



# Identification of non-accumulating intermediate compounds during estrone (E1) metabolism by a newly isolated microbial strain BH2-1 from mangrove sediments of the South China Sea

Amit Pratush<sup>1</sup> · Qi Yang<sup>1</sup> · Tao Peng<sup>1</sup> · Tongwang Huang<sup>1</sup> · Zhong Hu<sup>1</sup>

Received: 9 July 2019 / Accepted: 28 October 2019 / Published online: 17 December 2019

© Springer-Verlag GmbH Germany, part of Springer Nature 2019

## Abstract

Steroid estrogens are natural hormonal compounds produced by various animals and humans. Estrone (E1), estradiol (E2), and estriol (E3) are the most commonly known estrogens that are released into the environment along with human and animal excreta, which end up polluting water bodies. While these estrogens are usually biotransformed into their respective by-products by various microbial strains, E2 could also be transformed into E1 by 17 $\beta$ -hydroxysteroid dehydrogenases (17 $\beta$ -HSDs) under reducing environmental conditions. However, due to limited further biotransformation of E1, it accumulates to higher levels in water bodies compared to other natural estrogens in the aquatic environment. Given that E1 is one of the potential endocrine-disrupting compounds (EDCs), with several adverse effects on aquatic animals and consequently on the seafood industry, it is vital to remove E1 from the environment via improved steroid bioremediation. In the present study, we successfully isolated a potential E1-degrading microbial strain (named as BH2-1) from soil sediments collected from the Bai Hai mangrove region of the South China Sea. The strain BH2-1 has excellent E1-degrading potential and could degrade 89.5% of E1 after 6 days of incubation in a MSM-E1 medium containing 1% NaCl at pH 6. Besides, after 3 h and 6 h of extraction, two non-accumulating intermediate compounds [3-hydroxyandrost-5,7,9(11)-trien-17-one and androst-1,4,6-triene-3,17-dione (ATD)], respectively, were successfully identified using GC-MS analysis. These non-accumulating intermediate compounds have not previously been reported during E1 biodegradation and might be new intermediate metabolites. The identification of these new compounds also gives more insight into the mechanism of E1 metabolism and helps to establish a clear E1 biodegradation pathway, which further enriches our knowledge on the overall microbial steroid degradation pathway. Furthermore, whole-genome sequence analysis of strain BH2-1 revealed the presence of 46 genes that belong to 6 major steroid-degrading gene classes.

**Keywords** Biodegradation · Estrone · Endocrine-disrupting compound · Intermediate compounds · South China Sea · Steroid pollution

## Introduction

Steroid estrogens are also known as human sexual hormones (Qamar and Bhatt 2015). Natural and synthetic estrogens include estrone (E1), 17 $\beta$ -estradiol (E2), estriol (E3), 17 $\alpha$ -

ethinylestradiol (EE2), and mestranol (MeEE2), respectively (Ting and Praveena 2017). These hormones are metabolized inside the body, and their metabolites and conjugates (inactive estrogen) are excreted mainly in urine and feces, which get into wastewater and finally reach to the wastewater treatment plants (WWTPs), from where they are released into water bodies (fresh and marine), thereby contaminating water resources (Sang et al. 2012). Estrogen pollutants are termed as micropollutants, as they are present in minute quantities in water bodies but have significant effects on the aquatic ecosystem (Wedekind 2014). In aquatic systems, estrogens are also known as endocrine-disrupting compounds (EDCs) because they interfere with the normal functioning of the endocrine system of aquatic animals by mimicking and

---

Amit Pratush and Qi Yang contributed equally to this work.

---

Responsible editor: Robert Duran

---

✉ Zhong Hu  
hzh@stu.edu.cn

<sup>1</sup> Department of Biology, College of Science, Shantou University, Shantou 515063, China

antagonizing the effect of endogenous hormones as well as disrupting the anabolism and catabolism of endogenous hormones (Silva et al. 2012; Liu et al. 2016). To prevent the adverse effects of estrogen pollution on the aquatic environment, it is important to remove them. Removal of estrogen pollution from the aquatic environment can be carried out by physical (photocatalytic degradation), chemical (adsorption, advanced oxidation processes), and biological approaches (Silva et al. 2012; Zhang et al. 2015a). Biological degradation of steroid estrogen is one of the most common, successful, and economic approaches. There are some bacterial and white-rot fungi species, which efficiently degrade or transform steroid estrogens in contaminated environments. These microbial systems use estrogen as the source of carbon and energy by degrading or transforming them into other less harmful or neutral compounds (Kurisu et al. 2010; Horinouchi et al. 2012; Zhang et al. 2015b; Li et al. 2017). In wet and polluted environments, estradiol acts as a prevalent endocrine disruptor. E2 shows refractory degradation along with a wide pollution range and can easily bioconcentrate in human and animal bodies (Wang et al. 2019). Studies have revealed that E2 biotransformation is a multistep dehydrogenation reaction, which is catalyzed by 17 $\beta$ -hydroxysteroid dehydrogenase enzyme under reducing environmental conditions (Ye et al. 2019). It is believed that E2 degradation initiated by dehydrogenation at 17-position results in cleavage of the D ring (Yu et al. 2013; Lee and Liu 2002). E2 biotransformation leads to the formation of estrone. Generally, the microorganisms that degrade E2 are unable to biodegrade E1 further, which means that E1 removal from polluted water is relatively poor compared with E2 (Samir et al. 2006). It is partly for this reason that E2 biotransformation product (i.e., E1) starts to accumulate in water bodies and its concentration increases in the aquatic environment. Furthermore, it has been observed that E1 concentration in the surface water is much higher than that of other natural estrogens (Sami and Fatima 2019). E1 removal is, therefore, necessary to improve the overall bioremediation of steroid-polluted water bodies.

Studies have revealed that excessive exposure to E1 harms aquatic animals. Lei et al. (2013) showed the adverse effects on early life stages and estrogen receptor gene of Japanese medaka (*Oryzias latipes*) and concluded that E1 exposure resulted in sex reversal in males and unrecoverable impact on gonadal growth and development. In addition, Ankley et al. (2017) reported the adverse effects of E1 exposure on the male fathead minnows (*Pimephales promelas*) and concluded that E1 induces feminization in males. However, only a limited number of studies have reported on or explained the biological degradation of E1, with microbial strains using E1 as the sole carbon and energy source. Some studies have explained the consortia degradation of E1 (Thayanukul et al. 2010), along with the association of background carbon and nitrogen conditions (Du et al. 2017). Besides this, some

studies have also successfully degraded E1 from sludge samples under anaerobic conditions (Zhang et al. 2015b). Peterson et al. (2017) described E1 degradation (96%) in sewage wastewater during the nitrification process. Recently, Sami and Fatima (2019) explained E1 degradation by cyanobacterial species. Despite this, in-depth knowledge of E1 degradation mechanism is limited till date, for which reason many research groups are working to find out the intermediate steps in E1 degradation.

Herein, we isolated a microbial strain (BH2-1) from soil sediments collected from the Bai Hai mangrove area of Guangxi Province in Southern China, which is capable of degrading E1 by using it as the sole carbon and energy source. Microbiological and biochemical characterization along with whole-genome sequence analysis of the newly isolated strain was carried out. In addition, the intermediate compounds formed during the biodegradation reaction were also detected and characterized by HPLC and GC-MS techniques.

## Materials and methods

### Chemicals and reagents

All chemicals used in the present study were of analytical grade and more. E1 (C<sub>18</sub>H<sub>22</sub>O<sub>2</sub> > 98% pure) was procured from Sigma-Aldrich (Shanghai, China). E1 molecular weight, solvent solubility, vapor pressure, and melting point were 270.372 g mL<sup>-1</sup>, 30 mg L<sup>-1</sup> at 25 °C, 2.49 × 10<sup>-10</sup> mmHg at 25 °C, and 260.2 °C, respectively. All other organic solvents such as ethyl acetate and acetonitrile (HPLC grade) were purchased from M TEDIA (USA) and OceanPAK (Sweden), respectively. E1 stock solution (10 mg L<sup>-1</sup>) was prepared in HPLC grade methanol, and further, 2 mg mL<sup>-1</sup> was used for experimental purpose, whereas marine growth media (2216E), mineral salt medium (MSM), and Luria-Bertani (LB) broth components were purchased from Hangwei Ltd. (Honggu, China), BBI Life Sciences (Shanghai, China), and Oxoid Ltd. (England), respectively. Marine growth media (2216E) (pH 7.6–7.8) was composed of peptone powder (5.0 g L<sup>-1</sup>), yeast extract powder (1.0 g L<sup>-1</sup>), ferric chloride (0.01 g L<sup>-1</sup>), and NaCl (30%). MSM (pH 6.5–7.0) was composed of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (1.0 g L<sup>-1</sup>), Na<sub>2</sub>HPO<sub>4</sub> (0.8 g L<sup>-1</sup>), KH<sub>2</sub>PO<sub>4</sub> (0.2 g L<sup>-1</sup>), MgSO<sub>4</sub> (0.2 g L<sup>-1</sup>), FeCl<sub>3</sub> (1 mL L<sup>-1</sup>), and (NH<sub>4</sub>)<sub>2</sub>Mo<sub>7</sub>O<sub>24</sub> (1 mL L<sup>-1</sup>). Agar plates of both 2216E and MSM media were prepared by adding 15 g L<sup>-1</sup> agar powder in above composition. The MSM-E1 media (liquid) and MSM-E1 (agar plates) were prepared by adding 250  $\mu$ L of E1 stock solution in every 50 mL of MSM media and hot MSM agar media, respectively.

LB broth (pH 7.0) was composed of tryptone powder (10 g L<sup>-1</sup>), yeast extract powder (5.0 g L<sup>-1</sup>), and NaCl

(10 g L<sup>-1</sup>) in association with E1 and was used for the intermediate compound detection.

### Enrichment and isolation of E1-degrading bacterium

Soil samples collected from the Bai Hai mangrove area of Guangxi Province in Southern China (latitude 21° 24' 48" N and longitude 109° 10' 25" E) were used to screen a potential E1 degrader by using a standard enrichment technique (Veldkamp 1970). Studies revealed that E1 is photosensitive, and it undergoes photodegradation in the presence of light (Chowdhury et al. 2010; Zhao et al. 2019). To avoid any effect of light on microbial biodegradation reaction data, all E1 biodegradation experiments were carried out in dark conditions by incubating them in a dark room. For sample enrichment procedure, the collected sample (5 g) was inoculated with 2 mg mL<sup>-1</sup> E1 (E1 dissolved in ethyl alcohol) in 100 mL MSM media in a 250-mL volume Erlenmeyer flask and was incubated at 30 °C in an incubator shaker at the constant shaking speed (150 rpm) for 10 days in a dark room. After 10th day of incubation in the dark, 5% (v/v) of this mixture was further transferred to 100 mL fresh MSM medium having 2 mg L<sup>-1</sup> E1, the flask was again incubated at the same temperature and shaking conditions for the next 7 days in the dark room, and the process was repeated three times. After a time interval of more than 30 days, 1 mL volume of the last flask was serially diluted in sterile water and successively spread on MSM-E1 plates and, further, these plates were incubated at 30 °C for microbial growth. After sufficient colony growth on incubated MSM-E1 plates, every single colony was streaked on the new MSM-E1 plate for secondary screening. Further, each purified colony was grown in 2216E marine growth media up to 0.8 optical density (OD)<sub>600</sub>, the bacterial pellet was washed thrice with MSM media for the complete removal of growth medium components, and the washed bacterial culture was starved for the next 24 h before subjecting to E1 exposure for the complete consumption of any remaining medium component. MSM-E1 media were prepared by mixing 2 mg L<sup>-1</sup> E1 in the MSM medium. The 5% of starved cell inoculum was inoculated in different flasks having a 50 mL MSM medium along with 2 mg L<sup>-1</sup> E1 and was incubated at 30 °C for different time intervals up to 15 days under dark conditions. After a particular time interval (after every 2 days), the residual E1 was extracted by a two-phase extraction method by using ethyl acetate as an organic phase and the extracted E1 was further concentrated or dried on the 70 °C water bath and dissolved in HPLC running buffer (acetonitrile:water 1:1) and subjected to HPLC/UV. The whole experiment was carried out in triplicate. Microbial strains having E1 degradation potential were selected and preserved for further characterization.

### Morphological, physiological, and biochemical analysis of isolated strain

The isolated bacterial strain was further identified and characterized by using microbiological and molecular techniques. Gram staining was carried out by using a protocol given by John et al. (1994) to determine the Gram nature of the organism, and cells were observed under an optical microscope (Optec, Chongqing, China). A morphological study of the isolated bacterial strain was carried out with a scanning electron microscope (SEM) (JSM-6360LA, Japanese electronics; Japan). The isolated microorganism was analyzed for catalase and oxidase activities according to the protocol described by Cowan and Steel (1965).

### 16S ribosomal RNA gene sequence and phylogenetic analysis

The strain was characterized on the basis of the 16S ribosomal RNA (rRNA) sequence. The 16S rRNA gene was amplified and sequenced by Huada Gene Ltd. (Guangzhou, China), by using the following universal primers: forward primer 27F (5'-AGA GTTTGATCCTGGCTCAG-3') and reverse primer 1492R (5'-GGTTACCTTGTTACGACTT-3') in a PCR thermal cycler (LifeECO PCR Machines, Hangzhou, China) having the following reaction conditions: 95 °C for 5 min, 30 cycles of 45 s at 95 °C, 1 min at 55 °C and 1.5 min at 72 °C, and final extension cycle of 10 min at 72 °C. The amplified PCR product was loaded on the agarose gel (1%) and further purified by using AxyPrep kit (Beijing, China). The purified PCR product was sequenced, and the sequence homology was analyzed by a BLASTN tool. The nucleotide sequence of the amplified gene was aligned with the 16S rRNA gene sequences of various species already available in GenBank using the ClustalW program of the European Molecular Biology Laboratory (EMBL)-European Bioinformatics Institute (EBI). The phylogenetic tree was constructed by using the MEGA6.0 software.

### Quality control

Before E1 biodegradation experiments, quality controls were undertaken to ensure that E1 did not adsorb on any glassware. For that, all glassware was subjected to high temperature (450 °C) for 5 h in a muffin furnace to make them E1-free as this glassware was further used for E1 degradation analysis experiments. Further, the recovery efficiency of liquid-liquid extraction was evaluated by spiking the MSM medium with 500 ng of E1. The recovery efficiency of liquid-liquid extraction for E1 was > 90%. Moreover, there was no E1 peak detected from the blank sample extracts.

## E1 degradation analysis

The single pure colony of BH2-1 strain was selected from the MSM-E1 agar plate and further inoculated to 2216E medium and incubated in the dark at 30 °C for 12 h or until its OD<sub>600</sub> reaches to 0.6. The young BH2-1 cells were harvested using the same procedure as discussed above in the isolation section. The three reaction sets, i.e., two control sets (heat-killed control and cell-free control) and test set (MSM-E1 + live BH2-1 cells), were incubated under the same conditions. All reactions were carried out in triplicate. The test and control samples were extracted by using an aqueous two-phase extraction method where ethyl acetate was used as the organic phase. The residual E1 extraction was carried out at different time intervals of incubation (2 days, 4 days, 6 days up to 12 days), and the residual E1 was estimated by HPLC using an Alltech 3300 ELSD detector (Agilent 1100 Series Liquid Chromatography; Agilent Technologies, Palo Alto, CA, USA) by following the protocol established by Xu et al. (2014). E1 degradation efficiency (%) of the BH2-1 strain was calculated as follows (Li et al. 2017):

$$\text{Degradation efficiency (\%)} = \frac{C_{\text{initial}} - C_{\text{Residual}}}{C_{\text{initial}}} \times 100$$

where  $C_{\text{initial}}$  and  $C_{\text{residual}}$  represent the initial and final E1 concentrations in the reaction mixture, respectively.

## Growth and E1 degradation capability of strain

The growth curve and E1 degradation capability of the isolated strain were accessed by inoculating the overnight grown, pure, washed pre-culture of strain (OD<sub>600</sub> = 0.6) into the flasks having MSM media and a known concentration of E1 (2 mg L<sup>-1</sup>) as the sole carbon source. The inoculated flasks were incubated at 30 °C at 150 rpm. The OD and residual E1 concentration of the inoculated flasks were observed daily up to 12 days of incubation. The whole experiment was carried out in triplicate.

## Optimization of E1 degradation conditions

Newly isolated E1-degrading strain was further optimized for the maximum E1 degradation. To achieve maximum E1 degradation, the incubation temperature, medium pH, and NaCl concentration were optimized by using a one-variable-at-a-time (OVAT) strategy. For OVAT optimization, the initial value for temperature, medium pH, and NaCl concentration were 30 °C, 7.0, and 1%, respectively.

## Whole-genome analysis of BH2-1 strain

The strain BH2-1 was cultured up to exponential phase under optimum conditions, and its genomic DNA was extracted by using a DNA isolation kit (Dongsheng Biotech Co., Ltd., Guangzhou, China) having a concentration up to 50 ng μL<sup>-1</sup> (300 μL in total). The extracted DNA was sent to Huada Gene Ltd. (Guangzhou, China), under low-temperature conditions for the whole-genome sequence analysis. The sequencing results were assembled using SOAPdenovo software (Soapdenovo2). The genetic predictions of the strain were made on the basis of feedback data using the software Prokka, and to make use of predictive production to BlastKOALA (<http://www.kegg.jp/Blastkoala/>) based on KEGG, the metabolic pathway of strain was constructed from the database.

## Intermediate compound detection during E1 degradation reaction by HPLC and GC-MS

Detection of intermediate compounds during E1 degradation reaction carried out by the BH2-1 strain was also analyzed. New intermediate compounds were detected by reducing the residual E1 extraction time (extraction every 3 h and 6 h) and analysis of the reaction mixture by HPLC followed by GC-MS analysis. During this experiment, the BH2-1 biodegradation reaction procedure was exactly similar as described in the above section. The details of GC-MS sample preparation and sample running conditions are as follows: the residual E1 was extracted by the aqueous two-phase extraction method as discussed in the previous section; after that, the extracted sample was air-dried to evaporate the organic solvent (ethyl acetate) and the dried sample was further suspended in ultrapure acetonitrile, which was further subjected to GC-MS analysis. For GC-MS analysis, a 0.4 μL sample volume was injected to a HP-5MS-fused silica column (30 m × 0.25 mm, 0.25 μm) at the inlet and interface temperatures of 280 °C and 260 °C, respectively. Nitrogen was used as the carrier gas with a flow rate of 1.3 mL min<sup>-1</sup>.

## Results and discussion

### Isolation and identification of E1-degrading microbial strain

In the present study, the Bai Hai mangrove area of Southern China was selected for sampling because this area of the sea receives treated water from multiple WWTPs. As the end product of E2 biodegradation, E1 in biologically treated wastewater enters the sea upon discharge and therefore raises the levels of E1 in seawater at points where wastewater discharges are many compared to those in the other parts of the

sea. Thus, given the higher concentration of E1 in the Bai Hai mangrove area, the likelihood of isolating efficient E1-degrading microbial strains was much higher. In the present study, we isolated a large number of microbial strains (117) after the extensive primary screening, with all the bacterial colonies streaked on MSM-E1 agar plates for further screening. Secondary screening gives rise to 10 bacterial colonies, which could efficiently grow on the MSM-E1 medium. The residual E1 percentage of these 10 strains was determined by using the protocol reported by Xu et al. (2014) and Li et al. (2017). E1 degradation results revealed that strain no. 1 exhibited the highest E1 degradation potential as compared to the 9 other strains. Strain 1 was named BH2-1 and was selected for further studies. Since primary screening yielded a large number of colonies (117) that could grow on MSM-E1 agar plates, only 10 were capable of growing in the liquid MSM-E1 medium, suggesting that the growth of these strains on MSM-E1 agar plates may be that these strains use agar as the carbon source instead of E1. The use of agar as the carbon source by various microbial strains is supported by the findings of Hutcheson et al. (2011) and Imran et al. (2017), where marine bacterial strains such as *Saccharophagus degradans* and *Microbulbifer* sp. are able to produce agar-degrading carbohydrases and use the resultant galactose as the carbon source for their growth.

### Microbiological, biochemical, and molecular characterization of strain BH2-1

The strain BH2-1, which is capable of E1 degradation, is a rod-shaped bacterium, with physiological and biochemical analyses showing that it is Gram-positive (+ve) and aerobic. BH2-1 was positive for MR, VP, and gelatin liquefaction test but showed a negative test for hydrogen sulfide and starch hydrolysis. The 16S rRNA gene of strain BH2-1 amplified using universal primers, revealing that the gene sequence is about 1425 bp in length (GenBank accession number: MK587678). The BLASTN tool at NCBI showed that BH2-1 strain belongs to *Rhodococcus* sp. with 99.22% homology with the *Rhodococcus hoagii* strain DSSKP-R-001. The phylogenetic relationship of BH2-1 with related species was constructed by the neighbor-joining method using MEGA6.0 software (Fig. 1). While the newly isolated strain BH2-1 was found to be Gram-positive (+ve) and belonged to *Rhodococcus* sp., previously reported isolated E1-degrading bacteria were generally Alphaproteobacteria and Gram-negative (Thayanukul et al. 2010).

### Optimization of growth media and culture conditions for efficient E1 degradation

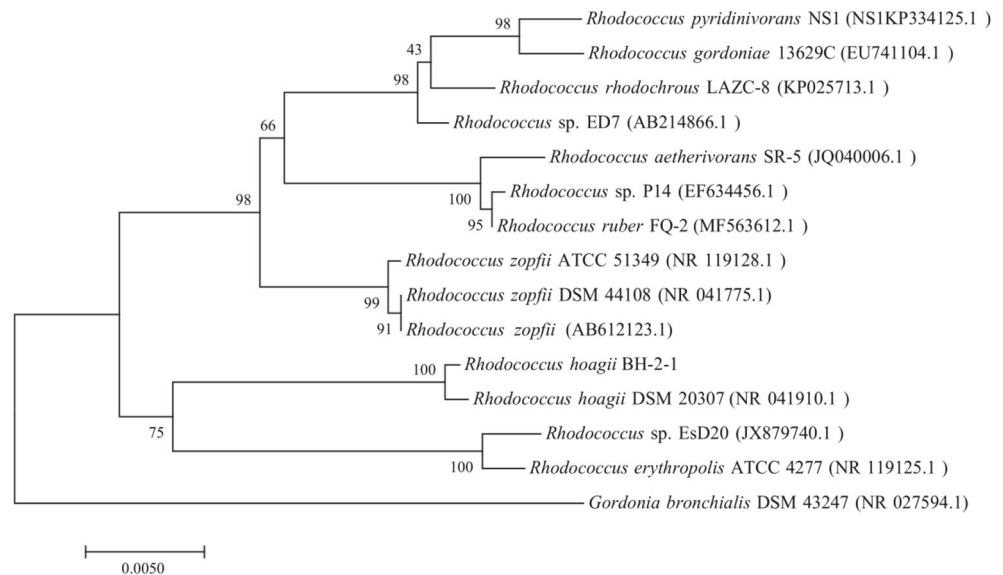
The strain BH2-1 was initially grown in the 2216E marine medium (pH 7.5) at different temperature ranges

(10–50 °C) at various pH (3–9) conditions and in different NaCl concentrations (0–5%). The optimization results showed that the strain BH2-1 grew well at temperatures ranging between 25 and 30 °C and exhibited the highest E1 removal of 79.4% at 25 °C with the highest growth ( $OD_{600} = 1.26$ ) (Fig. 2a). The pH of the medium also affects the growth and E1 degradation rate of strain BH2-1. The results revealed that the strain BH2-1 grew well at slightly acidic pH (6) and exhibited 83.7% removal of E1 with a maximum growth ( $OD_{600} = 1.19$ ) (Fig. 2b). Also, salt concentration (NaCl) in the medium had a significant effect on the growth and E1 degradation capabilities of this strain. The strain BH2-1 exhibited maximum growth ( $OD_{600} = 1.3$ ) and 89.5% E1 removal at 1% NaCl concentration (Fig. 2c). It was observed that changes in incubation temperature, medium pH, and salt concentration had dramatic effects on the growth and E1 degradation capabilities of this strain. The effect of temperature, pH, and NaCl concentration on the growth of strain BH2-1 and E1 metabolism is natural as in the mangrove areas, the optimum temperature varies between 25 and 30 °C with slightly acidic pH (Ukpong 1995). Moreover, mangroves are backwater areas and their seasonal salt concentration varies from 1 to 3.5% (D'Addezio 2015). Thus, the optimization results revealed that the strain BH2-1 grows well at 25 °C at slightly acidic medium pH (6) in the presence of 1% NaCl (Fig. 2a–c).

### E1 degradation by the strain BH2-1

To determine the E1 degradation capability of strain BH2-1, it was grown overnight and the cells harvested were further subjected to the MSM-E1 medium with 1% NaCl and pH 6. E1 degradation reactions were divided into 3 sets: i.e., test mixture (MSM-E1 + BH2-1 cells), negative control (MSM-E1 + heat-killed BH2-1 cells), and blank control (MSM-E1 without BH2-1 cells), which were placed in an incubator shaker at 25 °C at 150 rpm for different time periods (0 day, 2 days, 4 days, 6 days, 8 days, and 10 days). At 2-day intervals, the residual E1 from the test samples as well as two (heat-killed and without cell) control samples was extracted by using an aqueous two-phase extraction system as described by Xu et al. (2014). E1 extraction results showed that after 6 days, BH2-1 cells were removed or metabolized about 89.5% of E1 from the reaction mixture (Fig. 3). It was observed that the control samples with heat-killed cells showed a little reduction (1–2%) in E1 extraction after 8 days to 10 days of incubation. This minor change in E1 concentration might be due to the adsorption of E1 on the dead cell surface. However, in the case of control samples without microbial cells, complete E1 extraction took place (Fig. 3).

**Fig. 1** Phylogenetic tree showing the relationship of strain BH2-1 with other closely related *Rhodococcus* sp. strains based on 16S rRNA sequences. The tree was constructed by using a neighbor-joining method, and the bar indicates a genetic distance of 0.1



### Whole-genome sequence analysis of strain BH2-1

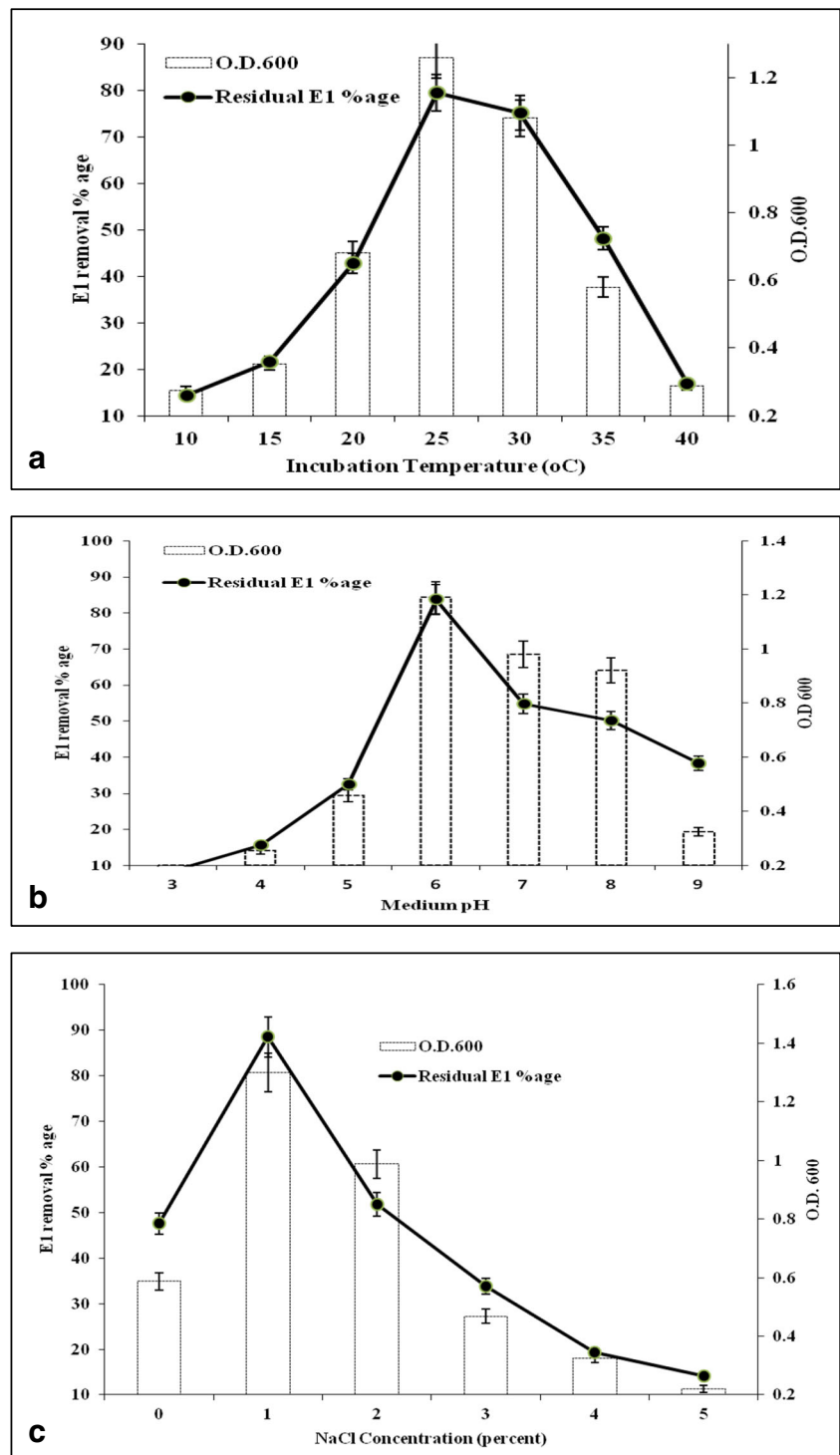
The whole-genome sequence analysis of strain BH2-1 revealed that it has a total of 2671 genes with various metabolic functions. Out of the total number of genes, 46 genes belong to 6 major steroid-degrading gene classes that produce enzymes such as cholesterol oxidase, steroid  $\Delta$ -isomerase, cytochrome P450 monooxygenase (SCO),  $3\delta$ -steroid-1-dehydrogenase, 3-steroid-9 $\alpha$ -hydroxylase (KSH), and  $3\alpha,20\text{-}\beta$ -hydroxysteroid steroid dehydrogenase (Fig. 4). These enzymes actively participate in steroid degradation reactions such as hydroxylation (Van der Geize et al. 2008), isomerization (Talalay and Wang 1955), oxylation (Samavat and Kurzer 2015), acylation, and hydrolysis. Besides, the strain BH2-1 expresses some regulatory proteins, which participate in steroid degradation (Penning 2003). Studies have revealed that the enzyme cholesterol oxidase (COX) is a bi-functional FAD-containing enzyme involved in the oxidation of cholesterol into 4-cholesten-3-one. This enzyme attacks sterols at the  $3\beta$ -hydroxyl positions to form 4-cholestenone and  $\text{H}_2\text{O}_2$ . This step is the first step in microbial degradation of cholesterol in the oxidation of the  $3\beta$ -hydroxyl group followed by the degradation of the side chain (Kumari and Kanwar 2012; MacLachlan et al. 2000). Further, steroid delta-isomerase, which has successfully been identified in *Comamonas testosterone* TA441, has steroid-degrading capability (Horinouchi et al. 2001). Cytochrome P450 is a typical heme protein that catalyzes the hydroxylation of aromatic and aliphatic substrates (Bernhardt and Urlacher 2014). Monooxygenase cytochrome P450 was discovered for the first time in 1963 to be involved in the aerobic hydroxylation of steroid hormones (Cooper et al. 1963). However, the first bacterial cytochrome P450 was discovered in *Bacillus*

*megaterium* in the mid-1970s (Berg et al. 1975). KSH was expressed in microbial species such as *Nocardia* (Strijewskim 1982), *Rhodococcus* sp. (Knol et al. 2008), and *Mycobacterium* (Yao et al. 2014) and was shown to actively participate in aerobic steroid degradation by initiating the alpha-hydroxylation of C9 position. Another steroid-degrading gene, which produces the enzyme  $3\alpha$ -hydroxysteroid dehydrogenase and is also expressed by the strain BH2-1, acts on a variety of steroid hormones by catalyzing the redox reaction of the hydroxyl ketone group at position 3 of C19–27 of steroids. The strain *Comamonas testosterone* (*Pseudomonas testis*) expresses this dehydrogenase (Abalain et al. 1995; Oppermann et al. 1993), which can reduce the ketone group present at position 3 of testosterone.

### Detection of intermediate compounds during E1 degradation reaction

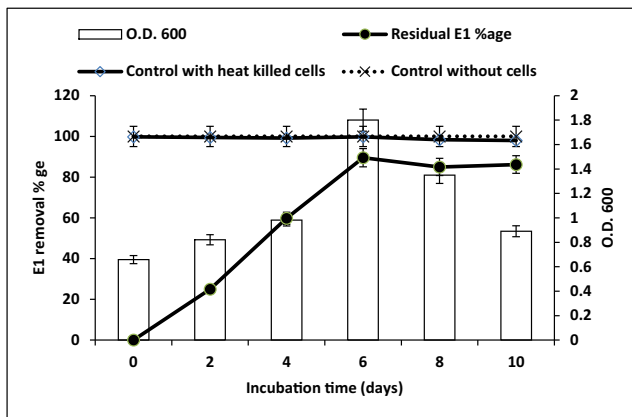
From the preceding, it was shown that strain BH2-1 could efficiently degrade E1 (up to 89.5%) after 6 days of incubation. Few reports have so far explained E1 biodegradation, but the exact metabolic pathway is still unclear. Here, we analyzed the intermediate compounds formed during E1 biotransformation/biodegradation carried out by the strain BH2-1. To achieve our set objectives, the residual E1 extraction time was reduced. Residual E1 extraction was carried out at 3-h intervals up to 6 days of incubation, with the collected extraction mixtures at each time point subjected to HPLC followed by GC-MS analysis. Two intermediate compounds, i.e., 3-hydroxyandrosta-5,7,9(11)-trien-17-one and androst-1,4,6-triene-3,17-dione (ATD), were found after 3 h and 6 h of extraction, respectively. The presence of these compounds

**Fig. 2** Growth and estrone degradation capabilities of BH2-1 bacterial strain. **a** BH2-1, growth, and estrone degradation optimization at various incubation temperatures (10–40 °C). **b** Optimization of media pH (3–9) for the growth and degradation of estrone. **c** Optimum NaCl concentration (0–5%) required for maximum growth and estrone degradation



was confirmed by GC-MS analysis by using the MS library (Fig. 5a, b). Both compounds were not found in the next phase of extraction, which confirms their non-accumulating nature. To validate our results, entire experiments were repeated three times, yielding similar results, therefore ascertaining our findings. These results indicate that the intermediate compounds formed during the first 6 h of biotransformation reactions were further consumed by the microbial cells as the reaction

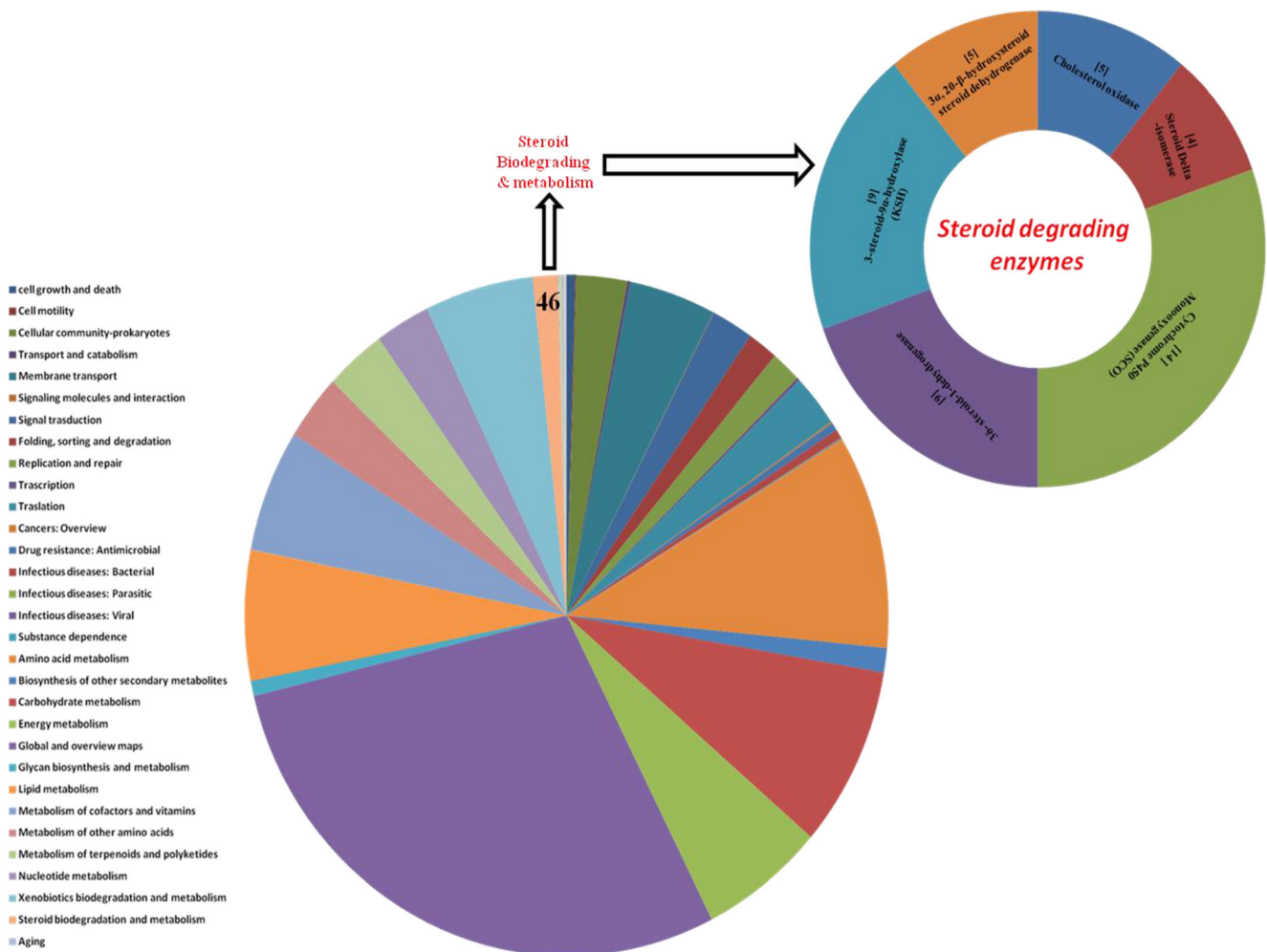
proceeds. Our findings were also validated by the whole-genome sequence analysis of strain BH2-1, which confirmed the expression of steroid  $\Delta$ -isomerase enzyme-producing gene in the strain BH2-1. This enzyme is responsible for the structural rearrangements in molecules, and the detected intermediate compound 3-hydroxyandrost-5,7,9(11)-trien-17-one is the result of only the structural rearrangements which took place in E1 in the first 3 h of incubation. The second



**Fig. 3** Estrone degradation profile of BH2-1 strain up to 10 days of the reaction mixture

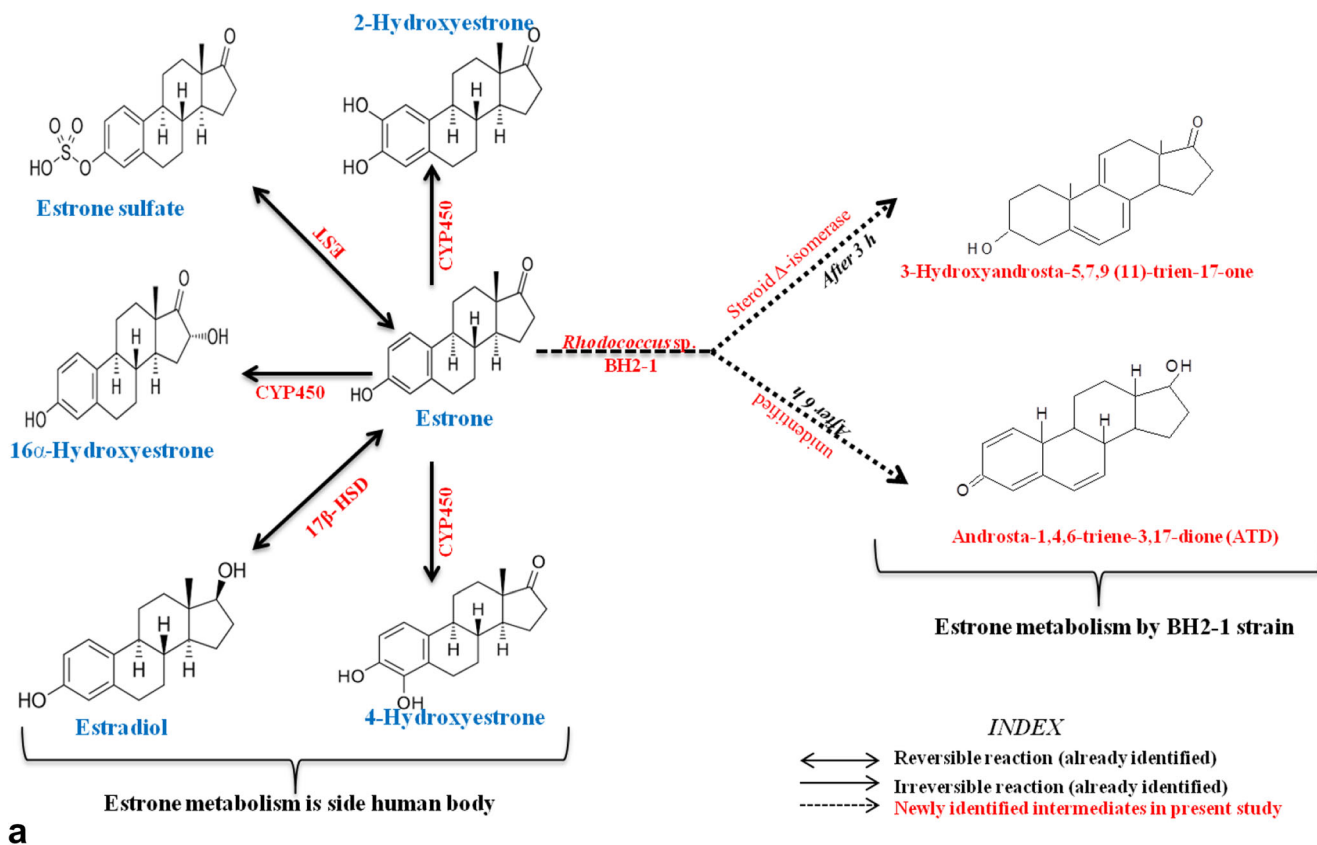
intermediate, i.e., ATD, is a well-known irreversible aromatase inhibitor, which reduces estrogen biosynthesis and enhances the levels of endogenous steroids like dihydrotestosterone and testosterone (Ellinwood et al. 1984; Kwok et al. 2015). ATD is also used as an illegal drug to enhance the

performance of sports persons (Kwok et al. 2015; Devlin 2010). In the study, we did not find any presence of testosterone in the reaction mixture even after 6 days of incubation. The reason behind this exception might be the absence of  $17\beta$ -hydroxysteroid dehydrogenase ( $17\beta$ -HSD) enzyme-producing genes in the genome of strain BH2-1, which is essential for the conversion of ATD into testosterone (Walter et al. 2014; Hilborn et al. 2017). Furthermore, Kuiper et al. (1997) reported that ATD has a very low affinity towards estrogen receptors and has a weak androgenic effect. Due to these reasons, ATD is less harmful to aquatic animals as compared to E1. In addition, studies have shown that in humans, E1 degradation takes place by various enzymes such as cytochrome P450 (CYP450), estrone sulfotransferases (ESTs), and  $17\beta$ -HSD. In the presence of these enzymes, E1 is conjugated into estrogen conjugates such as 2-hydroxyestrone and 4-hydroxyestrone,  $16\alpha$ -hydroxyestrone, estrone sulfate, and E2, respectively (Kuhl 2005). In the present study, the whole-genome analysis of strain BH2-1 revealed the presence of genes that are directly associated with steroid metabolism



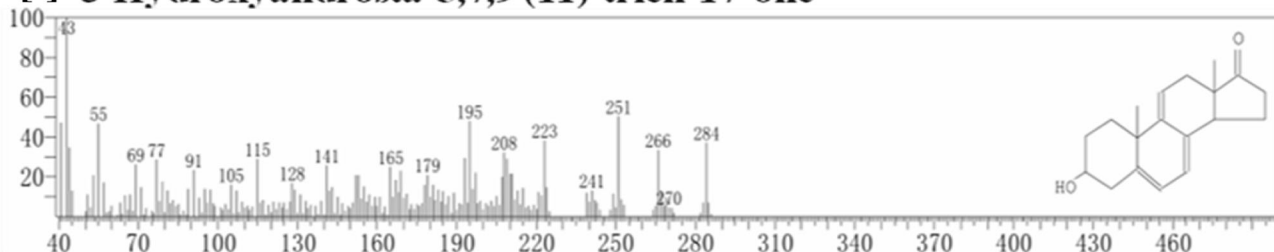
**Fig. 4** Genome analysis of BH2-1 strain along with the presence of steroid-degrading enzymes



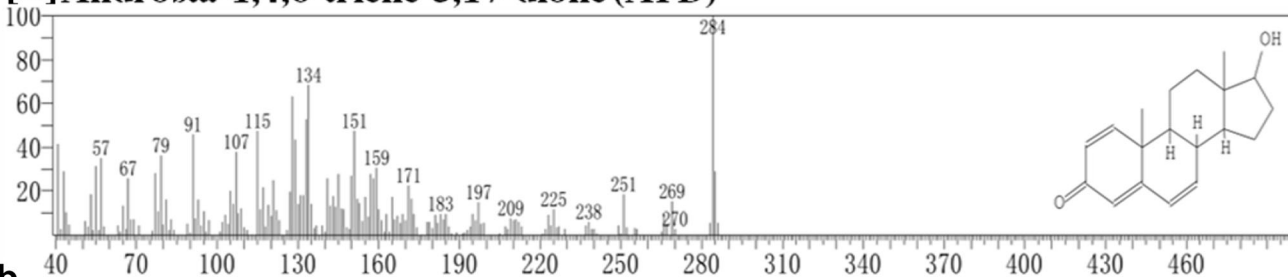


**a**

**[i] 3-Hydroxyandrosta-5,7,9 (11)-trien-17-one**



**[ii] Androsta-1,4,6-triene-3,17-dione (ATD)**



**b**

**Fig. 5 a** Intermediate compound detection during 3 h and 6 h of degradation reaction. Estrone metabolism by human enzymes (left) (Kuhl 2005) and by the strain BH2-1 (right). **b** GC-MS chromatograph

of both newly detected non-accumulating intermediates [3-hydroxyandrosta-5,7,9(11)-trien-17-one and androsta-1,4,6-triene-3,17-dione (ATD)] shown after 3 h and 6 h of reaction, respectively

such as cholesterol oxidase, steroid  $\Delta$ -isomerase, SCO,  $3\delta$ -steroid-1-dehydrogenase, KSH, and  $3\alpha,20\text{-}\beta$ -hydroxysteroid steroid dehydrogenase (Fig. 5a). The findings here suggest that given the expression of steroid-metabolizing genes by

the strain BH2-1, it could efficiently degrade E1. The detailed enzymatic metabolism of E1 in humans (left) (Kuhl 2005) and the proposed E1 metabolism by microbial strain BH2-1 (right) are given in Fig. 5 a. The GC-MS chromatograph of

intermediates (3-hydroxyandrosta-5,7,9(11)-trien-17-one and ATD) is shown in Fig. 5 b.

## Conclusion

In the present study, a new aerobic microbial strain was isolated from soil sediments collected from the Bai Hai mangrove area of Southern China. The isolated strain was capable of growing in minimal salt medium (MSM) with 2 mg L<sup>-1</sup> E1 as the sole carbon source. This isolated strain could remove about 89.5% of E1 under optimized conditions. The optimum growth conditions for maximum E1 degradation by the BH2-1 strain were as follows: incubation temperature of 25 °C, medium pH of 6.0, and NaCl concentration of 1%. Therefore, the strain BH2-1 could be a potential useful microorganism for E1 pollution treatment. The intermediate compounds detected during the E1 degradation reaction will further help to understand the complex E1 degradation mechanism in depth. In addition, the whole-genome analysis of strain BH2-1 has revealed the presence of various genes that actively participate in steroid degradation. The genome analysis of strain BH2-1 also helps to understand the role of various enzymes in E1 degradation. Further studies would seek to identify the enzymes which are responsible for E1 breakdown or degradation and the reaction mechanism behind this biodegradation process.

**Funding information** This work was supported by the grants from the National Natural Science Foundation of China (41706143, 31870104, and 31770130) and Guangdong Natural Science Foundation, China (2014A020217017).

## References

- Abalain JH, Di Stefano S, Abalain-Colloc ML, Floch HH (1995) Cloning, sequencing and expression of *Pseudomonas testosteroni* gene encoding 3 $\alpha$ -hydroxysteroid dehydrogenase. *J Steroid Biochem Mol Biol* 55:233–238. [https://doi.org/10.1016/0960-0760\(95\)00170-5](https://doi.org/10.1016/0960-0760(95)00170-5)
- Ankley GT, Feifarek D, Blackwell B, Cavallin JE, Jensen KM, Kahl MD, Poole S, Randolph E, Saari T, Villeneuve DL (2017) Re-evaluating the significance of estrone as an environmental estrogen. *Environ Sci Technol* 51:4705–4713. <https://doi.org/10.1021/acs.est.7b00606>
- Berg A, Carlstrom K, Gustafsson JA, Ingelman-Sundberg M (1975) Demonstration of a cytochrome P-450-dependent steroid 15 $\beta$ -hydroxylase in *Bacillus megaterium*. *Biochem Biophys Res Commun* 66:1414–1423. [https://doi.org/10.1016/0006-291X\(75\)90517-3](https://doi.org/10.1016/0006-291X(75)90517-3)
- Bernhardt R, Urlacher VB (2014) Cytochromes P450 as promising catalysts for biotechnological application: chances and limitations. *Appl Microbiol Biotechnol* 98:6185–6203. <https://doi.org/10.1007/s00253-014-5767-7>
- Chowdhury RR, Charpentier P, Ray MB (2010) Photodegradation of estrone in solar irradiation. *Ind Eng Chem Res* 49(15):6923–6930. <https://doi.org/10.1021/ie901796x>
- Cooper DY, Estabrook RW, Rosenthal O (1963) The stoichiometry of C21 hydroxylation of steroids by adrenocortical microsomes. *J Biol Chem* 238:1320–1323
- Cowan ST, Steel KJ (1965) Manual for the identification of medical bacteria. Cambridge University Press, London
- D’Addezio JM (2015) Seasonal variability of salinity and salt transport in northern Indian Ocean. *J Phys Oceanogr* 45:1947–1966. <https://doi.org/10.1175/JPO-D-14-0210.1>
- Devlin TM (2010) Textbook of biochemistry: with clinical correlations, 7th edn. John Wiley & Sons, Hoboken, p 432 ISBN 978-0-470-28173-4
- Du Z, Yinguang C, Li X (2017) Quantitative proteomic analyses of the microbial degradation of estrone under various background nitrogen and carbon conditions. *Water Res* 123:361–368. <https://doi.org/10.1016/j.watres.2017.06.070>
- Ellinwood WE, Hess DL, Roselli CE, Spies HG, Resko JA (1984) Inhibition of aromatization stimulates luteinizing hormone and testosterone secretion in adult male rhesus monkeys. *J Clin Endocrinol Metab* 59(6):1088–1096. <https://doi.org/10.1210/jcem-59-6-1088>
- Hilbom E, Olle S, Agneta J (2017) Estrogen and androgen-converting enzymes 17 $\beta$ -hydroxysteroid dehydrogenase and their involvement in cancer: with a special focus on 17 $\beta$ -hydroxysteroid dehydrogenase type 1, 2, and breast cancer. *Oncotarget* 2 8(18):30552–30562. <https://doi.org/10.18632/oncotarget.15547>
- Horinouchi M, Yamamoto T, Taguchi K, Arai H, Kudo T (2001) Metacleave enzyme gene tesB is necessary for testosterone degradation in *Comamonas testosteroni* TA441. *Microbiol* 147(Pt 12):3367–3375. <https://doi.org/10.1099/00221287-147-12-3367>
- Horinouchi M, Hayashi T, Kudo T (2012) Steroid degradation in *Comamonas testosteroni*. *J Steroid Biochem* 129:4–14. <https://doi.org/10.1016/j.jsbmb.2010.10.008>
- Hutcheson SW, Zhang H, Suvorov M (2011) Carbohydrase systems of *Saccharophagus degradans* degrading marine complex polysaccharides. *Mar Drugs* 9(4):645–665. <https://doi.org/10.3390/md9040645>
- Imran M, Poduval PB, Ghadi SC (2017) Bacterial degradation of algal polysaccharides in marine ecosystem. In: Naik MM, Dubey SK (eds) Marine pollution and microbial remediation. Springer, Singapore, pp 189–203
- John GH, Noel RK, Peter HAS, James TS, Stanley TW (1994) Bergey’s manual of determinative bacteriology, 9th edn. Lippincott Williams & Wilkins, p 11
- Knol J, Bodewits K, Hessels GI, Dijkhuizen L, van der Geize R (2008) 3-Keto-5 $\alpha$ -steroid delta(1)-dehydrogenase from *Rhodococcus erythropolis* SQ1 and its orthologue in *Mycobacterium tuberculosis* H37Rv are highly specific enzymes that function in cholesterol catabolism. *Biochem J* 410:339–346. <https://doi.org/10.1042/BJ20071130>
- Kuhl H (2005) Pharmacology of estrogens and progestogens: influence of different routes of administration. *Climacteric* 8(Suppl 1):3–63. <https://doi.org/10.1080/13697130500148875>
- Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson JA (1997) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* 138(3):863–870. <https://doi.org/10.1210/endo.138.3.4979>
- Kumari L, Kanwar SS (2012) Cholesterol oxidase and its applications. *Adv Microbiol* 2(2):49–65. <https://doi.org/10.4236/aim.2012.22007>
- Kurusu F, Oguru M, Saitoh S, Yamazoe A, Yagi O (2010) Degradation of natural estrogen and identification of the metabolites produced by soil isolates of *Rhodococcus* sp. and *Sphingomonas* sp. *J Biosci Bioeng* 109(6):576–582. <https://doi.org/10.1016/j.jbiosc.2009.11.006>
- Kwok WH, Leung GN, Wan TS, Curl P, Schiff PJ (2015) Metabolic study of androsta-1,4,6-triene-3,17-dione in horses using liquid chromatography/high resolution mass spectrometry. *J Steroid*

- Biochem Mol Biol 152:142–154. <https://doi.org/10.1016/j.jsbmb.2015.05.011>
- Lee HB, Liu D (2002) Degradation of 17 $\beta$ -estradiol and its metabolites by sewage bacteria. *Water Air Soil Pollut Focus* 134:351–366. <https://doi.org/10.1023/A:1014117329403>
- Lei B, Wen Y, Wang X, Zha J, Li W, Wang Z, Sun Y, Kang J, Wang Y (2013) Effects of estrone on the early life stages and expression of vitellogenin and estrogen receptor genes of Japanese medaka (*Oryzias latipes*). *Chemosphere* 93:1104–1110. <https://doi.org/10.1016/j.chemosphere.2013.06.025>
- Li S, Juan L, Sun M, Ling W, Zhu X (2017) Isolation, characterization, and degradation performance of the 17 $\beta$ -estradiol-degrading bacterium *Novosphingobium* sp. E2S. *Inter J Envi Res Public Health* 14(115):1–13. <https://doi.org/10.3390/ijerph14020115>
- Liu J, Liu J, Xu D, Ling W, Li S, Chen M (2016) Isolation, immobilization, and degradation performance of the 17 $\beta$ -estradiol-degrading bacterium *Rhodococcus* sp. JX-2. *Water Air Soil Pollut* 227(422):1–13. <https://doi.org/10.1007/s11270-016-3122-6>
- MacLachlan J, Wotherspoon ATL, Ansell RO, Brooks CJW (2000) Cholesterol oxidase: sources, physical properties and analytical applications. *J Steroid Biochem Mol Biol* 72(5):169–195. [https://doi.org/10.1016/S0960-0760\(00\)00044-3](https://doi.org/10.1016/S0960-0760(00)00044-3)
- Oppermann UCT, Netter KJ, Maser E (1993) Carbonyl reduction by 3 $\alpha$ -HSD from *Comamonas testosteroni*—new properties and its relationship to the SCAD family. In: Weiner H, Crabb DW, Flynn TG (eds) *Enzymology and molecular biology of carbonyl metabolism* 4. Springer US, Boston, pp 379–390
- Penning TM (2003) Hydroxysteroid dehydrogenases and pre-receptor regulation of steroid hormone action. *Hum Reprod Update* 9:193–205. <https://doi.org/10.1093/humupd/dmg022>
- Peterson KN, Tan DT, Bezares-Cruz C, Novak PJ (2017) Estrone biodegradation in laboratory scale systems designed for total nitrogen removal from wastewater. *Environ Sci Technol* 3(6):1051–1060. <https://doi.org/10.1039/C7EW00164A>
- Qamar A, Bhatt DL (2015) Effect of low cholesterol on steroid hormones and vitamin E levels: just a theory or real concern? *Circulation Res* 117:662–664. <https://doi.org/10.1161/CIRCRESAHA.115.307345>
- Samavat H, Kurzer MS (2015) Estrogen metabolism and breast cancer. *Cancer Lett* 356:231–243. <https://doi.org/10.1016/j.canlet.2014.04.018>
- Sami N, Fatima T (2019) Studies on estrone biodegradation potential of cyanobacterial species. *Biocatal Agric Biotechnol* 17:576–582. <https://doi.org/10.1016/j.bcab.2019.01.022>
- Samir KL, Xie B, Thompson ML, Sung S, Ong SK, Leeuwen JHV (2006) Fate, transport, and biodegradation of natural estrogens in the environment and engineered systems. *Environ Sci Technol* 40(21):6537–6546. <https://doi.org/10.1021/es0607739>
- Sang Y, Xiong G, Maser E (2012) Identification of a new steroid degrading bacterial strain H5 from the Baltic Sea and isolation of two estradiol inducible genes. *J Steroid Biochem Mol Biol* 129:22–30. <https://doi.org/10.1016/j.jsbmb.2011.01.018>
- Silva CP, Otero M, Esteves V (2012) Processes for the elimination of estrogenic steroid hormones from water: a review. *Environ Pollut* 165:38–58. <https://doi.org/10.1016/j.envpol.2012.02.002>
- Strijewskim A (1982) The steroid-9 alpha-hydroxylation system from *Nocardia* species. *Eur J Biochem* 128:125–135. <https://doi.org/10.1111/j.1432-1033.1982.tb06942.x>
- Talalay P, Wang VS (1955) Enzymic isomerization of delta5-3-ketosteroids. *Biochim Biophys Acta* 18(2):300–301
- Thayanukul P, Zhang K, Jonhom T, Kurisu F, Kasuga L, Furumai H (2010) Concentration-dependent response of estrone-degrading bacterial community in activated sludge analyzed by microautoradiography-fluorescence in situ hybridization. *Water Res* 44(17):4878–4887. <https://doi.org/10.1016/j.watres.2010.07.031>
- Ting YF, Praveena SM (2017) Sources, mechanisms, and fate of steroid estrogens in wastewater treatment plants: a mini review. *Environ Monit Assess* 189(178):1–19. <https://doi.org/10.1007/s10661-017-5890-x>
- Ukpong LE (1995) Vegetation and oil acidity of a mangrove swamp in Southeastern Nigeria. *Soil Use Manage* 11(3):141–144. <https://doi.org/10.1111/j.1475-2743.1995.tb00512.x>
- Van der Geize R, Hessels GI, Nienhuis-Kuiper M, Dijkhuizen L (2008) Characterization of a second *Rhodococcus erythropolis* SQ1 3-ketosteroid 9alpha-hydroxylase activity comprising a terminal oxygenase homologue, KshA2, active with oxygenase-reductase component KshB. *Appl Environ Microbiol* 74:7197–7203. <https://doi.org/10.1128/AEM.00888-08>
- Veldkamp H (1970) Chapter V. Enrichment culture for prokaryotic organisms. In: *Method microbiology* 3A edn. Elsevier Academic Press Inc, London, pp 305–361. [https://doi.org/10.1016/S0580-9517\(08\)70543-9](https://doi.org/10.1016/S0580-9517(08)70543-9)
- Walter L, Miller MD, Christa E, Fluck MD (2014) Chapter 13: Adrenal cortex and its disorders. In: *Pediatric endocrinology*, 4th edn, pp 471–532.e1. <https://doi.org/10.1016/B978-1-4557-4858-7.00022-6>
- Wang Y, Shao H, Zhu S, Tian K, Qiu Q, Huo H (2019) Degradation of 17 $\beta$ -estradiol and products by a mixed culture of *Rhodococcus equi* DSSKP-R-001 and *Comamonas testosteroni* QYY20150409. *Biotechnol Biotech Eq* 33(1):268–277. <https://doi.org/10.1080/13102818.2019.1568913>
- Wedekind C (2014) Fish populations surviving estrogen pollution. *BMC Biol* 12(10):1–3. <https://doi.org/10.1186/1741-7007-12-10>
- Xu RF, Sun MX, Liu J, Wang H, Li X, Zhu XZ, Ling WT (2014) Isolation, characteristics, and performance of a diethylstilbestrol-degrading bacteria strain *Serratia* sp. *Environ Sci* 35:328–333. (In Chinese). <https://doi.org/10.13227/j.hjcx.2014.08.047>
- Yao K, Xu LQ, Wang FQ, Wei DZ (2014) Characterization and engineering of 3-ketosteroid- big up tri, open1-dehydrogenase and 3-ketosteroid-9alpha-hydroxylase in *Mycobacterium neoaurum* ATCC 25795 to produce 9alpha-hydroxy-4-androstene-3,17-dione through the catabolism of sterols. *Metab Eng* 24:181–191. <https://doi.org/10.1016/j.ymben.2014.05.005>
- Ye X, Peng T, Feng J, Yang Q, Pratush A, Xiong G, Huang T, Hu Z (2019) Novel dehydrogenase 17 $\beta$ -HSDx from *Rhodococcus* sp. P14 with potential application in bioremediation of steroids contaminated environment. *J Hazard Mater* 362:70–177. <https://doi.org/10.1016/j.jhazmat.2018.09.023>
- Yu CP, Deeb RA, Chu KH (2013) Microbial degradation of steroidal estrogens. *Chemosphere* 91:1225–1235. <https://doi.org/10.1016/j.chemosphere.2013.01.112>
- Zhang C, Li Y, Wang C, Niu L, Cai W (2015a) Occurrence of endocrine disrupting compounds in aqueous environment and their bacterial degradation: a review. *Crit Rev Environ Sci Technol* 46(1):1–59. <https://doi.org/10.1080/10643389.2015.1061881>
- Zhang Z, Gao P, Su H, Zhan P, Ren N, Feng Y (2015b) Anaerobic biodegradation characteristics of estrone, estradiol, and 17 $\alpha$ -ethinylestradiol in activated sludge batch tests. *Desalin Water Treat* 53(4):985–993. <https://doi.org/10.1080/19443994.2013.848415>
- Zhao X, Grimes KL, Colosi LM, Lung WS (2019) Attenuation, transport, and management of estrogens: a review. *Chemosphere* 230:462–478. <https://doi.org/10.1016/j.chemosphere.2019.05.086>