300 mM, and 400 mM) (Guo et al. 2023). SDS-PAGE was performed to determine the optimal imidazole elution concentration.

Resting cell biotransformation and characterization of the substrate specificity

The recombinant strain *E. coli* BL21(DE3)-pETDuet-17β-HSD(HN14) was cultured and harvested with the same conditions as the preparation of protein purification. Then, the collected cells were washed four times and resuspended with M9 medium to prepare resting cells (Qian et al. 2022). The resting cells were transferred into 50 mL M9 medium containing a final concentration of 20 mg/L of E2, T, and E3, respectively, to achieve an initial OD_{600nm} of 0.4, and incubated at 30°C, 150 rpm for 2 d. *E. coli* BL21(DE3) strain carrying original plasmid pETDuet-1 served as the control group, and all treatments were identical to the experimental group. A triplicate of each of the treatments above was performed. The residue of E2, T, and E3 was measured by HPLC using the same method described above. Additionally, to detect intermediate metabolites produced during substrate degradation process, the 50 mL cultures were extracted with triple volumes of ethyl acetate. The residual water in the sample was removed by anhydrous sodium sulfate. Then, the extracts were evaporated to 5 mL using a rotatory evaporator at 55 °C. Furthermore, the extracts were dried in a vacuum and dissolved in 1 mL ethyl acetate for GC-MS analysis. The determination procedure of GC-MS is the same as described above. All experiments were conducted in three biological replicates.

Purified enzyme assays

The enzyme activity in vitro was determined using reaction mixtures containing 20 mM Tris-HCl, 250 mM NaCl, 0.25 mM E2, and 0.5 mM NAD^+. (Ye et al. 2019; Guo et al. 2023). Then, 100 μg purified 17β-HSD(HN14) protein was added to 1 mL above reaction mixtures to initiate the reaction at 30 °C and incubated for 1 h. After 1 h, 10 μL of 10 M HCl was added to terminate the reaction. The mixture was extracted with 500 μL of ethyl acetate, and the remaining E2 content was then detected by HPLC (Guo et al. 2023). To further characterize the characteristics and structural stability of 17β-HSD(HN14), the relative activity of 17β-HSD(HN14) under various salinity (0%, 3%,

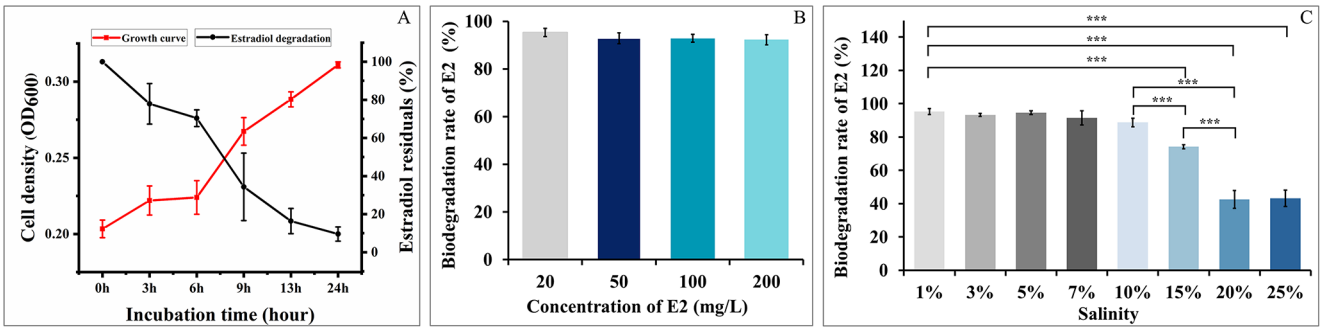

Fig. 1 E2 degradation analysis in *P. chungwhensis* HN14. (**A**) E2 degradation efficiency and growth of *P. chungwhensis* HN14 with 20 mg/L E2 as the sole carbon source. (**B**) The E2 degradation ability of strain HN14 in minimal salt medium with various E2 concentration within 7 d. (**C**) The E2 degradation ability of strain HN14 in minimal salt medium with 20 mg/L E2 under different salinity conditions within 7 d

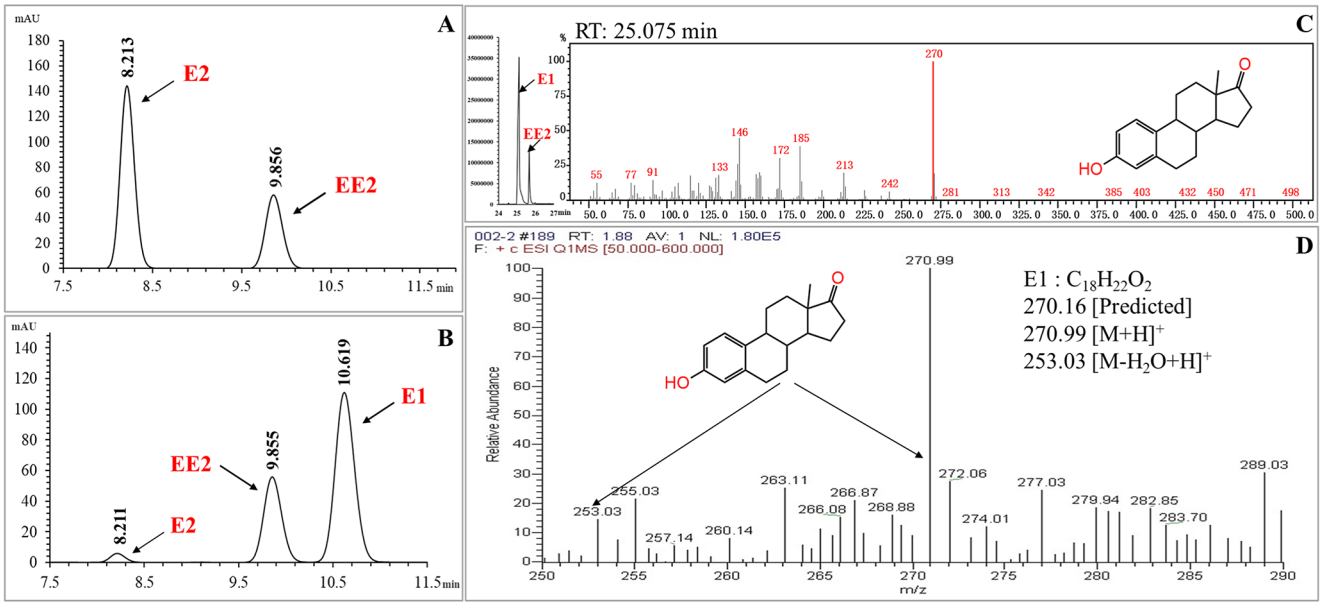

Fig. 2 Identification of the metabolic intermediates of E2 degradation in *P. chungwhensis* HN14 by HPLC (**A**: negative control; **B**: experimental group). (**C**) GC-MS. (**D**) LC-MS. E2: Estradiol; E1: Estrone; EE2: 17α-ethynylestradiol. EE2 is considered as an internal reference. The substrate concentration was 20 mg/L, and the culture time was 1 d

5%, 10%, 15%, 20%), temperature (0°C, 10°C, 20°C, 30°C, 40°C, 50°C, 60°C), and pH (5, 6, 7, 8, 9, 10, 11) conditions was characterized according to the change of absorbance at 340 nm in a microporous plate detector (SynergH1 H1MF), respectively.

Results

E2 degradation analysis in *P. chungwhensis* HN14

When E2 was used as the sole carbon source, the strain HN14 could degrade 87.8% of 20 mg/L E2 during 24 h. In addition, the value of OD_{600nm} of strain HN14 increased from the initial 0.20 to 0.33 (Fig. 1A). Compared with the detection results of the control group (Fig. 2A), one metabolic intermediate with a retention time of 10.594 min was detected by HPLC during the E2 degradation (Fig. 2B), which was speculated to be E1. GC-MS and LC-MS analysis were performed to further verify the accuracy of the predicted metabolic intermediate. The product was finally identified as E1 (retention time of 25.075 min with major ion peaks m/z of 270 (M, 100%), 271 (20%), 242 (7%), 213 (19%), 185 (46%), 172 (36%), 146 (51%), 133 (20%), 115 (20%), 91 (16%) in GC-MS, and retention time of 1.88 min and exhibited m/z values of 270.99 in LC-MS) (Fig. 2C and D).

In order to analyze the degradation characteristics of E2 by strain HN14 under various stresses, the E2 degradation efficiency was investigated under salinity gradient and various substrate concentration conditions. The results showed that strain HN14 can tolerate and efficiently degrade E2

from substrate concentration from 20 to 200 mg/L. Moreover, the phenomenon that the degradation efficiency was significantly inhibited by high E2 concentration was not observed (Fig. 1B). It is worth noting that strain HN14 harbored a high E2 degradation ability in the salinity range from 1 to 10% (Fig. 1C). Even under extremely high salinity concentration (salinity of 25%), it still maintained its ability to degrade E2 with a degradation rate of over 40% during 7 d (Fig. 1C).

Bioinformatics analyses of 17β-HSD(HN14)

Through whole genome retrieval, a protein belonging to the short-chain dehydrogenase (SDR) family oxidoreductase was identified and named as 17β-HSD(HN14) (Table. S3). A neighbor-joining phylogenetic tree based on proteins sequences, including 17β-HSD(HN14), has been constructed (Fig. 3). Building upon sequence homology analysis, 17β-HSD (HN14) shares approximately 38% protein homology with the dehydrogenation-competent 17β-HSDx identified from *Rhodococcus* sp. P14 (Ye et al. 2019). Furthermore, a multiple sequence alignment was performed based on the protein sequences of 17β-HSD (HN14) with other reported 17β-HSD proteins. An activity center (Tyr-xxx-Lys) and an NADH binding region (Gly-xxx-Gly-x-Gly) were identified (Fig. S1). Furthermore, molecular docking analysis was performed to evaluate the affinity of substrate E2 to protein17β-HSD(HN14). The docking result showed that there may be a substrate binding pocket on the surface of protein 17β-HSD(HN14). Based on molecular docking models, protein 17β-HSD (HN14) was found to bind to the substrate E2 through strong electrostatic interactions and hydrogen bonds. The potential key amino acids are GLN-30, TYR-104, ILE-197, THR-199, ALA-202, and TYR-212 (Fig. S2). The binding energy was − 9.3 kcal/mol, which indicates that the binding of 17β-HSD(HN14) to substrate E2 is stable. Additionally, the 17β-HSD proteins from diverse biological sources have different protein isoelectric point (pI).

Function verification of 17β-HSD(HN14)

To explore the dehydrogenation ability of 17β-HSD(HN14) towards steroid hormones, it was cloned into the expression vector pETDuet-1 (Fig. S3A). Furthermore, the recombination strain carrying the *17β-HSD(HN14)* gene was constructed and used for resting cells biotransformation and characterization of the substrate specificity. 17β-HSD(HN14) was expressed under IPTG induction conditions. The protein molecular mass of 17β-HSD(HN14) was about 33 kDa on SDS-PAGE and Western blotting (Fig. S3B and Fig. S3C), consistent with the molecular mass of the calculated polypeptides. The function of 17β-HSD(HN14) was detected within resting cells biotransformation. Subsequently, we sought to further identify the substrate specificity of 17β-HSD(HN14) towards various steroid hormones, including E2, E3, and T. The results of the biotransformation rate measurement indicate that E2 and T can be efficiently converted by 17β-HSD(HN14) with biotransformation ratios of 76.88% and 86.26% within 48 h, respectively (Fig. 4A). However, the ability to convert E3 is poor (data not shown). The metabolites by 17β-HSD(HN14) were detected by HPLC and GC-MS. By comparing with the standard substance, the metabolites of E2 and T were identified as E1 and AD, respectively (Fig. 4B, C, E, and F). However, the conversion product of E3 was not detected. The findings showed that the engineered strain with the recombinant 17β-HSD(HN14) protein could catalyze the

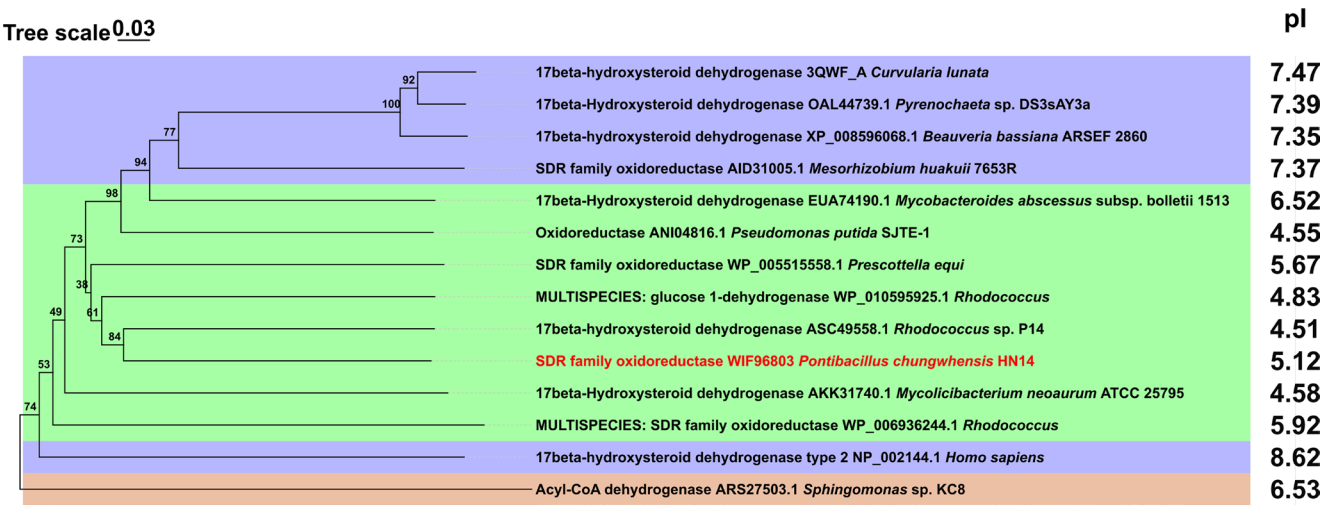

Fig. 3 Neighbor-joining phylogenetic trees of 17β-HSD(HN14) from *P. chungwhensis* HN14 based on protein sequence. *pI* protein isoelectric points

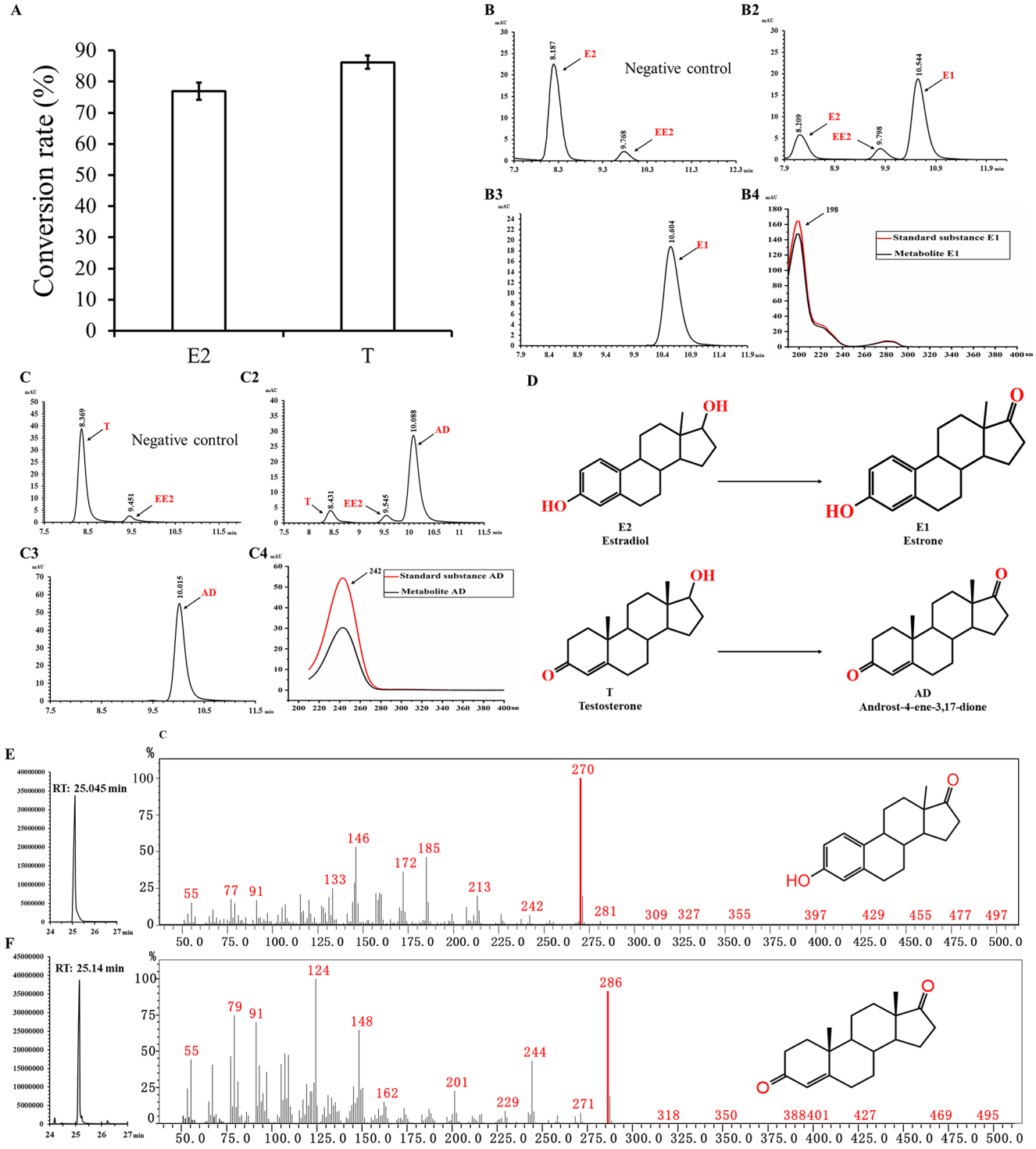

Fig. 4 Conversion of 20 mg/L E2 and T by 17β-HSD(HN14) in recombinant strain *E. coli* BL21-pETDuet-*17β-HSD(HN14)*. (**A**) Substrate conversion efficiency of recombinant strain *E. coli* BL21-pETDuet-*17β-HSD(HN14)*. (B) HPLC profile of E2 in negative control group. **B**) Analysis of metabolic intermediates of E2 by HPLC in experimental group. (B2) HPLC profile of standard-sample of E1. (B3) The UV-vis spectrum of metabolic intermediate and standard-sample of E1. (**C**) HPLC profile of T in negative control group. (C2) Analysis of metabolic intermediates of T by HPLC in experimental group. (C3) HPLC profile of standard-sample of AD. (C4) The UV-vis spectrum of metabolic intermediate and standard-sample of AD. (**D**) The transformation process of E2 and T by recombinant strain *E. coli* BL21-pETDuet-*17β-HSD(HN14)*. (**E**) Analysis of metabolic intermediates of E2 by GC-MS. (**F**) Analysis of metabolic intermediates of T by GC-MS. E2: Estradiol; E1: Estrone; EE2: 17α-ethynylestradiol; T: testosterone; AD: androst-4-ene-3,17-dione; Negative control: *E. coli* BL21 with pETDuet-1 empty vector; Experimental group: *E. coli* BL21 with pETDuet-*17β-HSD(HN14)*; EE2 is considered as an internal reference. The substrate concentration was 20 mg/L, and the culture time was 2 d

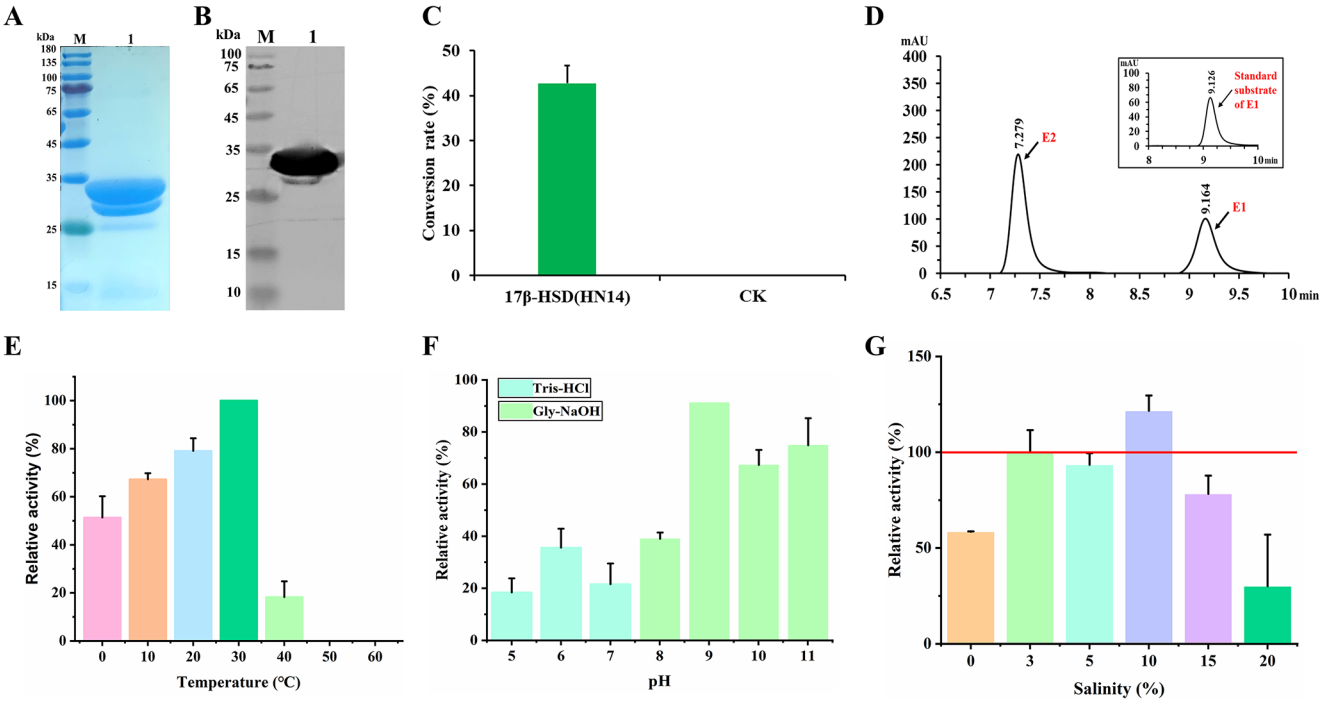

Fig. 5 The purification and characterization of 17β-HSD(HN14). (**A**) SDS-PAGE of purifide17β-HSD(HN14). (**B**) Western blotting of purifide17β-HSD(HN14). (**C**) The degradation of 0.25 mM E2 by purified 17β-HSD(HN14) within 1 h. 17β-HSD(HN14): the reaction mixture contained purified 17β-HSD(HN14); CK: the reaction mixture did not contain purified 17β-HSD(HN14). (**D**) HPLC profile of E2 degraded by purified 17β-HSD(HN14). (**E**) The catalytic temperature range for 17β-HSD(HN14) when E2 was used as the substrate. (**F**) The catalytic pH range for 17β-HSD(HN14) when E2 was used as the substrate. (**G**) The catalytic salinity gradient for 17β-HSD(HN14) when E2 was used as the substrate

dehydrogenation process on E2 and T at the C-17 position (Fig. 4D).

Properties of purified 17β-HSD(HN14)

Moreover, the recombinant protein 17β-HSD(HN14) with 6×his tag on the N terminal was purified using Ni-NTA affinity chromatography. The optimum concentration of elution buffer is 200 mM imidazole buffer (data not shown). According to the SDS-PAGE results, the purity of recombinant protein 17β-HSD(HN14) met the requirements of subsequent experiments (purity > 90%) (Fig. 5A). The molecular mass of purified protein was about 33 kDa and consistent with Western blotting results (Fig. 5A and B). Within 1 h, 42.7 ± 4% of 0.25 mM E2 is transformed and forms metabolic intermediate E1 by 100 µg purified 17β-HSD(HN14) protein, indicating that 17β-HSD(HN14) has the catalytic ability in vitro (Fig. 5C and D). Furthermore, the effect of temperature, pH, and salinity on the activity of the enzyme 17β-HSD(HN14) was investigated (Fig. 5E-G). The results showed that the optimal catalytic temperature of 17β-HSD(HN14) is 30°C. 17β-HSD(HN14) is sensitive to high temperature. When the temperature exceeds 30°C, the enzyme activity of 17β-HSD(HN14) rapidly reduces as the temperature increases. When the temperature exceeds 50°C, 17β-HSD(HN14) is completely inactivated (Fig. 5E). The optimal catalytic pH of 17β-HSD(HN14) is 9. 17β-HSD(HN14) exhibits high activity at alkaline conditions compared to acidic conditions and can maintain weak enzyme activity in weakly acidic environments (Fig. 5F). Additionally, 3–10% salinity conditions have almost no negative effects on 17β-HSD(HN14). Additionally, enzyme 17β-HSD(HN14) still maintains certain enzyme activity under extremely high salinity conditions (15–20%) (Fig. 5G). It is worth noting that compared with the enzyme activity under the condition of 3% salinity, when the salinity is 0, the enzyme activity of 17β-HSD(HN14) is only about 50% (Fig. 5G), suggesting that a certain concentration of salt ions is necessary for 17β-HSD(HN14) to play a catalytic role. Strong substrate catalytic activity under high salinity conditions suggests that 17β-HSD(HN14) may be a halophilic dehydrogenase.

Discussion

The PAH-degrading bacteria *P. chungwhensis* HN14 was initially isolated from surface sediments in Hainan mangroves (Qian et al. 2022). In this study, the steroid degradation ability of *Pontibacillus* strains was investigated for

the first time. Previous studies have demonstrated that high salinity environments render the vast majority of organic pollutant-degrading bacteria unable to survive due to the high osmotic pressure (Zhao et al. 2023). Additionally, the efficiency of organic pollutant removal was significantly limited by bacteria's low tolerance to high substrate concentrations and the toxic effects of organic pollutants on them (Lin et al. 2022). The coexistence of high salinity, alkalinity, and high substrate concentrations typically leads to a notable decrease in degradation rates and inhibition of strain growth (Li et al. 2023). Therefore, halotolerant degraders with high environmental resistance are the most suitable choice for improving the performance of organic pollutants in harsh environments. As a natural degrader of organic pollutants, the degradation characteristics of E2 present here suggest that the strain HN14 could degrade E2 to maintain the growth of the strain and demonstrated efficient substrate degradation efficiency under high salinity and high substrate concentration stress conditions (Fig. 1). Compared with the previously reported efficient steroid degrading strains, including *Rhodococcus* sp. P14, *Rhodococcus equi* DSSKP-R-001, and *Comamonas testosteroni* ATCC11996, the strain HN14 has comparable substrate degradation efficiency and more robust environmental adaptability (Ye et al. 2017; Tian et al. 2022; Xiong et al. 2009; Gong et al. 2011). The considerable efficiency in degrading E2 indicates that the strain HN14 possesses the ability to sense, respond to, and degrade E2 rapidly. The strain HN14 has the characteristics of resisting and degrading high concentrations of E2, which enables it to withstand the adverse impact of E2, thereby achieving effective E2 bioremediation in the in-situ environment. Consequently, the degradation characteristics under extreme conditions suggest that compared to other E2-degrading strains, strain HN14 may have more substantial competitiveness in E2 degradation in high-salinity environments, such as high-salinity organic wastewater.

Numerous studies have indicated that steroid degradation typically initiates substrate dehydrogenation, which reduces the toxicity of the original substrate and facilitates its degradation (Donova et al. 2005; Kristan and Rižner 2012; Yu et al. 2013; Swizdor et al. 2012). E2 is the natural estrogen with the highest estrogenic activity, so the transition from E2 to E1 is considered biological detoxification process due to E1 is generally less potent in terms of estrogenic activity compared to E2 (Tian et al. 2022; Deich et al. 2021). It is similar to our study. Additionally, a metabolite formed by the dehydrogenation suggested that the dehydrogenase may play an essential role in E2 degradation by strain HN14. Combining bioinformatics analysis with molecular biology validation, a 17β-HSD was identified and named 17β-HSD(HN14), which can catalyze the dehydrogenation reaction at the C-17 position of the substrate and convert E2 and T to E1 and AD, respectively. Since strain HN14 is a typical moderately halophilic microorganism, it is speculated that 17β-HSD(HN14) may have unique biochemical properties compared with other dehydrogenases. The purified enzyme catalytic reaction assays suggested that 17β-HSD(HN14) still has the dehydrogenation ability in vitro. Moreover, 17β-HSD(HN14) exhibits a preference for tolerance to alkalinity and salinity and sensitivity to high temperatures. These characteristics are similar to the physical and chemical parameters of the marine environment (Kenigsberg et al. 2022; Walden et al. 2019). Therefore, we speculate that the marine environment will have a specific impact on the characteristics of strains and even functional proteins. The degradation characteristics of the strain and enzyme showed that the enzyme 17β-HSD(HN14) exhibits similar high salinity tolerance characteristics to strain HN14. Overall, 17β-HSD(HN14) exhibits similar substrate catalytic ability and comparable catalytic efficiency to other reported 17β-HSDs (Ye et al. 2017, 2019; Wu et al. 2019; Peng et al. 2022). The effects of temperature and pH on enzyme activity for 17β-HSD(HN14) are similar to other 17β-HSD enzymes (Ye et al. 2017, 2019). However, 17β-HSD(HN14) maintains better protein structural stability and catalytic activity under high salinity conditions. These advantages mean that the application of strain HN14 or enzyme 17β-HSD(HN14) may be a better choice to improve the removal efficiency of steroids in high-salinity environments.

Extreme high salinity condition generally leads to denaturation, aggregation, and precipitation of the majority of proteins. Currently, massive evidence has indicated that that halophilic microorganisms harbor robust enzymes capable of preserving both protein structural integrity and catalytic activity under high osmotic pressure (DasSarma 2015; Kennedy et al. 2001; Karan et al. 2012). Sequence and structural analyses have demonstrated that these enzymes from halophilic microorganisms have been found to be negatively charged due to a surplus of acidic over basic residues and alteration in hydrophobicity. These differences enhance the protein solubility and promote the maintenance of enzyme function in low water activity conditions (DasSarma 2015). DasSarma (2015) further found that the average pI of halophilic proteins was about 5.0. On the contrary, the average pI of non-halophilic proteins tend to be neutrality. In this study, we summarized and described the pI of 17β-HSD with the same function but different geographic sources (Fig. 3). The comparison results show that the pI of 17β-HSD from terrestrial organisms are neutral or weakly alkaline except for 17β-HSD from Homo sapiens (3KM0_A), in stark contrast to 17β-HSD from ocean organisms, which is consistent with previous study results (Kennedy et al. 2001; Karan et al. 2012). The pI of 17β-HSD(HN14) is 5.12, indicating that

17β-HSD(HN14) may be a halophilic 17β-HSD. In general, the surface composition of halophilic proteins is more prone to contain small and borderline hydrophobic amino acids including alanine (A), glycine (G), threonine (T), and serine (S) than residues with bulky hydrophobic side chains, encompassing isoleucine (I), phenylalanine (F), and leucine (L) (Kastritis et al. 2007). In this study, we performed a comparative analysis of the amino acid composition of 17β-HSD(HN14). From the perspective of amino acid residue composition, the sum of G, A, S, and T was 86, accounting for about 33% of the total amino acid residues, which is slightly higher than the proportion of F, I, and L in 17β-HSD(HN14), which is consistent with characteristic of halophilic proteins (Table. S4). Additionally, the amino acid residue ratio of (G + A + S + T) to (F + I + L) in HN14 is greater than 17β-HSD that a non-halophilic protein found in Homo sapiens (NP_002114.3) (Table. S5). In summary, the differences in the composition and structure of these amino acid residues might endow 17β-HSD(HN14) with the ability to maintain stable protein structure under high salinity conditions. Further experimental verification is necessary for a deeper understanding of the underlying salt-tolerant molecular mechanisms of halophilic 17β-HSD(HN14).

Previous studies have reported that E1 and E2 solutions have a severe inhibitory effect on seed germination. Still, when the wheat seeds are treated with E1 or E2 solutions treated with estrogen-degrading strains, the inhibitory impact will be significantly reduced, indicating the vital role of degrading strains or degrading enzymes in actual bioremediation (Miao et al. 2023). The effective degradation capability and high salinity tolerance of strain HN14, as well as its enzyme 17β-HSD(HN14), bestow them with broader application potential and promising prospects in complex ecological environments. In the future, combined with substrate molecule and protein co-crystallization technology, saturation mutagenesis, and visualization high-throughput methods (Peng et al. 2023; Chayen and Saridakis 2008), the application of 17β-HSD(HN14) in steroids bioremediation will be further expanded.

Conclusions

In this study, the degradation ability of *P. chungwhensis* HN14 towards E2 under high salinity stress or high substrate concentration conditions was first characterized. Additionally, 17β-HSD(HN14) from strain HN14 was identified and characterized, which is capable of transforming a variety of steroid hormones. Depending on the degradation characteristics, 17β-HSD(HN14) removes the C-17 hydrogen atoms of E2 and T. Through purified enzyme degradation assays, we demonstrated that 17β-HSD(HN14) has excellent structural stability and enzyme catalytic activity under high salinity conditions and explored its potential molecular mechanisms by bioinformatics. In summary, this study provides a new perspective on bioremediation strategies for steroid pollution specifically in high-salt environments.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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