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Research Paper

Metabolism analysis of 17α-ethynylestradiol by *Pseudomonas citronellolis* SJTE-3 and identification of the functional genes

Wanli Peng , Yali Fu, Ben Jia, Xin Sun, Yanqiu Wang, Zixin Deng , Shuangjun Lin , Rubing Liang *

State Key Laboratory of Microbial Metabolism, Joint International Research Laboratory of Metabolic & Developmental Sciences, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, China

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ABSTRACT

Synthetic estrogens are the most hazardous and persistent environmental estrogenic contaminants, with few reports on their biodegradation. *Pseudomonas citronellolis* SJTE-3 degraded natural steroids efficiently and metabolized 17α-ethynylestradiol (EE2) with the addition of different easily used energy sources (glucose, peptone, ethanol, yeast extract, fulvic acid and ammonia). Over 92% of EE2 (1 mg/L) and 55% of EE2 (10 mg/L) in culture were removed in seven days with the addition of 0.1% ethanol, and the EE2-biotransforming efficiency increased with the increasing ethanol concentrations. Two novel intermediate metabolites of EE2 ($C_{22}H_{22}O$ and $C_{18}H_{34}O_2$) were identified with high-performance liquid chromatography (HPLC) and GC-Orbitrap/MS. Comparative analysis and genome mining revealed strain SJTE-3 contained a unique genetic basis for EE2 metabolism, and the putative EE2-degrading genes exhibited dispersed distribution. The EE2 metabolism of strain SJTE-3 was inducible and the transcription of eight genes were significantly induced by EE2. Three genes (*sdr*3, *yjcH* and *cyp*2) encoding a short-chain dehydrogenase, a membrane transporter and a cytochrome P450 hydroxylase, respectively, were vital for EE2 metabolism in strain SJTE-3; their over-expression accelerated EE2 metabolic processes and advanced the generation of intermediate metabolites. This work could promote the study of bacterial EE2 metabolism mechanisms and facilitate efficient bioremediation for EE2 pollution.

1. Introduction

Among the emerging contaminants (ECs), endocrine-disrupting compounds (EDCs) have drawn much attention since the 1990 s, due to their adverse health effects on humans and wildlife (Petrie et al., 2015). Environmental estrogens (EEs), the most concerning EDCs, are distributed in various aquatic and terrestrial environments and cause a serious global public health hazard (Santoro et al., 2016; Adeel et al., 2017). In addition to the natural estrogens like 17β-estradiol (E2) and estrone (E1), 17α-ethynylestradiol (EE2), the most commonly used synthetic estrogen in oral contraceptive pills in animal husbandry and human medicine (700 kg/year), shows strong estrogenic activity and causes severe disorders in animals and human beings (Aris et al., 2014). It enters into the environment mainly through the human urine in domestic wastewater discharge, and has been found in wastewater, sewage sludge, surface and underground water, drinking water, soils, and sediments. Its concentration was found to be up to 230 ng/L in surface water and 133.64 ng/g in sediment; its concentrations could even reach 4100 ng/L in effluents and 943 ng/g in sludge (Aris et al., 2014; Zhao et al., 2019; Dodgen et al., 2014; Tan et al., 2021). As EE2 contains an alkynyl group at the C17 position, it shows more stable chemical properties than the natural estrogens and persists in the environment for a long time, which is quite difficult to remove (Tan et al., 2021). Therefore, EE2 is considered as one of the priority ECs in the latest Japanese Drinking Water Quality Standard and the EU Watch list in Decision 2015/495/EU (Nguyen et al., 2020).

Biodegradation is considered to be a simple and effective method to remove the EEs and remedy the polluted environments (Zhao et al., 2019; Dodgen et al., 2014; Tan et al., 2021; Nguyen et al., 2020; Olivera and Luengo, 2019). Some microorganisms have been found to be able to utilize estrogen as a carbon or energy source and decompose estrogens into harmless or low-estrogenic products (Zhao et al., 2019; Olivera and Luengo, 2019; Yu et al., 2013). Although these estrogen-degrading strains can degrade natural estrogen efficiently, it is too difficult for them to utilize EE2 (Olivera and Luengo, 2019; Cajthaml et al., 2009). Only a few microorganisms have been identified with the capabilities to transform or co-metabolize EE2, including the bacteria strains *Nitrosomonas europaea, Sphingomonas* spp., *Enterobacter tabaci*, and

* Corresponding author.
E-mail address: icelike@sjtu.edu.cn (R. Liang).

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Pseudomonas spp.; the cyanobacteria strain *Microcystis novacekii*; the microalgae strain *Selenastrum capricornutum*; the fungi strain *Phoma* sp. and the mushroom strain *Pleurotus ostreatus* (Cajthaml et al., 2009; Skotnicka-Pitak et al., 2009; Haiyan et al., 2007; Gaulke et al., 2008; Khunjar et al., 2011; Forrez et al., 2009; Zheng et al., 2016; Sedighi et al., 2019; Fioravante et al., 2012; Hom-Diaz et al., 2015; Hofmann and Schlosser, 2016; Sheng et al., 2021; Della Greca et al., 2008; Yi and Harper, 2007; Pauwels et al., 2008). However, EE2 cannot be used by these strains as a sole energy source to support cell growth. For example, *Pseudomonas* sp. and *Acinetobacter* sp. were reported to metabolize 95% of 1 mg/L EE2 in five days together with E2 (Pauwels et al., 2008). *N. europaea* could transform 90% of 1 mg/L EE2 in nine days and ammonia was required for the initiation of EE2 biotransformation (Skotnicka-Pitak et al., 2009; Sheng et al., 2021). EE2 is a typical recalcitrant chemical when transformed by microorganisms. Its biotransformation efficiency is quite low and the period is very long (Olivera and Luengo, 2019; Cajthaml et al., 2009; Pratush et al., 2020). The co-metabolic biodegradation of EE2 by ammonia-oxidizing bacteria (AOB) represents the major pathway for EE2 removal in natural and engineered systems, and requires a high concentration of ammonia to initiate the removal of EE2; no EE2 removal was observed at a relatively low concentration of ammonia (Gaulke et al., 2008; Khunjar et al., 2011; Forrez et al., 2009; Sheng et al., 2021). Only the sulfo-EE2, 4-hydroxy-EE2 and nitrated EE2, have been identified as the intermediate metabolites of EE2 (Skotnicka-Pitak et al., 2009; Gaulke et al., 2008; Khunjar et al., 2011; Della Greca et al., 2008); only the ammonia monooxygenase (AMO) and some enzymes for denitrification have been proposed to participate in EE2 co-metabolism in the AOB, with an unknown mechanism (Khunjar et al., 2011; Sheng et al., 2021; Yi and Harper, 2007). Currently, the molecular mechanism of EE2 transformation or co-metabolism in bacteria remains elusive and unclear. The scarcity of the identified intermediate metabolites, the genome sequence of the EE2-degrading strain, and the genes and enzymes with characterized functions, all hindered the mechanism study of EE2 bio-utilization and the related application for environment remediation (Tan et al., 2021; Olivera and Luengo, 2019; Pratush et al., 2020).

Pseudomonas citronellolis SJTE-3 has been found to have a versatile and efficient ability to degrade natural steroids (E2, E1, and testosterone) and synthetic estrogen (EE2). The whole genome sequence of this strain has been obtained and deposited in GenBank (GenBank NZ_CP015878.1/CP015879.1) (Zheng et al., 2016). It is also the first sequenced EE2-utilizing strain. Characterization of the intermediate metabolites of EE2 and the functional genes for EE2 metabolism in this strain can help to reveal the metabolic mechanism of EE2 in bacteria. In this work, the metabolic properties and the intermediate metabolites of EE2 in *P. citronellolis* SJTE-3 were determined and its genetic basis and the key functional genes for EE2 metabolism were analyzed. The identification of two novel intermediate metabolites of EE2 and three new genes responsible for EE2 metabolism in strain SJTE-3 could promote the study of EE2 metabolism mechanism and facilitate EE2 pollution bioremediation procedures.

2. Materials and methods

2.1. Chemicals, strains, and cultures

17α-ethynyl estradiol (EE2, $C_{20}H_{24}O_2$, Mw = 296.40, > 98% purity), 17β-estradiol (E2, $C_{18}H_{24}O_2$, Mw = 272.38, > 99% purity), estrone (E1, $C_{18}H_{22}O_2$, Mw = 270.37, > 99% purity), testosterone (TES, $C_{19}H_{28}O_2$, Mw = 288.42, > 99% purity), Tween-80 ($C_{24}H_{44}O_6$, Mw = 428.60), and Triton X-100 ($C_{34}H_{62}O_{11}$, Mw = 646.86) were the product of Sigma-Aldrich (Allentown, PA, USA). The reagents were dissolved in DMSO to 10 mg/mL and stored at − 20 °C. Acetonitrile and ethyl acetate of HPLC grade were obtained from EMD (Gibbstown, NJ, USA). All the strains and plasmids used in this study were listed in Table S1. LB medium (tryptone 10.0 g/L, yeast extract 5.0 g/L, NaCl 8.0 g/L) and the minimal medium (KH_2PO4 4.5 g/L, $K_2H_PO_4$•$3H_2O$ 13.75 g/L, $(NH_4)_2SO_4$ 2.0 g/L, $MgSO_4$•$7H_2O$ 0.16 g/L, $FeSO_4$ 5.0 μg/L, $CaCl_2$•$2H_2O$ 1.0 μg/L, $MnCl_2$•$4H_2O$ 2.0 μg/L, pH 7.4) were used for strain culture. Solid plates were prepared by supplying agar (15.0 g/L) to the liquid medium.

2.2. Standard DNA manipulation

All of the oligonucleotides used in this study were listed in Table S2, designed with the Oligo 7 software and synthesized by Invitrogen Ltd. (Shanghai, China) (Rychlik, 2007). DNA polymerases were purchased form TaKaRa Co. (Dalian, China); T4 DNA ligases and all of the restriction endonucleases were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). All of the plasmids were constructed by the Gibson assembly method and verified by sequencing at Invitrogen Ltd. (Shanghai, China). Chemical and electroporation-mediated transformation were used for plasmids transforming into E. coli strains and P. *citronellolis* strains, respectively. The genome DNA and the plasmid DNA were isolated with the TIANamp Bacteria DNA Kit and TIANprep Mini Plasmid Kit (Tiangen, Beijing, China); the PCR fragments were purified with TIANquick Midi Purification Kit (Tiangen, Beijing, China). Other general techniques were carried out following the standard protocols.

2.3. Detection of steroid chemicals with high-performance liquid chromatography (HPLC)

A reverse-phase HPLC system was used to detect the residues of steroids using an Agilent 1260 Infinity LC system equipped with a fluorescence detector (FLD) and a SB-C18 reverse-phase column (4.6 × 150 mm, 5 μm particle size, Agilent, CA, USA). Steroids (E1, E2, EE2, and TES) dissolved by acetonitrile into different concentrations (0.1 μg/mL, 0.5 μg/mL, 1 μg/mL, 10 μg/mL, 25 μg/mL, and 50 μg/mL) and the standard curves were determined. The column was maintained at 30 °C, and the injection volume was 10 μL. The mobile phase consisted of acetonitrile and water (55/45, v/v) and the flow rate was 1.0 mL/min. Testosterone was detected using a Photo-Diode Array (DAD) detector at the wavelength of 245 nm; the estrogens (E1, E2, and EE2) were detected with the Fluorescence Detector (FLD detector), with the excitation and emission wavelengths at 280 and 315 nm, respectively. The steroids in culture were detected after treatment and the residues were calculated according to the standard curves of each chemical with all the R^2 values over 0.99.

2.4. Enrichment, isolation, and property analysis of P. citronellolis SJTE-3

The samples were collected from activated sludge obtained from of a wastewater treatment plant in China (no specific permissions were required, and this work did not involve any endangered or protected species). Ten grams of the sample was inoculated into a 500 mL flask with 100 mL minimal medium containing EE2 (10 mg/L) and the flask was shaken at 30 °C for seven days. Ten milliliters of the culture was added into 90 mL fresh medium containing EE2 (10 mg/L) and cultured for another seven days. After being enriched three times, the cultures were plated onto MM plates containing EE2 (10 mg/L) and cultivated for three days. The colonies grown on the plates were harvested and re-cultured; their EE2-transforming abilities were detected by HPLC as described above. One strain able to transform EE2 was isolated and named as SJTE-3, deposited in the China General Microbiological Culture Collection Center (CGMCC, No. CGMCC15428); its whole genome was sequenced and submitted to the National Center for Biotechnology Information (NCBI) database (GenBank No. NZ_CP015878.1 and CP015879.1) (Zheng et al., 2016). The morphological and physiological properties of strain SJTE-3 were characterized following Bergey's Manual of Determinative Bacteriology (Hitchens et al., 1944). The evolutionary status was analyzed by constructing a phylogenetic tree

based on the 16S rDNA sequence of strain SJTE-3 (GenBank No. KU860463.1) and those of other related strains with MEGA 7.0 (Kumar et al., 2016). Neighbor-Joining method was used to build the unrooted trees with 1000 replications, and the evolutionary distances were calculated with the Kimura two-parameter distance model.

2.5. Comparative genome analysis and genome mining of P. citronellolis SJTE-3

The average nucleotide identity (ANI) values of the whole genome sequence of *P. citronellolis* SJTE-3 compared to seven other *Pseudomonas* strains were analyzed with JSpeciesWS online software (http://jspecies.ribohost.com/jspeciesws/#home). The comparative genome analysis of strain SJTE-3 and six other estrogen-degrading strains was performed with TBtools software (Chen et al., 2020). The putative gene homologues were identified using BLAST analysis in NCBI and the RAST software (Aziz et al., 2008). Multiple sequence alignments (MSA) of the potential metabolic genes and proteins in strain SJTE-3 and the other estrogen-degrading strains were analyzed with DNAman software with default parameters. The visualization analysis of the homologous gene relationships in strain SJTE-3 and other estrogen-degrading strains was performed with MAUVE v2.4.0 (Darling et al., 2004).

2.6. Determination of the utilization efficiencies of different steroids by P. citronellolis SJTE-3

The utilization efficiencies of different steroids (E1, E2, EE2, and TES) by strain SJTE-3 were determined by detecting the chemical residues in culture and calculating the loss of the chemical. A single colony of strain SJTE-3 was inoculated into 20 mL LB medium and cultured at 30 °C overnight. Cells were harvested and washed three times with sterilized water before being re-suspended with the minimal medium. The cell inocula (initial OD_{600} = 0.05) were added into 1 L minimal medium containing E1 (10 mg/L), E2 (10 mg/L), TES (10 mg/L), or EE2 (1, 5, 10 and 50 mg/L), respectively; the media with the addition of 0.1% ethanol were also supplied. The groups without steroids, without cells or with the boiled cells were used as controls to assess the abiotic loss. All cultures were cultivated at 30 °C for seven days, and 3 mL of culture was collected every 4 h to detect cell density (1 mL) and steroid residues (2 mL) using HPLC. Two milliliters of culture was acidified by adding 20 μL HCl (1 M). With the addition of 1 mL ethyl acetate and the oscillation for 1 min, the mixture was then centrifuged at 2000 rpm for 15 s to collect the organic layer. The aqueous layer was extracted four times with 1 mL ethyl acetate each time, and all the organic layers were collected. The organic layers were dehydrated with anhydrous sodium sulfate, dried with nitrogen at 45 °C, and dissolved in 200 μL of acetonitrile for HPLC detection as above. Five independent experiments were performed and the data were the average values with standard errors. Statistical analysis was performed using SPSS 24 (IBM, US) for the *t*-test and the *p* value was calculated.

2.7. Detection of the EE2-utilizing efficiency of P. citronellolis SJTE-3 supplied with different additives

The EE2-utilizing efficiency of strain SJTE-3 supplied with different additives (easily used carbon sources, nitrate, or ammonia) as extra energy sources was determined by detecting the cell growth and the residual EE2 concentration. Strain SJTE-3 were cultured in LB medium overnight and the cell inocula were inoculated into the minimal medium containing 10 mg/L of EE2, and different carbon or nitrogen sources (0.1% glucose, 0.1% peptone, 0.1% yeast extract, 0.1% glycine, sodium nitrate, 0.1% fulvic acid, 0.1% ammonium chloride, 0.1%glycerol, 0.01% ethanol, 0.1% ethanol, and 1% ethanol) with initial 0.05 OD_{600}. The cultures with the supplied energy sources and no EE2 were used as controls. All the cultures were cultured at 37 °C for seven days and every 12 h, 3 mL of culture was collected to monitor the cell density (1 mL) and detect the residual EE2 with HPLC (2 mL). Three replicates were used for each sample and five independent experiments were performed; data were all the average values with standard errors.

2.8. Determination of the intermediate metabolites of EE2 by P. citronellolis SJTE-3 with gas chromatography mass spectrometry (GC-MS) and GC-Orbitrap/MS

The intermediate metabolites of EE2 degraded by strain SJTE-3 and the constructed strains were determined by GC-MS and the GC-Orbitrap/MS system, according to the protocol described in previous report (Beck et al., 2008). Briefly, strain SJTE-3 was cultured in the minimal medium with 10 mg/L of EE2 and 0.1% ethanol for seven days; the culture was sampled every 30 min. One milliliter of the culture was extracted three times with equal volumes of ethyl acetate, and the extract was completely evaporated under a gentle stream of nitrogen. The evaporated extract was derivatized at 70 °C for 40 min with a mixture containing 50 μL of N, O-Bis (trimethyl silyl) trifluoroacetamide (BSTFA): trimethyl chlorosilane (TMCS) (99:1, v/v), and 50 μL of pyridine. The samples were evaporated with nitrogen, and 1 mL hexane with Mirex (1 μg/mL) was added as an internal standard to calibrate the systematic errors (Beck et al., 2008). The treated samples were detected using a GC-MS system (Agilent 7890B-GC equipped with a 5977B-MS detector employing an electron impact ion source, CA, USA). One microliter of the sample was analyzed with an HP-5MS UI capillary column (0.25 μm × 30 m, Agilent, CA, USA) in splitless mode, and 1 mL/min high purity helium was used as the carrier gas in linear velocity mode. The GC oven temperature program started at 50 °C for 2 min, then heated to 280 °C at 20 °C/min and held there for 10 min; the total analysis time was 23.5 min. The source and transfer line temperatures were 250 °C and 280 °C, respectively; the mass spectrometer was operated in the scanning mode in the range of 33–550 *m/z* and recorded at an electron energy of 70 eV. The GC-Orbitrap/MS detection system used for high-resolution MS analysis, it was equipped with a TRACE 1310 GC system and EI source (Thermo Fisher, MA, USA). The samples were analyzed with a TG-5SILMS column (0.25 μm × 30 m, Thermo Fisher, MA, USA) with 1.2 mL/min helium used as the carrier gas. The EI was also performed at 70 eV with the source temperature set at 250 °C; the full scan MS acquisition was performed in profile mode using a *m/z* range of 50–650. For each sample, five independent experiments were performed and the data were the average values with standard errors.

2.9. Reverse transcription and quantitative PCR (RT-qPCR) analysis of the potential genes for EE2 metabolism in P. citronellolis SJTE-3

After several putative estrogen-degrading genes in strain SJTE-3 were predicted by genome mining and sequence alignment, the transcription levels of these genes in the culture containing EE2 were analyzed by RT-qPCR. Strain SJTE-3 was cultured in the minimal medium containing 0.1% ethanol with or without 10 mg/L of EE2 for 24 h, and the cultures were sampled every hour. The RNA of all of the samples was extracted with the RNAprep Pure Bacteria Kit (Tiangen, Beijing, China). The reverse transcription was performed with 1 μg RNA and 20 ng random primers using a PrimeScript Reverse Transcriptase Kit (Takara, Dalian, China), and quantitative PCR was performed using Premix ExTaq (Takara, Dalian, China) with a QuantStudio 3 RealTime PCR Apparatus (Thermo Fisher, MA, USA). The oligonucleotides used for RT-qPCR detection were listed in Table S2. The qPCR conditions were set at 95 °C for 3 min, 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s; a final melting analysis was performed by slow heating with 10 s increments of 0.5 °C from 60 °C to 95 °C. The relative fold changes in mRNA quantity of the target genes were calculated using the $2^{-\Delta\Delta Ct}$ algorithm. Three replicates were sampled at each time point and all the data were the average values of five independent experiments with standard errors.

2.10. Construction of the recombinant over-expressing strains and detection of their EE2 utilization efficiencies

To determine the genes participating in EE2 metabolism by strain SJTE-3, the genes induced by EE2 were amplified and cloned into the broad-host-range plasmid pBSPPc-Gm under the control of the P_{lac} promoter, generating a series of the recombinant plasmids, which were then electro-transformed into strain SJTE-3 to construct the corresponding recombinant strains (Table S1) (Xu et al., 2013). The transcription and expression of the cloned genes were detected by RT-qPCR analysis and SDS-PAGE electrophoresis. The recombinant strains and the strain S3-vector (strain SJTE-3 with empty plasmid pBSPPc-Gm) were cultured in minimal medium (with 10 mg/L of EE2 and 0.1% ethanol) at 37 °C for seven days. Every 6 h, 2 mL of the cultures were taken out to detect the residual EE2 and the intermediate metabolites using HPLC, GC-MS and GC-Orbitrap/MS. Three replicates were set for each sample and all the data were the average values of five independent experiments with standard errors.

3. Results

3.1. P. citronellolis SJTE-3 could metabolize the natural steroids and the synthetic estrogen efficiently

After several rounds of enrichment, *P. citronellolis* SJTE-3 (Deposit No. CGMCC15428) was isolated and found to have the ability to degrade estrogens and other steroid chemicals; its 16S rDNA sequence (GenBank No. KU860463.1) and its whole genome sequence (GenBank NZ_CP015878.1 and CP015879.1) were deposited to NCBI database. The phylogenetic tree demonstrated that strain SJTE-3 was quite similar to those of *P. citronellolis* strains (> 98% identity); the genome of strain SJTE-3 showed a high ANI value of 97.58% compared to that of *P. citronellolis* P3B5, much higher than the average 70% value of all the aligned genomes (Fig. S1, Table S3). Strain SJTE-3 was a Gram-negative, aerobic, asporous short rod whose colonies on LB plates were circular, yellowish, and flat with smooth edges. It could oxidize L-arabinose, ribose, D-xylose, galactose, glucose, glycerol, D-fucose, gluconate and 2-keto-gluconate, and its reactions to ornithine decarboxylase (ODC), citrate utilization (CIT), urease (URE), tryptophane deaminase (TDA), and Voges–Proskauer (VP) were positive (Table S4).

The ability of *P. citronellolis* SJTE-3 to utilize different steroids (E1, E2, EE2 and TES) was determined by detecting the cell growth and the removal efficiency. This strain could use the natural steroids as its sole carbon source and degrade them efficiently. Nearly 100% of E2 (10 mg/L), 77% of E1 (10 mg/L), and 72% of TES (10 mg/L) were degraded in seven days (Fig. 1A). EE2 was difficult for strain SJTE-3 to utilize as the sole carbon source; however, the addition of 0.1% ethanol could initiate the metabolism of EE2 effectively. In 24 h, over 68% of EE2 (1 mg/L) and 53% of EE2 (10 mg/L) were consumed by this strain; no removal of EE2 was observed in the control group inoculated with the boiled cells (Figs. 1B, S2A). Interestingly, the extra addition of 2% ethanol into the culture at 36 h could increase the cell growth, and the EE2 utilization was also stimulated again after 48 h; the removal rate of EE2 by strain SJTE-3 was enhanced from 55% to 65% at 84 h, implying that the initiation of the easily used carbon source may have been indispensable for EE2 metabolism (Fig. 1B). After seven-day culture with the initiation of 0.1% ethanol, 92% of EE2 (1 mg/L), 75% of EE2 (5 mg/L), 55% of EE2 (10 mg/L), and 41% of EE2 (50 mg/L) were metabolized by strain SJTE-3 (Fig. S2). The utilization of 10 mg/L E1 (half-life 0.730 d) and E2 (half-life 0.252 d) by strain SJTE-3 was more efficient and faster than its utilization of EE2 (half-life 2.183 d) and testosterone (half-life 2.776 d). A longer time was needed for strain SJTE-3 to metabolize EE2 at higher concentrations, rising from 0.311 d (1 mg/L) to 1.426 d (5 mg/L) and 2.183 d (10 mg/L). Compared with other reported strains, strain SJTE-3 metabolized EE2 thoroughly and rapidly (Skotnicka-Pitak et al., 2009; Haiyan et al., 2007; Sedighi et al., 2019; Fioravante et al., 2012; Sheng et al., 2021; Pauwels et al., 2008; Sabirova et al., 2008; Wang et al., 2019; Roh et al., 2010; Ye et al., 2017) (Table S5).

3.2. Different energy sources could favor the EE2 utilization of P. citronellolis SJTE-3

Various energy sources existing in real environments influence the cell survival and chemical utilization of microorganisms. The effects of different carbon sources, nitrate and ammonia on the EE2 utilization efficiency of strain SJTE-3 were analyzed. The results showed that most of the easily used carbon sources (glucose, peptone, ethanol, yeast extract, and fulvic acid) could promote the cell growth of strain SJTE-3; while little growth improvement was found in the cultures supplied with glycerol, glycine, sodium nitrate or ammonium chloride. Supplying glucose and ethanol in culture enhanced the cell growth efficiently, and the highest cell density was observed in the culture supplied with glucose at 12 h (Fig. 2A). The removal efficiency of EE2 by strain SJTE-3 was promoted by almost all the additions, except for glycerol and sodium nitrate. Ethanol was the most effective in promoting the removal rate of EE2 in a concentration-dependent mode; with the addition of 1% ethanol, the utilization efficiency of 10 mg/L EE2 by strain SJTE-3 reached about 58% in three days and about 72% in seven days (Fig. 2B). This means that supplementation with easily used energy sources may strengthen the central metabolism, increase cell growth, and generate more reducing power to initiate EE2 metabolism (Gaulke et al., 2008; Khunjar et al., 2011; Forrez et al., 2009; Fioravante et al., 2012; Sheng et al., 2021; Pauwels et al., 2008; Wang et al., 2019a; Roh and Chu, 2010; Ye et al., 2017).

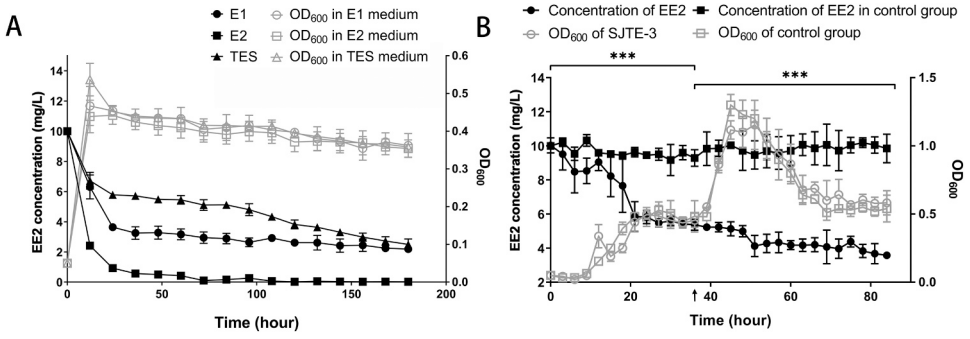


Fig. 1. The steroid utilization efficiency and cell growth of *P. citronellolis* SJTE-3. (A) The degradation efficiency of 10 mg/L natural steroids (E1, E2, and testosterone) by *P. citronellolis* SJTE-3 and the cell growth of this strain. Strain SJTE-3 was cultured in the minimal medium with the steroid chemical for seven days; the concentrations of the chemical residues in culture were detected by HPLC and plotted. (B) The utilization efficiency of 10 mg/L EE2 by strain SJTE-3 and the cell growth of this strain. Strain SJTE-3 was cultured in the medium with 10 mg/L EE2 and 0.1% ethanol for seven days (only presented the curves to 84 h); 2% ethanol was supplied into the 36 h culture (labeled with arrow). The boiled cells of strain SJTE-3 were used as the control group. The concentrations of EE2 residues were detected by HPLC and plotted. The error bars represented standard errors of five independent experiments; the statistical analysis was performed with SPSS 24 software for the *t*-test and the *p* value was calculated (*, *p* value < 0.05; **, *p* value < 0.01; ***, *p* value < 0.001).

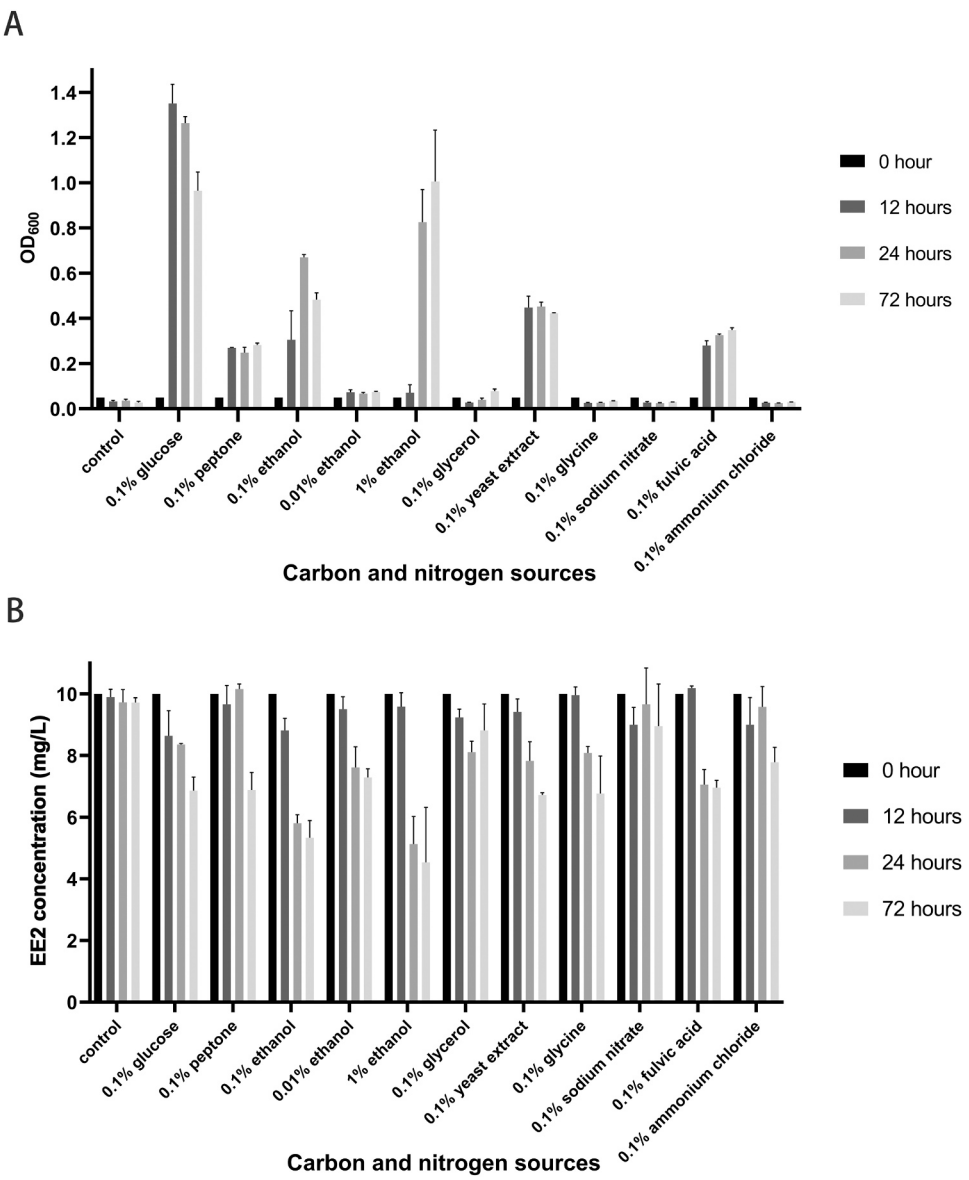


Fig. 2. The effect of different additives on cell growth and EE2 utilization efficiency of *P. citronellolis* SJTE-3. Different additives including carbon sources (0.1% glucose, 0.1% peptone, 0.1% yeast extract, 0.1% glycine, 0.1% fulvic acid, 0.1% glycerol, 0.01% ethanol, 0.1% ethanol, and 1% ethanol), nitrate (0.1% sodium nitrate) and ammonia (0.1% ammonium chloride) were supplied into the minimal medium with 10 mg/L EE2. Strain SJTE-3 was inoculated and cultured in different media for seven days, respectively. The cell growth (A) and the EE2 residues in culture (B) were detected at different time points (0 h, 12 h, 24 h, and 72 h) and plotted. The error bars represented standard errors of five independent experiments.

3.3. Two novel intermediate metabolites of EE2 degraded by P. citronellolis SJTE-3 were characterized

To identify the intermediates of EE2 degraded by *P. citronellolis* SJTE-3, this strain was cultured in minimal medium with EE2 and 0.1% ethanol as the carbon source, and samples were taken every 30 min for the HPLC and GC-MS detection. Two peaks, compound 1 (14.13 min) and compound 2 (13.22 min), were observed in the HPLC map in the 14 h culture (Fig. 3A, 3B); the formulas of compound 1 ($C_{25}H_{30}OSi$, −1.01960 ppm) and compound 2 ($C_{21}H_{42}O_2Si$, −0.21345 ppm) were given according to the high-resolution mass results (Fig. 3C, 3D). As all the samples were derived via silylation, the *m/z* values of compound 1 and compound 2 were 302 and 282, respectively, and their original formulas should be $C_{22}H_{22}O$ and $C_{18}H_{34}O_2$, respectively. The structures of the two compounds were predicted based on the molecular formulas, while the positions of the new double bond and ethyl group (compound 1) and the broken site of the carbon-carbon bond (compound 2) were uncertain (Fig. 3C, 3D). The two identified intermediates of EE2 by strain SJTE-3 are novel and different from the reported metabolites of EE2 (Skotnicka-Pitak et al., 2009; Khunjar et al., 2011; Sheng et al., 2021; Della Greca et al., 2008; Yi and Harper, 2007).

3.4. Comparative genome analysis and genome mining of P. citronellolis SJTE-3 contributed to the identification of putative estrogen-degrading genes

The general features of the whole genome of *P. citronellolis* SJTE-3 (GenBank No. NZ_CP015878.1) were analyzed and are summarized in Table S6. The genome contains one circular chromosome DNA and a large plasmid DNA, and the top functional clusters of orthologous groups (COG) categories of this strain were general function (10.54%), amino acid transport and metabolism (9.46%), transcription (7.03%), and inorganic ion transport and metabolism (5.68%) (Fig. S3). As strain SJTE-3 was the only sequenced EE2-utilizing bacteria, the comparative genome analyses of *P. citronellolis* SJTE-3 and several E2-degrading bacteria were performed to identify the conserved or specific putative coding sequences (CDSs) and explore the genetic basis for synthetic estrogen metabolism. The genome sequence of strain SJTE-3 was relatively similar to those of *P. putida* B6–2 (84.94%) and *P. putida* SJTE-1 (84.23%), but dissimilar to that of *Rhodococcus* sp. strain P14 (81.78%) (Fig. 4A). As the genes responsible for estrogen degradation were distributed not clustered in the bacterial genome, it was quite difficult to label the accurate positions of the genes involved in the

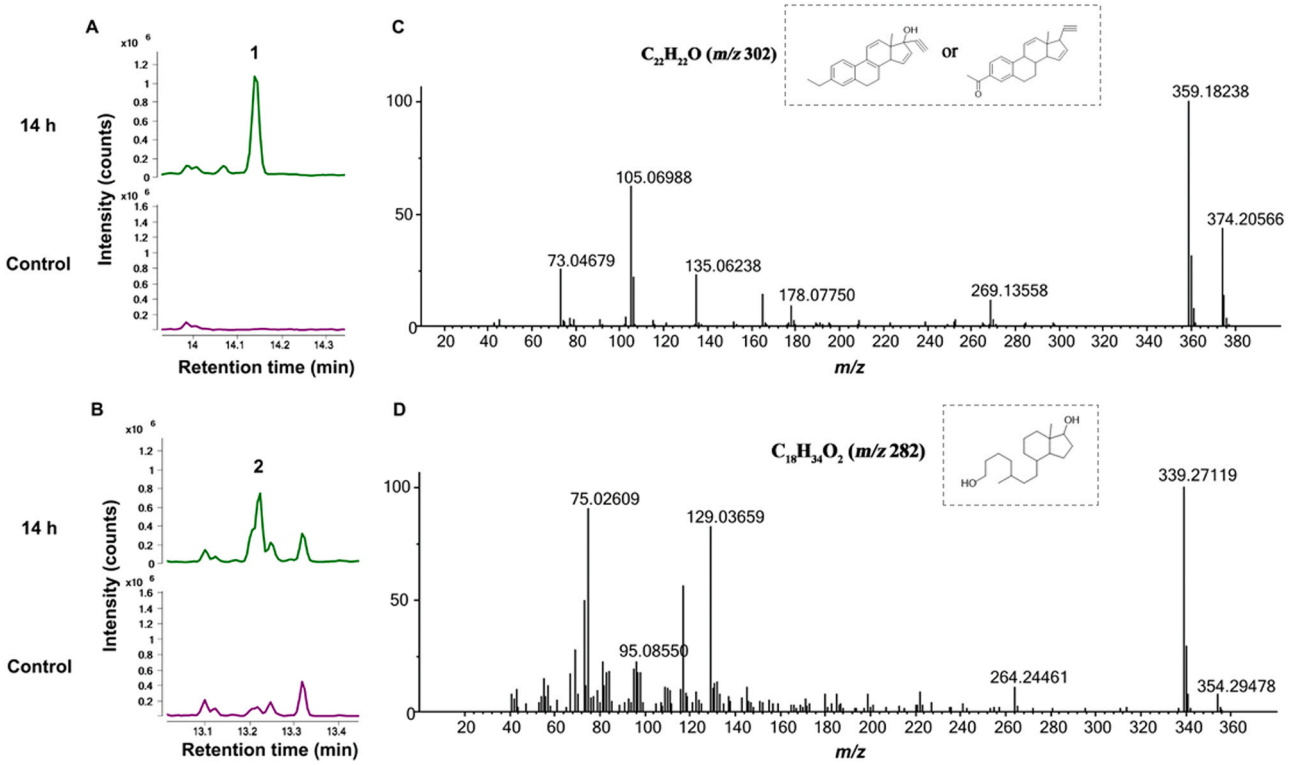


Fig. 3. The intermediate metabolites of EE2 transformed by *P. citronellolis* SJTE-3. Strain SJTE-3 was cultured in the minimal medium with 10 mg/L EE2 and 0.1% ethanol, and the samples was picked every 30 min for detection of HPLC, GC-MS and GC-Orbitrap/MS. The culture with no EE2 was set as negative control. The cultures were detected at 14 h, and the peak map of two peaks at 14.13 min (A) and at 13.22 min (B) were presented. The mass spectra of the two peaks observed in GC-Orbitrap/MS system were shown, compound 1 (C) and compound 2 (D). Their formulas and their proposed structure were also presented in the dotted box. The positions of new double bond and ethyl group in compound 1 and the broken site of carbon-carbon bond in compound 2 were uncertain.

estrogen degradation on the genome maps. Further Mauve visualization of the whole genome of six strains, *P. citronellolis* SJTE-3, *P. putida* B6–2, *P. putida* SJTE-1, *Rhodococcus* sp. P14, *Sphingomonas* sp. KC8 and *C. testosteroni* P19, also supported the dispersed distribution status of the estrogen-degrading genes; some homologous genes could be aligned in all the strains (Fig. 4B). Furthermore, the unique genes in strain SJTE-3 and genes with the low or high sequence similarities to their homologues in other strains were aligned and identified. Therein, 124 CDSs were only found in the genome of strain SJTE-3; 437 CDSs in strain SJTE-3 were identified with similarities of less than 10% to their homologues in the six strains with the natural estrogen degrading capabilities (*P. putida* SJTE-1, *P. putida* B6–2, *C. testosteroni* P19, *C. testosteroni* ATCC 11996, *Sphingomonas* sp. KC8, and *Rhodococcus* sp. P14); and 346 CDSs in strain SJTE-3 were different from those in the three *P. citronellolis* strains (*P. citronellolis* P3B5, TTU2014–008ASC, and TTU2014–011ASC) (Table S7). This means that the strains with the estrogen-utilizing capabilities may possess a similar genetic basis and strain SJTE-3 may have its own degradation specificity. To our knowledge, the gene responsible for the EE2 metabolism has never been identified. Although the *amoA* gene and *nirK* cluster genes in AOB may participate in the initiation of EE2 transformation, other genes encoding the catalytic enzymes in the EE2-metabolic pathway or in other EE2-transforming strains remain unknown (Tan et al., 2021; Olivera and Luengo, 2019; Sheng et al., 2021). Based on the sequence alignment with the reported estrogen-degrading genes, 634 genes encoding oxidoreductases, dioxygenases, isomerases and transferases were found in the genome of strain SJTE-3, and 163 CDSs were predicted to be associated with the potential EE2 metabolism (Table S7). Twenty-four genes encoding the putative short chain dehydrogenases (SDR), three genes encoding the putative P450 hydroxylase and one gene encoding the putative dioxygenase were highlighted as having relatively high sequence similarity. These results demonstrated that *P. citronellolis* SJTE-3 contained a series of function genes and specific metabolic pathways responsible for estrogen degradation.

3.5. The EE2 metabolism and the transcription of eight putative genes in *P. citronellolis* SJTE-3 were induced by EE2

As the EE2 utilization by *P. citronellolis* SJTE-3 began after the exhaustion of the easily used energy sources, experiments were performed to determine whether the EE2 metabolism in this strain was inducible. Strain SJTE-3 was first cultured in the minimal medium with 10 mg/L of EE2 or 0.1% ethanol for 12 h, respectively, before the cell inocula were inoculated into the minimal medium with 10 mg/L of EE2 and 0.1% ethanol. The detection of the EE2 removal residues in the first 30-min culture indicated that the EE2 removal efficiency of the EE2-pretreated cells was significantly higher than that of the ethanol-pretreated cells. This meant that the metabolism of EE2 by strain SJTE-3 could be induced by EE2 via the induction of the transcription of related function genes (Fig. 5A).

Although a series of dehydrogenases and reductases in the genome of strain SJTE-3 were annotated, only a subset of these enzymes have participated in estrogen degradation. Based on the genome mining and sequence alignment, 35 genes in the genome of *P. citronellolis* SJTE-3 were proposed to be related to EE2 metabolism (Table S8). The results of the transcription analyses of these 35 genes showed that after strain SJTE-3 was cultured in the minimal medium with EE2 and ethanol for 12 h, the transcription of eight genes (*sdr*1, *fabG*, *sdr*2, *sdr*3, *benD*, *yjcH*, *cyp*2, and *cyp*3) was significantly upregulated by about 3–38 fold, and the transcription of other genes was not changed significantly (Fig. 5B). Among them, the transcription levels of four genes (*sdr*3, *yjcH*, *cyp*2, and *cyp*3) increased over 10 fold; the induction time point of these genes exactly corresponded to the initiation time of EE2 removal by strain SJTE-3 (Fig. 6A). This implies that the eight upregulated genes may be

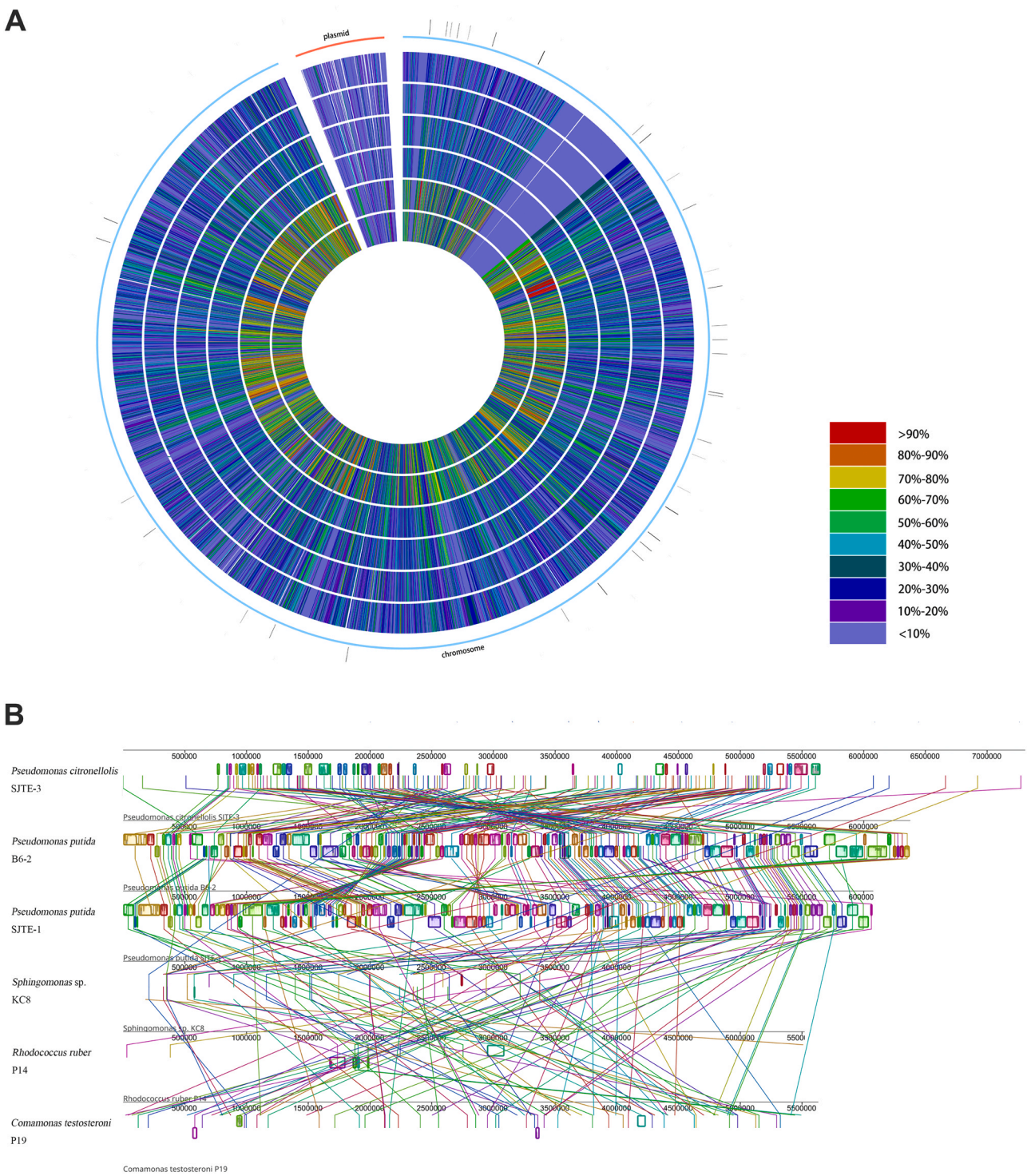
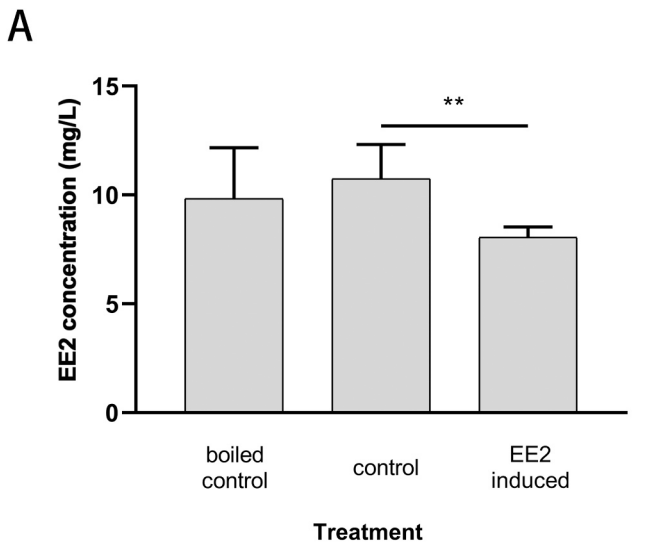
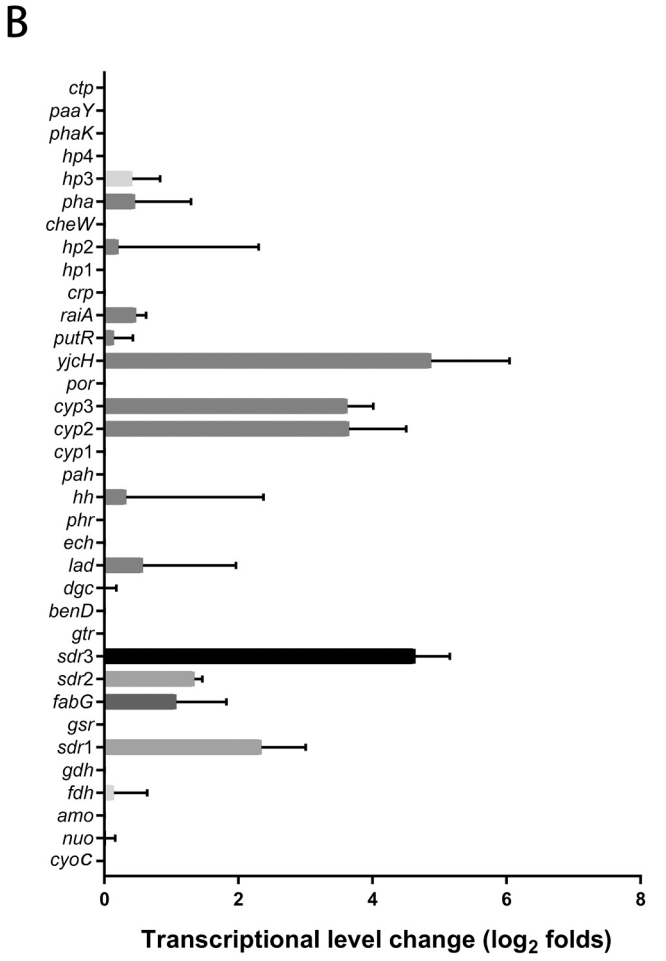


Fig. 4. Comparative analyses and Mauve visualization of the whole genome of *P. citronellolis* SJTE-3. (A) The genome sequence comparison of *P. citronellolis* SJTE-3 with those of six other estrogen-degrading bacteria. The outermost circle (circle 1) represented the positions of thirty-five genes potentially associated with EE2 metabolism, which was found by sequence alignment. Circle 2, the open reading frames (ORFs) in the chromosome (blue) and plasmid (orange) of *P. citronellolis* SJTE-3. Circle 3, the ORFs in the genome of *Rhodococcus* sp. P14, circle 4, the ORFs in the genome of *Sphingomonas* sp. KC8, circle 5, the ORFs in the genome of *C. testosteroni* ATCC 11996, circle 6, the ORFs in the genome of *C. testosteroni* P19, circle 7, the ORFs in the genome of *P. putida* B6–2, circle 8, the ORFs in the genome of *P. putida* SJTE-1. The ORFs were represented by sticks in different colors; from red to blue were assigned according to the similarities of the homologues in six other strains to the ORFs in strain SJTE-3. (B) The Mauve visualization of the whole genomes of *P. citronellolis* SJTE-3 and those of five other estrogen-degrading bacteria (*P. putida* B6–2, *P. putida* SJTE-1, *Rhodococcus* sp. P14, *Sphingomonas* sp. KC8, and *C. testosteroni* P19). The genome of *P. citronellolis* SJTE-3 was set as reference genome. The homologues distributed in these genomes were showed in colored rectangles and connected with lines, implying the corresponding positions of these homologous gene blocks in different genomes. The blocks under the center line represented the regions in inverse orientation, and the unique segments were showed with white areas. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)





(caption on next column)

Fig. 5. Induction of EE2 on the EE2 utilization and the transcription of putative functional genes of *P. citronellolis* SJTE-3. (A) Effect of EE2 pretreatment on the utilization efficiency of EE2 by strain SJTE-3 in the first 30 min. Cells of strain SJTE-3 and the boiled cells of strain SJTE-3 were cultured in the minimal medium with 10 mg/L of EE2 for 12 h; cells of strain SJTE-3 cultured in the minimal medium with 0.1% ethanol was used as control. After being harvested and re-suspended, the cells of the three groups were cultured in the minimal medium with 0.1% ethanol and 10 mg/L EE2 for 30 min. The EE2 residues in cultures were detected by HPLC and plotted. The error bars represented standard errors of five independent experiments. Statistical analysis was performed with SPSS 24 software for the *t*-test; the *p* value was calculated and marked with star (*, p value < 0.05; **, p value < 0.01; ***, p value < 0.001). (B) The transcription of 35 putative genes relate to EE2 metabolism in strain SJTE-3. Strain SJTE-3 was cultured in the minimal medium with 0.1% ethanol and 10 mg/L of EE2 for 12 h, and strain SJTE-3 cultured in the minimal medium with 0.1% ethanol was set as control. The transcription levels of 35 putative genes related to EE2 metabolism were detected by RT-qPCR. The error bars represented standard errors of five independent experiments.

involved in the EE2 metabolism of strain SJTE-3.

3.6. Three genes (sdr3, yjcH, and cyp2) participated in the EE2 metabolism of P. citronellolis SJTE-3

To determine the functional genes participating in the EE2 metabolism of *P. citronellolis* SJTE-3, the eight EE2-induced genes were cloned into the expression vector and transformed into strain SJTE-3 to generate eight recombinant over-expressing strains (Table S1). After being induced with IPTG, the transcription of the eight genes was found to be upregulated about 30–500 folds (Fig. S4). The cell growth and the EE2 removal rate by the eight recombinant strains and strain SJTE-3 with the empty plasmid (S3-vector) were detected, and results indicated that the EE2 removal of three recombinant strains (S3-SDR3, S3-YjcH, and S3-CYP2) was much faster and more efficient than that of strain S3-vector, in spite of their similar growth curves (Fig. 6B). This meant the improvement of the EE2-utilizing efficiency by strain SJTE-3 was due to the over-expression of the three genes (*sdr*3, *yjcH* and *cyp*2). Furthermore, the intermediate metabolites of EE2 degraded by the three recombinant strains (S3-SDR3, S3-YjcH, and S3-CYP2) were analyzed. The results showed that over-expression of gene *sdr*3 accelerated the appearance of compound 1 (at 14.13 min, *m/z* 302) for about 1.5 h, from 14.5 h to 13 h (Fig. 6C, 6F). The over-expression of the gene *yjcH* also sped up the appearance of this intermediate metabolite by about 1.0 h, from 14.5 h to 13.5 h (Fig. 6D, 6F). However, the two intermediate metabolites could not be detected when the gene *cyp*2 was over-expressed, probably due to its acceleration of the further transformation of the intermediates though the ring-opening reaction (Fig. 6E, 6F). The EE2 degradation rates of the three recombinant strains were calculated, and the degradation kinetics after 12 h were fitted to the first-order equations (Table 1). These results demonstrated that the three genes, *sdr*3, *yjcH*, and *cyp*2 participated in the EE2 metabolism of *P. citronellolis* SJTE-3 and may play an important role in the side modification and ring-opening reaction. In addition, the comparison analyses of the three genes (*sdr*3, *yjcH*, and *cyp*2) and their adjacent genome regions in strain SJTE-3 and other estrogen-degrading strains indicated that the three genes were far from each other in the genome of strain SJTE-3 and their surrounding regions varied in the other degrading strains (Fig. S5). The homologues of the three genes and their adjacent genes in the other six estrogen-degrading strains showed relatively low sequence identities to their homologous genes in strain SJTE-3 (Fig. 7). This meant that the genes associated with EE2 metabolism in *P. citronellolis* SJTE-3 exhibited dispersed distribution, and the three genes (*sdr*3, *yjcH*, and *cyp*2) may possess unique characteristics.

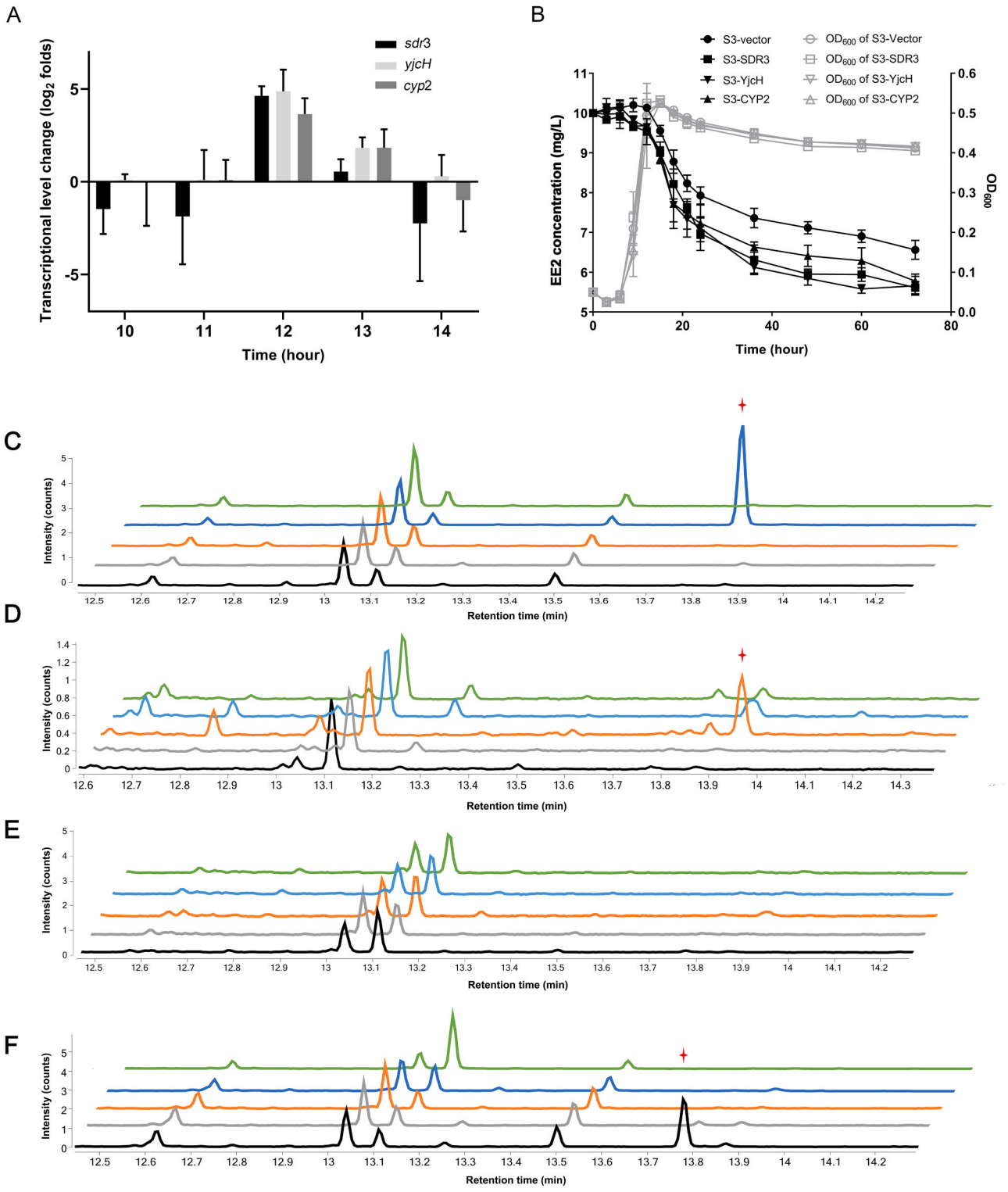


Fig. 6. Characterization of the three genes participated in EE2 metabolism in *P. citronellolis* SJTE-3. (A) The transcription analysis of three genes (*sdr*3, *yjcH*, and *cyp*2) at different time points. Strain SJTE-3 was cultured in the minimal medium with 0.1% ethanol and 10 mg/L of EE2, and strain SJTE-3 cultured in the minimal medium with 0.1% ethanol was set as control. The cells were collected every 30 min from the 10 h culture to the 14 h culture, and the transcription levels of the three genes (*sdr*3, *yjcH*, and *cyp*2) were detected by RT-qPCR. The error bars represented standard errors of five independent experiments. (B) Cell growth and EE2 utilization efficiency of four recombinant strains. Four recombinant strains (S3-SDR, S3-YjcH, S3-CYP, and S3-vector) were cultured in minimal medium with 10 mg/L EE2 and 0.1% ethanol; the EE2 residues in culture and cell growth of strain SJTE-3 were detected every 3 h for three days. The error bars represented standard errors of five independent experiments. (C-F) The HPLC chromatograms of the intermediate metabolites of EE2 transformed by four recombinant strains. The chromatograms of strain S3-SDR (C), strain S3-YjcH (D), strain S3-CYP (E) and strain S3-vector (F) were presented. The chromatograms of samples at different time points (from top to bottom: 12.5 h, 13 h, 13.5 h, 14 h, and 14.5 h) were presented in different panels. The intermediate metabolites were marked with stars, and all the experiments were repeated for five times.

Table 1
The degradation kinetics of EE2 by different recombinant strains.

Strains	EE2 concentration	Equation	R^2	Kinetic constant
S3-vector	10 mg/L	$y = 9.3226e^{-2.0298x}$	0.9825	2.0298
S3-SDR3	10 mg/L	$y = 10.4793e^{-1.9556x}$	0.9874	1.9556
S3-YjcH	10 mg/L	$y = 12.3538e^{-2.2125x}$	0.9830	2.2125
S3-CYP2	10 mg/L	$y = 12.5394e^{-2.5505x}$	0.9639	2.5505

y represented the concentration of EE2 in medium (mg/L), and x represented the culture time (days). The coefficient was calculated after the 12 h culture with the exhaustion of 0.1% ethanol in medium.

3.7. Proteins S3_SDR3, S3_YjcH, and S3_CYP2 were the members of classical SDR, membrane transporter, and cytochrome P450

The three EE2-metabolizing genes, *sdr*3, *yjcH*, and *cyp*2, encoded protein S3_SDR3 (a putative reductase, ANI18437.1), protein S3_YcjH (a putative membrane transporter protein, ANI18573.1), and S3_CYP2 (a putative cytochrome P450 hydroxylase, ANI18619.1), respectively. The MSA analysis showed that protein S3_SDR3 contained the conserved sites of classical SDR, the YXXXK active site (158–162 aa), and the VXGXXXGXG cofactor-binding site (14–22 aa) (Fig. 8A). It shared over 50% identity with the 17β-HSD (ANI04816.1) from *P. putida* SJTE-1, which was responsible for the transformation of estradiol into estrone, suggesting its potential role in the reduction and desaturation of EE2 (Fig. 8A). Evolutionary analysis showed that it was very close to protein WP_061563290.1 in *P. citronellolis* P3B5, implying the potential estrogen degradation ability of strain P3B5 (Fig. S6A). The putative membrane transporter S3_YjcH was a DUF485 domain-containing protein homologue with two trans-membrane sequences (Fig. 8B). It was distributed in different species and was very close to its homologue in *P. citronellolis* P3B5 (Fig. S6B). It was highly conserved in *Pseudomonas*, showing 86.41% and 66.67% identity with the inner membrane protein YjcH

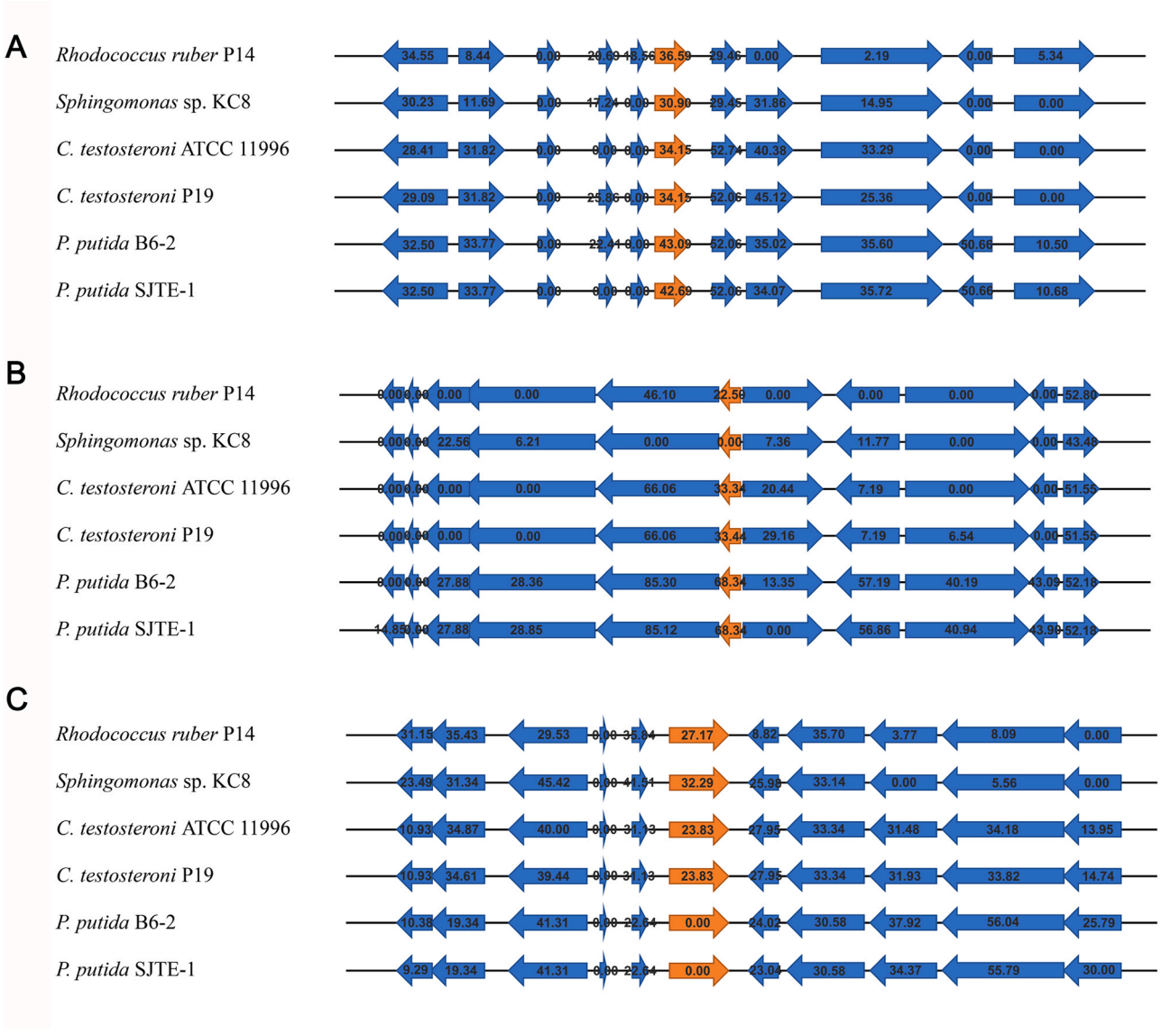


Fig. 7. Sequence alignments of three EE2-metabolism related genes and their adjacent ORFs in *P. citronellolis* SJTE-3 and other estrogen-degrading strains. Three genes, *sdr*3 (A), *yjcH* (B), and *cyp*2 (C), and their adjacent ORFs of *P. citronellolis* SJTE-3 were aligned with their homologues and neighbouring ORFs in six other estrogen-degrading strains (*P. putida* B6–2, *P. putida* SJTE-1, *Rhodococcus* sp. P14, *Sphingomonas* sp. KC8, *C. testosteroni* ATCC 11996, and *C. testosteroni* P19). The ORFs were presented in arrows with their sequence identities aligned with the corresponding genes in strain SJTE-3. The homologous genes of *sdr*3, *yjcH*, and *cyp*2 genes were assigned with orange color. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 8. Multiple sequence alignment (MSA) of three proteins involved in EE2 metabolism in *P. citronellolis* SJTE-3. Proteins S3_SDR3 (A), S3_YjcH (B), and S3-CYP2 (C) were aligned with their homologues in other degrading strains. The aligned proteins included protein CAD85559.1 from *N. europaea* ATCC19718, protein WP_008027336.1 and WP_003252591.1 from *P. putida* B6–2, proteins ABQ79984.1 and ABQ80101.1 from *P. putida* F1, proteins WP_012271104.1 and WP_012271014.1 from *P. putida* GB-1, proteins WP_010123494.1, WP_010128053.1 and WP_010124721.1 from *Sphingomonas* sp. KC8, proteins WP_061563290.1, WP_024128557.1 and WP_009613127.1 from *P. citronellolis* P3B5, proteins WP_010595991.1, WP_010593093.1, and WP_099528111.1 from *Rhodococcus ruber* P14, proteins WP_057091786.1, WP_003061686.1, and WP_057093896.1 from *C. testosteroni* P19, and proteins ANI04816.1, and WP_014754112.1 and WP_003252591.1 from *P. putida* SJTE-1. The secondary structures were shown in helices (α-helices) and arrows (β-strands). The amino acids were shaded with colors according to sequence similarity; the conserved amino acids were shaded with black, and the homologous extent over 75%, 50% and 33% were shaded with pink, blue, and yellow, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

from *P. aeruginosa* and *E. coli*, respectively. YjcH in *E. coli* is normally co-transcribed with the acetyl-CoA synthetase (encoded by the *acs* gene) and the acetate symporter (encoded by the *actP* gene) (Fig. 8B) (Riley et al., 2006). The S3_YjcH protein was likely responsible for the trans-membrane transportation of EE2 inside the cells of strain SJTE-3. Protein S3_CYP2, the putative cytochrome P450 hydroxylase, contained the conserved heme-binding motif FXXGXHXCXG (392–401 aa) and a unique EXXR motif at the SRS5 position (Fig. 8C). It was distributed in all the selected estrogen-degrading strains, and had 25.26% identity to protein WP_010123494.1 from *Sphingomonas* sp. KC8 with unknown function, which was quite close (Fig. S6C). As the SRS5 position is considered to be associated with substrate selectivity, this variation in this enzyme may lead to the different substrate bias and the catalytic ability toward estrogens (Gricman et al., 2014, 2015).

4. Discussion

Synthetic estrogen EE2, a typical chemical among the new ECs, has strong estrogenic activity, stable chemical properties, and potentially severe adverse health effects (Aris et al., 2014; Tan et al., 2021; Lei et al., 2020; Yuan et al., 2018). Although biodegradation with microorganisms is considered an effective method for the removal of steroid chemicals, it is extremely difficult for microorganisms to utilize EE2 as their energy source; existing reports on the microbial transformation of EE2 indicate that this process is slow and inefficient (Tan et al., 2021; Olivera and Luengo, 2019). Accordingly, the intermediate metabolites of EE2, the genome sequences of EE2-degrading strains and the genes involved in EE2 metabolism are seldom reported. Thus, the metabolic mechanism of EE2 in microorganisms remains unclear, greatly impeding the efficient remediation of EE2-polluted environments (Tan et al., 2021; Olivera and Luengo, 2019; Pratush et al., 2020).

In this work, the utilization properties and genetic basis of EE2 metabolism in *P. citronellolis* SJTE-3 were studied, as this strain was the only sequenced bacteria with an efficient EE2 utilization capability. The metabolic properties and two novel intermediates of EE2 degraded by *P. citronellolis* SJTE-3 were characterized, and three genes encoding an SDR, a membrane transporter, and a cytochrome P450 hudroxylase were identified to participate in the EE2 metabolism in this strain. These

findings could shed a light on the metabolic mechanism study of EE2 in bacteria and contribute to the subsequent environmental bioremediation of EE2 pollution.

P. citronellolis SJTE-3 was found to be capable of degrading natural steroids (TES, E1, and E2) as the sole carbon source, and metabolized the synthetic estrogen EE2 with the addition of the easily used energy sources. With 0.1% ethanol added, almost all the EE2 (1 mg/L) or over 55% of the EE2 (10 mg/L) was metabolized in 24 h and the biomass increased by about 0.45 OD. Compared with other reported EE2-transforming strains, *P. citronellolis* SJTE-3 showed high efficiency and transformed EE2 in a short period (Skotnicka-Pitak et al., 2009; Haiyan et al., 2007; Sedighi et al., 2019; Fioravante et al., 2012; Sheng et al., 2021; Pauwels et al., 2008; Sabirova et al., 2008; Wang et al., 2019; Roh et al., 2010; Ye et al., 2017) (Table S5). For example, *N. europaea* could transform 90% of 1 mg/L EE2 in nine days with an almost negligible OD increase; *Sphingobacterium* sp. JCR5 could eliminate 87% of EE2 (30 mg/L) in ten days with an OD increase of about 0.18; and *Enterobacter tabaci* S1 could remove 90% of EE2 (4 mg/L) in four days with only a 0.05 OD increase (Skotnicka-Pitak et al., 2009; Haiyan et al., 2007; Sedighi et al., 2019) (Table S5).

Environment conditions have strong effects on the metabolic properties and degrading efficiency of microorganisms. In aquatic environments, the concentration of EE2 is usually at the level of ng/L; however, the distribution of EE2 is uneven and sometimes the concentrations of EE2 are relatively high (Aris et al., 2014; Zhao et al., 2019; Dodgen et al., 2014; Tan et al., 2021). Environmental EE2 at very low concentrations was not enough to support the cell growth of microorganisms alone, while the addition of co-existing energy sources improved cell growth and initiated EE2 metabolism. Therefore, *P. citronellolis* SJTE-3, with high suitability and degradation efficiency, may be fit for the removal of EE2 in real environments.

Several factors, such as the redox conditions and the initial concentration of organic matter, can influence the initiation and metabolism of EE2 in bacteria (Tan et al., 2021; Sheng et al., 2021). The growth restriction observed in the metabolism of EE2 by strain SJTE-3 and other reported strains may be caused by the limited amount and bioavailability of EE2 and the possible cytotoxicity effect of intermediates (Table S5). Reductants generated from primary substrate utilization, e.g., nicotinamide-adenine dinucleotide (NADH), also represent a limiting factor for co-metabolism of organic contaminants. A supply of easily used carbon sources may induce the basic metabolic pathways and accumulate more enzymes and more reducing power in cells. The culture of *P. putida* LMG2321 was supplied with 0.5-g yeast extract, 0.5-g casamino acids, and 5 mM glucose per liter, and an increase of about 0.3 OD_{600} was observed. The culture of *Sphingobacterium* sp. JCR5 was supplied with 0.5-g yeast extract per liter and an increase of about 0.18 OD_{600} was observed. The culture of *E. tabaci* S1 was also supplied with 0.05-g yeast extract per liter, and an increase of about 0.05 OD_{600} was observed (Table S5) (Haiyan et al., 2007; Sedighi et al., 2019; Sabirova et al., 2008). Meanwhile, the addition of ammonia initiated the EE2 co-metabolism in the nitrifying activated sludge, and the initial concentration of ammonia was positively correlated with the EE2 removal efficiency (Jantanaprasartporn et al., 2018). The co-metabolism of EE2 by nitrifying bacteria *N. europaea* occurred when the initial ammonia concentration was in the range of 38–180 mg N/L, and there was a complex relationship existed between the primary metabolism (ammonia oxidation) and EE2 co-metabolism; no EE2 removal was observed at relatively low initial ammonia concentrations (7 and 14 mg N/L) (Table S5) (Sheng et al., 2021). The addition of alternative energy sources such as hydrazine can also enhance EE2 biotransformation by alleviating the reducing power deficiency (Vader et al., 2000).

In *P. citronellolis* SJTE-3, the supply of different easily used carbon sources (glucose, peptone, ethanol, yeast extract, and fulvic acid) and ammonia initiated its EE2 metabolism. Ethanol was the most efficient additive for the EE2 utilization of strain SJTE-3; the addition of a very low concentration of ethanol (0.01%) and a relatively high concentration of ethanol (1%) both initiated the EE2 metabolism of strain SJTE-3 efficiently. The consumption of carbon sources accumulated enough biomass and reducing force and helped cells adjust their metabolic network for EE2 transformation. Ammonium chloride initiated the EE2 transformation of strain SJTE-3, while sodium nitrate did not, implying that ammonia probably also acted as the extra reductant for EE2 metabolism in strain SJTE-3, not only in the AOB. The efficient metabolism of EE2 by *P. citronellolis* SJTE-3 under the cultures with various carbon and nitrogen sources demonstrated its robustness and fitness in the bio-transformation of synthetic estrogens and implied its potential utility in bioremediation. In addition, although the initiation of EE2 metabolism could be achieved by supplying energy sources, the pretreatment with EE2 also induced a much higher EE2 removal rate of strain SJTE-3. This meant that some particular metabolic pathways and the transcription of the potential functional genes were induced by EE2. In other words, the co-metabolism of energy sources and the induction of EE2 may be vital for the efficient transformation of EE2 in *P. citronellolis* SJTE-3. This finding may provide a new idea for the study of the EE2 metabolic network in bacteria.

To date, only a few possible metabolic pathways of natural estrogens in bacteria have been proposed, and there is a dearth of data on EE2 biodegradation (Chen et al., 2017; Kurisu et al., 2010; Ma et al., 2018). The EE2 metabolism in microorganisms has been speculated to occur via sulfonylation, hydroxylation, glucosylation, or carboxylation (Cajthaml et al., 2009; Skotnicka-Pitak et al., 2009; Gaulke et al., 2008; Khunjar et al., 2011; Forrez et al., 2009; Sheng et al., 2021; Della Greca et al., 2008). However, these reactions only modify EE2 from an active form to an inactive form, and the four-ring structures retain their integrity. In the study of EE2 transformation by nitrifying bioreactor, 3-sulfate-EE2 and 2-OH-EE2 were identified and the cleavage at ring A of EE2 occurred prior to other rings (Yi and Harper, 2007). The 3-glucose-EE2 was found in the transformation by *S. capricornutum*, 6-OH-EE2 was produced by *A. braunii*, and 10-OH-EE2 was generated by *S. quadricauda* (Della Greca et al., 2008). In AOB and heterotrophic bacteria, the 3-sulfo-EE2, 2-OH-EE2, 4-OH-EE2, 4-nitro-EE2 and 2-nitro-EE2 were identified (Skotnicka-Pitak et al., 2009; Haiyan et al., 2007; Gaulke et al., 2008; Khunjar et al., 2011; Forrez et al., 2009). In the batch and continuous flow bioreactors containing *N. europaea*, the carboxyl-EE2 was identified at the B ring and alkynyl group (Sheng et al., 2021; Yi and Harper, 2007).

In this work, we attempted to determine the intermediate metabolites of EE2 transformed by *P. citronellolis* SJTE-3. Although HPLC and HPLC-MS detection have been used many times in attempts to identify the metabolite intermediates, no intermediate has been found, even in the concentrated solutions of 5 L fermentation liquor. The mass spectra with the electrospray ionization (ESI) source and atmospheric pressure chemical ionization (APCI) source have proven to be inefficient to identify the intermediates of EE2, implying that the metabolites of EE2 are reluctant to be ionized probably due to the impedance of the alkynyl group at the C_{17} position. The intermediates were also undetectable in HPLC system, suggesting that the conjugated system in the A ring may be broken and the ultraviolet absorption decreased. Finally, two intermediate metabolites of EE2 were detected by the electron ionization (EI) source GC-MS and high-resolution GC-Orbitrap/MS. Based on the *m/z* values, the compound 1 (*m/z* 302, $C_{22}H_{22}O$) and compound 2 (*m/z* 282, $C_{18}H_{34}O_2$) have not yet been reported. Compound 1 was more unsaturated than EE2 and had two more carbons, implying that certain reduction reactions occurred with the addition of an unknown group. This modification may be similar to that in *S. capricornutum*, which reduces the toxicity of EE2 to cells (Hom-Diaz et al., 2015). One oxygen atom was lost in the compound 1, probably due to the removal of a hydroxyl group in the C_3 or C_{17} site, or the removal of two hydroxyl groups and the addition of an acetyl group. A similar desaturation reaction was reported in a study on EE2 reduction by *N. europaea* (Skotnicka-Pitak et al., 2009). In contrast, compound 2 was highly saturated and had two fewer carbon atoms than EE2, which may have been

generated by the removal of an alkynyl group or the destruction of the four-ring-structure. A similar ring-broken reaction was reported in *Sphingobacterium* sp. JCR5 (Haiyan et al., 2007). However, the two intermediates only existed in cultures for 30 min, and no further intermediates were detected. Therefore, the tetracyclic structure of EE2 was considered to be broken and the undetectable products were probably metabolized by strain SJTE-3. The accumulation of the two intermediates indicated that the transformation of these intermediates may be key or restriction steps in the EE2 metabolite process; the accumulated intermediates were probably caused by a relatively low enzymatic activity of the downstream step or an efficient transformation of the upstream step. The over-expression of related enzymes may improve the transformation speed of these two metabolites and shorten the EE2 degradation speed. According to the reported intermediate metabolites of EE2 in fungi and AOB, the primary products of EE2 reserved the alkynyl group, such as OH-EE2, sulfate-EE2, and glucose-EE2. Considering the structural similarity between EE2 and natural estrogen, the alkynyl group may be an important barrier to EE2 metabolism in microorganisms.

The bacterial degradation of natural estrogen began with the side modification and then the breaking of the rings. Different estrogen-degrading strains may achieve the metabolism of natural estrogens via various predicted pathways, including the 4,5-seco pathway (hydroxylation at C4 and broken between C4/C5), 9,10-seco pathway (broken between C9/C10 and ketonization at C9), 2,3-seco pathway (broken between C2/C3, ketonization at C1 and carboxylation at C3), and the oxidation at the C16 site (oxidization at C16 and broken of the D ring) (Chen et al., 2017; Horinouchi et al., 2018; Wang et al., 2013; Lee and Liu, 2002). Short chain dehydrogenase (SDR), 17β-hydroxysteroid dehydrogenase (17β-HSD), 3α-hydroxysteroid dehydrogenase (3α-HSD), hydroxylase, cytochrome P450, and ring cleavage dioxygenase have been found to be able to catalyze the transformation of natural estrogens effectively (Chen et al., 2017; Yu et al., 2015; Wang et al., 2018, 2019b; Ye et al., 2019; Xiong et al., 2020; Li et al., 2021). Horseradish peroxidase has been reported to function in the modification and degradation of EE2, and the supplied natural organic matter influenced its activity (Hofmann and Schlosser, 2016; Golveia et al., 2018; Yang et al., 2018). However, although EE2 could induce the transcription of some bacterial genes, the enzymes responsible for its degradation have rarely been characterized, due to the scarcity of genome sequences and intermediate metabolites. Currently, only a few of steriod-degrading bacteria have been sequenced, and *P. citronellolis* SJTE-3 in this work was the only sequenced strain with the EE2-utilizing capability. The ammonia monooxygenase (AMO) was thought to be the key enzyme for the EE2 co-metabolism in AOB, supported by the evidence of the mono-hydroxylated intermediates. While no differential expression of the *amoA* gene between the "no EE2″ and "with EE2″ groups was observed, and the expression of the *amoA* gene decreased significantly several days after the addition of EE2 in the chemostat (Khunjar et al., 2011; Sheng et al., 2021). The other 34 genes involved in electron transfer, energy metabolism, transcriptional regulation, and substrate transport were found to be differentially expressed in the co-metabolism of EE2 in *N. europaea*, including nitrite reductase, cytochrome, and multicopper oxidase (Sheng et al., 2021). This suggests that the biotransformation of EE2 may depend on an intact and well-organized metabolic network, and a series of enzymes may participate in this process.

Comparative genome analysis and genome mining of *P. citronellolis* SJTE-3 and other estrogen-degrading bacteria could promote the exploitation of putative function genes for EE2 metabolism. Strain SJTE-3 was found to possess a series of unique genes for EE2 utilization. Three genes, *sdr*3, *yjcH* and *cyp*2, were confirmed to participate in and play an important role in the metabolic process of EE2 in strain SJTE-3, which encode a classical SDR, a membrane transporter and a cytochrome P450 hydroxylase, respectively. The EE2 removal rates of the recombinant strains with the empty plasmid (S3-vector) were lower than that of the wide-type strain SJTE-3, probably because of the growth burden caused by plasmids, antibiotics and inducer toxicity. The two metabolic intermediates of EE2 degraded by the strains S3-SDR3 and S3-YjcH appeared in advance for approximately 1–1.5 h, suggesting that the two genes (*sdr*3 and *yjcH*) may function in the upstream of the EE2 metabolic process in strain SJTE-3. Protein S3-SDR3 probably functions in the reduction and desaturation of EE2, and protein S3-YjcH may be responsible for the transportation of EE2. However, the compound 1 (*m/z* 302 intermediate) was not observed in the culture of strain S3-CYP2 and no alternative MS peak was found. This implies that the gene *cyp*2 may function in the transformation of this intermediate through a ring-opening reaction. The potential metabolic pathways of EE2 in strain SJTE-3 were proposed based on the possible structures of the two intermediates and the putative function of three genes (Table S9).

SDRs are believed to participate in the hydroxylation, dehydration, and side-modification of estrogenic chemicals. The 17β-HSD can convert estradiol into estrone for the initiation of estradiol degradation, and the 3α-HSD can reduce the ketone group at the C3 site of testosterone (Horinouchi et al., 2018; Yu et al., 2015; Ye et al., 2019; Wang et al., 2019b; Xiong et al., 2020). The S3_SDR3 is a putative HSD sharing 50% identity with the ANI04816.1 in *P. putida* SJTE-1 for the reduction of E2 at the C_{17} site, implying that it is probably associated with the dehydrogenation reaction of EE2. As its over-expression accelerated the appearance of compound 1, S3_SDR3 may have been responsible for the reduction and desaturation of EE2 in strain SJTE-3.

As estrogenic chemicals are relatively large and have low solubility in water, they are difficult to transmit through the cell membrane directly; a transporter may facilitate their transportation. The *yjcH* gene in strain SJTE-3 encoded a DUF485 domain-containing protein with two trans-membrane sequences, and with its over-expression the two intermediates appeared earlier. Protein S3_YjcH was highly conserved in *Pseudomonas* and similar to the inner membrane protein YjcH from *P. aeruginosa* and *E. coli*, which are involved in organic acid transport along with the acetate symporter ActP in *P. putida* and in alkane degradation in *P. aeruginosa* (Rand et al., 2017; Xu et al., 2020). In strain SJTE-3, S3_YjcH may facilitate the trans-membrane transportation of EE2 and its metabolites via the acetate transport system.

The CYP450s are considered versatile in the transformation of organic chemicals and some of them can catalyze the estrogen metabolism (Bernhardt and Urlacher, 2014; Schmitz et al., 2014; Makino et al., 2014; Bracco et al., 2013). The CYP106A2 from *Bacillus megaterium* ATCC 13368 can hydroxylate DHEA at the C15 position (Schmitz et al., 2014). The CYP154C5 from *Nocardia farcinica* IFM 10152 and the CYP154C3 from *Streptomyces griseus* can hydroxylate testosterone at the C16 position (Makino et al., 2014; Bracco et al., 2013). The S3-CYP2 in strain SJTE-3 possessed a special SRS5 position, which may have endowed this enzyme with special substrate selectivity and achieved its catalyzing activity with regard to EE2 and its metabolites.

In conclusion, *P. citronellolis* SJTE-3 can metabolite EE2 efficiently with the slight addition of some easily used energy sources and produce two novel intermediate metabolites of EE2. In addition, three newly identified genes played the important and diverse roles in the metabolism of EE2. As no additional intermediates were obtained and the putative function genes for estrogen degradation were scattered, there may still be other genes involved in the EE2 utilization of this strain. Further studies concerning the intermediate metabolites, gene function, and catalytic mechanism of EE2 are in progress.

5. Conclusion

P. citronellolis SJTE-3 was found to be versatile in utilizing various kinds of steroid contaminants, especially in metabolizing the synthetic estrogen 17α-ethynyl estradiol with the initiation of several easily used additives. The characterization of two novel intermediate metabolites of EE2 and the identification of three genes for EE2 transformation could enrich the EE2-utilizing strain pool and gene resources, as well as

promote the study of the biodegrading mechanism of synthetic estrogens.

Compliance with Ethical Standards

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CRediT authorship contribution statement

Wanli Peng: Genome analysis, RT-qPCR, HPLC, GC-MS and GC-Orbitrap/MS detection and all the data analysis; Writing of the original draft preparation and the manuscript modification. **Yali Fu**: part of the RT-qPCR, HPLC, GC-MS detection. **Ben Jia**: Genome analysis. **Xin Sun**: part of the RT-qPCR and the data analysis. **Yanqiu Wang**: part of the HPLC detection. **Shuangjun Lin**: Supervision and support. **Zixin Deng**: Supervision and support. **Rubing Liang**: Conceptualization, Supervision, Writing – review & editing.

Author contributions

R L designed the experiments and P W and R L wrote the manuscript. P W and F Y performed all of the experiments, and P W and B J performed the genome analysis. X S and Y W participated in the quantitative PCR and LC-MS analysis. S L, Z D and R L gave support and advice to the experiments. All the authors discussed the results and commented on the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethical approval

This article does not contain any studies with human participants performed by any of the authors.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.127045.

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