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Bioaugmentation removal and microbiome analysis of the synthetic estrogen 17α-ethynylestradiol from hostile conditions and environmental samples by *Pseudomonas citronellolis* SJTE-3

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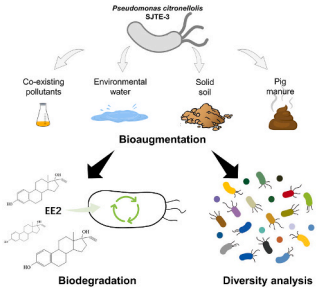
HIGHLIGHTS

- *Pseudomonas citronellolis* SJTE-3 can tolerate high concentration of Zn^{2+} and Cr^{3+}.
- Tween 80 promoted EE2 removal by strain SJTE-3 as surfactant or co-utilized energy.
- Strain SJTE-3 removed EE2 effectively from solid soil, lake water and manure samples.
- Strain SJTE-3 is ecologically safe to the indigenous bacteria community in manure.
- Abundance of Proteobacteria and Actinobacteria enhance for EE2-utilizing potential.

GRAPHICAL ABSTRACT



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ABSTRACT

Synthetic estrogens are emerging environmental contaminants with great estrogenic activities and stable structures that are widespread in various ecological systems and significantly threaten the health of organisms. *Pseudomonas citronellolis* SJTE-3 is reported to degrade the synthetic estrogen 17α-ethynylestradiol (EE2) efficiently in laboratory conditions. In this work, the environmental adaptability, the EE2-degrading properties, and the ecological effects of *P. citronellolis* SJTE-3 under different hostile conditions (heavy metals and surfactants) and various natural environment samples (solid soil, lake water, and pig manure) were studied. Strain SJTE-3 can tolerate high concentrations of Zn^{2+} and Cr^{3+}, but is relatively sensitive to Cu^{2+}. Tween 80 of low concentration can significantly promote EE2 degradation by strain SJTE-3, different from the repressing effect of Triton X-100. High concentration of Tween 80 prolonged the lagging phase of EE2-degrading process, while the final EE2 removal efficiency was improved. More importantly, strain SJTE-3 can grow normally and degrade estrogen stably in various environmental samples. Inoculation of strain SJTE-3 removed the intrinsic synthetic and natural estrogens (EE2 and estrone) in lake water samples in 4 days, and eliminated over 90% of the amended 1 mg/L EE2 in 2 days. Bioaugmentation of strain SJTE-3 in EE2-supplied solid soil and pig manure samples achieved a removal rate of over 55% and 70% of 1 mg/kg EE2 within 2 weeks. Notably, the bioaugmentation of extrinsic

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strain SJTE-3 had a slight influence on indigenous bacterial community in pig manure samples, and its relative abundance decreased significantly after EE2 removal. Amendment of EE2 or strain SJTE-3 in manure samples enhanced the abundance of Proteobacteria and Actinobacteria, implying their potential in utilizing EE2 or its metabolites. These findings not only shed a light on the environment adaptability and degradation efficiency of strain SJTE-3, but also provide insights for bioremediation application in complex and synthetic estrogen polluted environments.

1. Introduction

Estrogens are emerging contaminants of high environmental concern and are considered as typical endocrine-disrupting compounds (EDCs), with adverse effects on normal physiology of organisms even at extremely low concentrations (Adeel et al., 2017; Petrie et al., 2015). Compared with natural estrogens, including 17β-estradiol (E2), estrone (E1), and estriol (E3), synthetic estrogens are crucial EDC pollutants that are drawing increasing attention for their more stable chemical structures, much stronger estrogenic activities, and longer environmental persistence (Aris et al., 2014). The manufacture and usage of synthetic estrogens have increased because of their wide use in birth control pills and other medications used in human society and livestock husbandry (Aris et al., 2014). The most widely used synthetic estrogen, 17α-ethinylestradiol (EE2), is a basic component of oral contraceptives and is also included in prescription medication such as hormone replacement therapies, palliative treatments for breast cancer in postmenopausal women, prostate cancer, and lotions used to prevent androgen-dependent hair loss in women, which is normally discharged into environment through the municipal wastewater treatment plants (WWTPs) (Klaic and Jirsa, 2022; Tang et al., 2021). Animals are administered synthetic estrogens to promote milk production and control reproduction. And the primary sources of estrogenic compounds into environment are the excrement waste of intensive farmed poultry, cattle, pigs, and sheep (Abdellah et al., 2020; Pollard and Morra, 2017). Human activities for its use in oral contraceptives discharged approximately 700 kg/year of EE2 globally, while livestock practices led to the discharge of close to 80,000 kg/year (Adeel et al., 2017).

Because of its low bioavailability and high pKa value, EE2 is poorly absorbed in animal bodies and is excreted into the environment after being treated in WWTPs (He et al., 2017; Olivera and Luengo, 2019). The widely adopted agricultural practice of applying animal manure or sludge bio-solids to farmlands as an alternative fertilizer has heightened the contamination of groundwater, surface water, and soil by estrogen (Abdellah et al., 2020; Lange et al., 2002; Pollard and Morra, 2017). Different estrogenic chemicals and their metabolites widely distributed in various environments due to insufficient treatment and environmental dispersion (Casey et al., 2020; Klaic and Jirsa, 2022). Although the concentration of EE2 in most natural ecological systems is in trace amounts (ng/L), it is enough to disrupt the normal function of endocrine system severely. EE2 can also accumulate to high concentrations (μg L^{-1}) in certain circumstances, such as farm waste, animal manure, lake sediments, and crop field soils, forming great threat to environmental organisms (Olivera and Luengo, 2019; Pratush et al., 2020; Tang et al., 2021).

Removal of estrogens in the environment can be achieved through physical adsorption to soils, water matrices, or activated carbon; chemical redox reactions with strong oxidizers; and microbial degradation using estrogen-degrading strains or biota (Liu et al., 2009; Olivera and Luengo, 2019; Petrie et al., 2015; Pratush et al., 2020). Among these methods, microbial degradation of estrogens is vital and effective due to its easy operation, low energy consumption, and lack of secondary pollution (Olivera and Luengo, 2019; Pratush et al., 2020). The carbon-rich, highly reduced estrogens may provide carbon and energy sources for microorganisms. Some strains of bacteria, yeast, fungi, or microalgae have been reported to completely degrade natural estrogens (Chiang et al., 2019). However, microorganisms are quite reluctant to use the synthetic estrogen EE2, which is likely due to its complicated scaffold structures and extremely low aqueous solubility (Olivera and Luengo, 2019). Earlier studies indicated that EE2 was mainly degraded aerobically in mixed cultures; a few bacterial strains partially transformed or co-metabolized EE2 together with ammonia, dissolved organic matter, easily-used carbon sources, or other natural estrogens (Forrez et al., 2009; Gaulke et al., 2008; Haiyan et al., 2007; Hom-Diaz et al., 2015; Khunjar et al., 2011; O'Grady et al., 2009; Pauwels et al., 2008; Sabirova et al., 2008; Sedighi et al., 2019; Sheng et al., 2021; Skotnicka-Pitak et al., 2009; Syed et al., 2022; Yi and Harper, 2007). For example, several heterotrophic nitrifying bacteria and ammonia oxidizing strains can co-metabolize EE2 (Castellanos et al., 2021; Forrez et al., 2009; Gaulke et al., 2008; Khunjar et al., 2011; Sheng et al., 2021). In the presence of dissolved organic matter, *Rhodococcus equi* can remove 39% of 1.4 mg L^{-1} EE2 in 65 h, and *Shewanella oneidensis* MR-1 can remove 41.6% of 0.5 mg L^{-1} EE2 after 132 h (He et al., 2018; O'Grady et al., 2009). However, when used as a sole carbon source, EE2 can not be degraded by *Rhodococcus* even after 100-h cultivation. Most confirmed reactions of EE2, including sulfonylation, hydroxylation, glucosylation, and carboxylation, are the modifications of EE2 from an active form to an inactive form, while its four-ring structure retains its integrity. As a result of strain scarcity, the understanding of EE2 degradation in bacteria is yet to be established.

Moreover, only a small number of degrading strains that work efficiently in laboratory cultures are applicable in actual environment. It is difficult for many strains to maintain their degrading capacity in harsh and complex natural environment. Limited energy sources, complex buffer systems, and various coexisting pollutants may reduce growth, impair degradation, and hasten degeneration (Fischer and Majewsky, 2014; He et al., 2018; Muller et al., 2010; Zhang et al., 2019). Some strains can degrade natural estrogens under complex conditions. *Sphingomonas* strain KC8 can degrade E2 and E1 when growing on complex nutrients (Roh and Chu, 2010). Strain SJTH-1 maintained a stable removal of E2 from simulated stress conditions and solid soil samples (Xiong et al., 2020). Also supplying nutrients may promote the estrogen-degrading rate of inherent or extrinsic strains. Fe (III) and dissolved organic matter promoted the microbial degradation of E2 in aqueous media (Gu et al., 2018; He et al., 2018). Yet, strains with stable estrogen-degrading efficiency and great stress tolerance are rare and are still being explored. Furthermore, the effect of exogenous degrading strains on indigenous microbial communities in the bioremediation process has raised great concerns (Lara-Moreno et al., 2022; Zhang et al., 2019). The preservation of intrinsic microbial communities is important for the balance of ecological system. Although the relevance of natural estrogen degradation and microbial community change were concerned, few studies have focused on the degrading efficiency and ecological effects of EE2-degrading strains bioaugmented into complex contaminated environments.

In our previous study, we isolated and identified *Pseudomonas citronellolis* SJTE-3, which metabolizes EE2 efficiently with a trace amount of ethanol or other energy sources, and characterized its degrading properties, genetic background, and functional genes. Two intermediate metabolites ($C_{22}H_{22}O$ and $C_{18}H_{34}O_2$) of EE2 were identified and three genes (*sdr3*, *yjcH*, and *cyp 2*) were confirmed to be involved in the EE2-degrading process (Peng et al., 2022; Zheng et al., 2016). In the current study, the EE2-degrading efficiency of strain SJTE-3 under various hostile conditions and natural environmental samples were studied. The

ecological effect of its inoculation in pig manure samples on the intrinsic microbial community was also analyzed. The findings from this work can deepen the understanding of environmental suitability of this strain, and support its further application for bioremediation in natural and complex estrogen-polluted environments.

2. Material and methods

2.1. Chemicals and media

17α-ethynylestradiol (EE2, $C_{20}H_{24}O_2$, >98%), Tween-80 ($C_{24}H_{44}O_6$), and Triton X-100 ($C_{34}H_{62}O_{11}$) were purchased from Sigma-Aldrich (Allentown, PA, USA). The EE2 solution was prepared by dissolving EE2 in dimethyl sulfoxide (DMSO) to a concentration of 10 mg mL^{-1} and storing it at −20 °C. Methanol, acetonitrile, and ethyl acetate of high-performance liquid chromatography (HPLC) grade were purchased from Macklin Biochemical Co., Ltd. (Beijing, China). The Luria-Bertani (LB) medium (tryptone 10.0 g L^{-1}, yeast extract 5.0 g L^{-1}, and NaCl 8.0 g L^{-1}) and the minimal medium (KH_2PO_4 4.5 g L^{-1}, $K_2HPO_4{\cdot}3H_2O$ 13.75 g L^{-1}, $(NH_4)_2SO_4$ 2.0 g L^{-1}, $FeSO_4$ 5.0 μg L^{-1}, $MgSO_4{\cdot}7H_2O$ 0.16 g L^{-1}, $CaCl_2{\cdot}2H_2O$ 1.0 μg L^{-1}, and $MnCl_2{\cdot}4H_2O$ 2.0 μg L^{-1}, pH 7.4) were used for strain culture. Solid media were prepared by supplying agar (15 g L^{-1}) to liquid media.

2.2. Growth and degradation assays of strain SJTE-3 under different conditions

Strain SJTE-3 was cultured using previously described process (Peng et al., 2022). A single colony of strain SJTE-3 was inoculated into 3 mL LB medium and cultured overnight, and then inoculated into 100 mL LB medium at 37 °C and 200 rpm. The cells were then harvested at the exponential phase and washed with Millipore water thrice. The cell inocula were diluted to 2.0 OD_{600}, and were inoculated into the minimal medium containing EE2 (10 mg L^{-1}) with initial OD_{600} at 0.05. The medium was added with heavy metals (Zn^{2+}, Cu^{2+}, and Cr^{3+} in the form of chloride salt) of different concentrations (0.05–6.25 mg L^{-1}) or surfactants (Tween 80 and Triton X-100) ranging from 0.1 to 5.0 critical micelle concentration (CMC). The cultures were incubated at 37 °C for 7 days in a shaker set at 200 r min^{-1}, and were sampled everyday to detect the biomass (OD_{600}) and the EE2 residues using HPLC as follows. The average values of five independent experiments were calculated with the mean and standard deviation.

2.3. Remediation analysis of EE2 in natural lake water samples using strain SJTE-3

To test the estrogen-degrading efficiency of strain SJTE-3 in natural water, real lake water samples were collected from Lake Taihu in Jiangsu Province, China (no specific permissions were required and this work did not involve any endangered or protected species), and the components of the natural lake water were analyzed as follows. The water samples were sterilized through filtration using a 0.22-μm filter (Millipore, MA, USA). Next, 200 mL of the lake water samples was transferred into 500-mL flasks. For the bioaugmentation test, the cell inocula of strain SJTE-3 were directly added to the sterilized lake water samples at a cell density of 0.05 OD_{600} per mL. The lake water samples added with the boiled cells of strain SJTE-3 (the dead cells of strain SJTE-3 after 15 min-boiling at 100 °C) was used as control. For the simulated remediation assay, the lake water samples were amended with the appropriate amount of EE2 stock solution, ethanol, and the cell inocula of strain SJTE-3, with the final concentration of EE2 at 1 μg per mL, ethanol at 1 μL per mL, and cell density at 0.05 OD_{600} per mL. The lake water samples inoculated with the boiled cells of strain SJTE-3, EE2 and ethanol, were set as the control. All the treatments were cultured at 37 °C for 14 days in a shaker set at 200 r min^{-1}. Every 2 days, the biomass of the lake water samples was determined by counting the colony-forming units (CFUs), and the EE2 residues were determined using HPLC or gas chromatography-mass spectrometry (GC-MS) system. Three parallel samples were set at each time point and five independent experiments were performed. The average values were calculated with the mean and standard deviation.

2.4. Simulated remediation test of EE2 using strain SJTE-3 in solid soil samples

To test the EE2-degrading efficiency of strain SJTE-3 in simulated solid soil, the fresh solid soil samples were collected from the soil region (0–10 cm) under the lawn on the campus of Shanghai Jiao Tong University, China (no specific permissions were required and this study did not involve any endangered or protected species). The composition analysis of solid soil samples was performed as follows, and the simulated biodegradation assay was performed similar to previous report (Xiong et al., 2020). Briefly, the soil samples were autoclaved, dried, and sieved (<2 mm). Ten grams of the sieved soil was amended with 10 mL Milli-Q water and EE2 of 10 μg to simulate the estrogen-contaminated soil. Ethanol of 1 μL was added into per gram soil as a co-metabolic carbon source and 0.05 OD_{600} cells of strain SJTE-3 were inoculated in per gram soil. The soil samples inoculated with the boiled cells of strain SJTE-3, EE2 and ethanol were set as the control. Vigorous stirring was performed thoroughly to achieve sufficient dispersion of the amended substrates. Every 10 g of soil sample was spread on plates at 37 °C and cultured for 14 days. All samples were overturned for aeration and weighed to replenish evaporated water every day. Three parallel plates were set at each time point and five independent experiments were repeated. Every 2 days, the biomass of the soil samples was determined by counting the CFUs and the EE2 residues was detected with HPLC system. The average values were calculated with the mean and standard deviation.

2.5. Removal assay of EE2 by strain SJTE-3 in actual pig manure samples

To test the EE2-degrading efficiency of strain SJTE-3 in real animal manure, the fresh pig manure samples in a solid–liquid mixture were collected from a livestock farm in Jiang Xi province of China (no specific permissions were required, and this work did not involve any endangered or protected species). The fresh pig manure was sampled without further treatment and its composition was determined as follows. Approximately 200 g of fresh manure samples in a 500 mL flask was amended with the appropriate amount of EE2 and the cell inocula of strain SJTE-3, with the final concentration of EE2 at 1 μg and the initial cell density at 0.05 OD_{600} in 1 g manure samples. The manure samples inoculated with the boiled cells of strain SJTE-3 and EE2 were set as control. The mixture was cultured at 30 °C in the shaker with rotation speed of 200 r min^{-1}, and was sampled every 2 days to determine the biomass (counting the CFUs) and EE2 residues using HPLC. Five independent experiments were performed and three parallels were set at each time point. The average values were calculated with the mean and standard deviation.

2.6. Composition analysis of different environmental samples

For the solid soil and the pig manure samples, the physical and chemical properties were determined according to the Austrian and European standards (ÖNORM). Briefly, the soil water content (SWC) was measured gravimetrically after drying in aluminum dishes for 24 h at 105 °C. The pH values of the samples were measured in ultrapure water with a ratio of 1:2.5 using a pH sensor. The water-holding capacity (WHC) was determined by repeatedly saturating 10 g of the field-moist soil with water and draining it. The total carbon (TC) and total nitrogen (TN) contents were measured with an elemental analyzer (ELEMENTAR, DE). The dissolved organic carbon (DOC) was measured by removing the carbonate from the samples using 6 M HCl and quantifying with the

elemental analyzer (ELEMENTAR, DE). For the natural lake water samples, the chemical oxygen demand (COD) was determined using the potassium dichromate method on HACH DRB200 digester and HACH DR2800 multi parameter water quality analyzer (HACH, CO, USA). The total phosphorus (TP), NH_4–N, and NO_2–N were determined using the colorimetric method, and the NO_3–N and total nitrogen (TN) were determined using the ultraviolet spectrophotometric method (Rice et al., 2012).

2.7. Pretreatment of different samples for EE2 residues detection

For the laboratory liquid cultures, the mixtures were treated as described (Xiong et al., 2020). Briefly, the collected samples were acidified by adding 1% (v/v) 1 M HCl, and then extracting thrice with an equal volume of ethyl acetate through oscillation. The organic layer was collected and dried under gentle nitrogen, and then dissolved with acetonitrile for further detection. For the solid soil, pig manure, and lake water samples amended with extra EE2 and strain SJTE-3, the samples were freeze-dried and ultrasonically extracted with methanol thrice. The collected organic layers were dried under gentle nitrogen and dissolved in acetonitrile for further detection. The solid-phase extraction to the lake water for ex-situ bioaugmentation test was performed with the HyperSep C18 cartridges according to the instruction (Thermo Fisher Scientific Inc., MA, USA). Briefly, the cartridges were pretreated with 7 mL acetonitrile, 5 mL methanol, and 5 mL ultrapure water. The collected lake water samples (200 mL) were passed through the cartridges at speed of 4 mL min^{-1} and dried under gentle nitrogen, and then were redissolved with 1 mL acetonitrile for further detection. The mean recovery efficiency of EE2 in different samples was calculated based on the calibration curve of EE2, ranging from 86% to 100% (Fig. S1).

2.8. Detection of EE2 using HPLC or GC-MS analyses

The HPLC or GC-MS analyses were performed to determine the EE2 residues in different cultures or environmental samples as described before (Peng et al., 2022). Except for the ex-situ bioaugmentation samples of natural lake water, which were detected using the GC-MS system, all other pretreated samples were detected using the HPLC system (Agilent 1260 Infinity LC system equipped with a fluorescence detector, Agilent, CA, USA). For the HPLC detection, the separation was achieved on an SB-C18 column (4.6 × 150 mm, 5 μm, Agilent, CA, USA) with 1 mL min^{-1} flow rate (55/45, acetonitrile/water) at 30 °C. The excitation and the emission wavelengths were 280 nm and 315 nm, respectively. The GC-MS detection was performed using Agilent 7890 B-GC equipped with a 5977 B-MS detector employing an electron impact ion source (Agilent, CA, USA). The extracted estrogens (E1, EE2) were completely evaporated under gentle nitrogen. The evaporated extracts were derivatized with 50 μL N, *O*-Bis (trimethyl silyl) trifluoroacetamide (BSTFA): trimethyl chlorosilane (TMCS) (99:1, v/v) and 50 μL pyridine by incubating at 70 °C for 40 min. The derivatized samples were evaporated under gentle nitrogen, and 1 mL Mirex in hexane (1 μg mL^{-1}) was added as an internal standard to calibrate the systematic errors. The separation was achieved on an HP-5MS UI capillary column (0.25 μm × 30 m, Agilent, CA, USA) in splitless mode with 1 mL min^{-1} helium as carrier gas; the GC oven temperature program started at 50 °C for 2 min, was heated to 280 °C at 20 °C/min, and held for 10 min. The source and transfer line temperatures were 250 °C and 280 °C. The quantities of estrogens in the culture were calculated according to the standard curves of each chemical with all the R^2 values over 0.99.

2.9. Microbial diversity analysis of simulated and bioaugmented pig manure samples

The effect of EE2 amendment and bacteria inoculation on the intrinsic microbial community of pig manure samples was studied through the analysis of bacterial diversity. The manure samples were mixed with EE2 and cell inocula of strain SJTE-3 by adding 10 μg EE2 and 0.5 OD_{600} cells per 10 g manure waste, and cultured for 14 days. The controls were the manure samples added with EE2 without cell inocula. All the manure samples were collected on days 0, 4, 8, and 14, and the microbial diversity analysis was performed by Shanghai Personalbio Technology Co., Ltd. (Shanghai, China). Each group included three parallels, and three parallel samples were taken at each time point. Total DNA was extracted using the QIAmp DNA Stool Mini kit (Qiagen, CA, USA). The V3–V4 regions of bacterial 16S rDNA were amplified with primers 341 F and 806 R (CCTAYGGGRBGCASCAG; GGACTACNNGGGTATCTAAT). The amplicons were purified with the Agencourt AMPure Beads (Beckman Coulter, DE), quantified and sequenced on the Illumina MiSeq platform. The average values and the *p*-value were calculated with the mean and standard derivation of triplicate repeated samples. A total of 1,942,667 bacterial 16S rDNA reads were obtained, with 1,070,422 in the experimental groups and 872,245 in control groups. The sequences with 97% similarity were clustered into operational taxonomic units (OTUs) with the Greengenes database (DeSantis et al., 2006). The QIIME2 process was used to analyze the alpha diversity of microbial community structure. The microbial community composition in different samples was analyzed using the GENESCLOUD online platform with default parameters (www.genescloud.cn).

3. Results

3.1. Strain SJTE-3 could tolerate high concentration of Zn^{2+}/Cr^{3+} and grew normally

Heavy metals are common coexisting pollutants of estrogenic contamination, causing a heavy burden to growth and degradation of microorganisms (Kyakuwaire et al., 2019; Zhang et al., 2021). Three typical heavy metals (Zn^{2+}, Cu^{2+}, or Cr^{3+}) were added into the minimal medium with EE2 to detect their effect on cell growth and the removal rate of EE2 by strain SJTE-3. The results showed that strain SJTE-3 still exhibited normal cell growth even when exposed to Zn^{2+} or Cr^{3+} of 6.25 mg L^{-1}, approximately 100-fold of the concentration in naturally polluted environments (Fig. 1A and C) (Kumar et al., 2019). However, when exposed to Cu^{2+} of more than 0.05 mg L^{-1}, the cell growth and EE2 degradation by strain SJTE-3 were significantly inhibited (Fig. 1B). The exposure to Zn^{2+} or Cr^{3+} over 0.25 mg L^{-1} also impaired the EE2 removal by strain SJTE-3 (Fig. 1A and C). The great tolerance of strain SJTE-3 to Zn^{2+} and Cr^{3+} of high concentration makes it possibly fit for use in heavy metal and estrogen combined pollution environments.

3.2. Strain SJTE-3 degraded EE2 efficiently in the culture with surfactant Tween 80

Surfactants can enhance the solubility and bioavailability of some organic pollutants and accelerate the biodegradation process (Paria, 2008). Two typical surfactants, Tween-80 and Triton X-100, were supplied to study their effects on EE2 degradation by strain SJTE-3. The results show that Tween 80 of low concentration (0.1 CMC) significantly enhanced the EE2 removal by strain SJTE-3. Although high concentration of Tween 80 (1.0–5.0 CMC) prolonged the lagging phase of the EE2-degrading period, the final EE2 removal efficiency by strain SJTE-3 was all improved (Fig. 2A). Although strain SJTE-3 could not use Tween 80 as sole carbon source, the biomass was elevated when the strain was culture in minimal medium added with Tween 80 (1.0–5.0 CMC) and 0.1% ethanol (Fig. S2A). It suggested that Tween-80 at low concentration may facilitate transportation and utilization of carbon source, while it may be co-metabolized by strain as energy at high concentration.

In contrast, Triton X-100 significantly inhibited cell growth and EE2 degradation by strain SJTE-3 even at low concentration (0.1 CMC) (Fig. 2B). As Triton X-100 could not be utilized by strain SJTE-3 no matter as a sole or co-metabolized carbon source, the promoted EE2

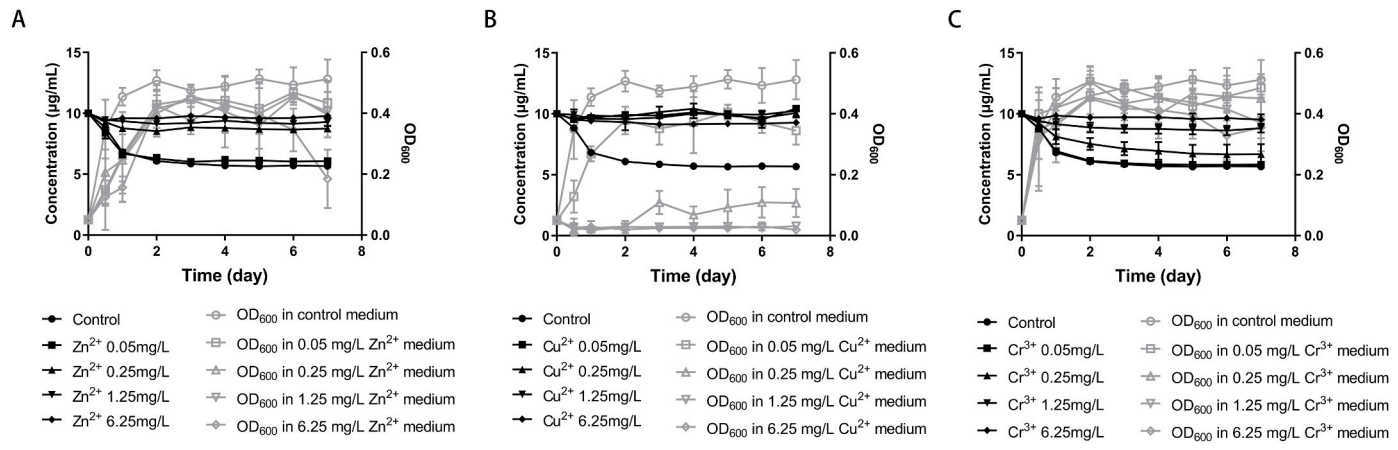

Fig. 1. Effect of three heavy metals on EE2 degradation and cell growth of *P. citronellolis* SJTE-3. *P. citronellolis* SJTE-3 were cultured in minimal medium with EE2 (10 mg L^{-1}), or supplied with Zn^{2+}, Cu^{2+} or Cr^{3+}(0.05 mg L^{-1}, 0.25 mg L^{-1}, 1.25 mg L^{-1}, 6.25 mg L^{-1}), respectively. The residual EE2 and cell growth were detected everyday for seven days with HPLC system and were plotted. The mean values were calculated and the error bars represented the standard errors of five independent experiments.

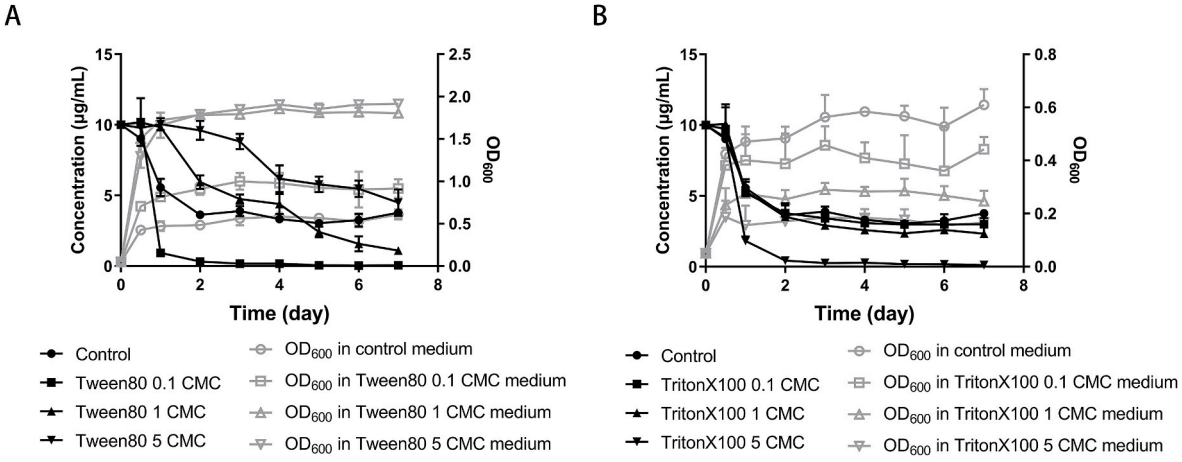

Fig. 2. Effect of two surfactants on EE2 degradation and cell growth of strain SJTE-3. Strain SJTE-3 were cultured in minimal medium with EE2 (10 mg L^{-1}), or supplied with Triton X-100 or Tween 80 of different concentration (0.1 CMC, 1.0 CMC, 5.0 CMC). The residual EE2 and cell growth were detected everyday for seven days with HPLC system and were plotted. The mean values were calculated and the error bars represented the standard errors of five independent experiments.

removal by strain SJTE-3 with Triton X-100 of high concentration (5.0 CMC) is likely due to the release of cellular enzymes as a result of membrane disruption caused by Triton X-100. These results indicated that Tween-80 and Triton X-100 affect EE2 degradation by strain SJTE-3 in different ways.

3.3. Strain SJTE-3 diminished the intrinsic EE2 and E1 in the natural lake water

The natural aquatic environment is a major estrogen discharge and pollution site with great concerns (Gu et al., 2018; Klaic and Jirsa, 2022). The Taihu Lake is the third largest freshwater lake in China and serves as an indispensable water source for a population of 20 million. The lake is severely eutrophicated and the quality of the water supply is threatened (Yan et al., 2014). To investigate the estrogen removal capability of strain SJTE-3 in natural lake water, the water from Taihu Lake was sampled, the composition and properties were determined (Table S1). Approximately E1 of 7.7 ng L^{-1} and EE2 of 17.2 ng L^{-1} were detected in the lake water samples, implying that the lake was contaminated with estrogens by urban human activity or livestock industry discharge. After inoculating strain SJTE-3 with the initial OD_{600} of 0.05 and cultivating it for 4 days, approximately 28% of EE2 and 26% of E1 were removed from the lake water samples. It was significantly higher than the non-biological removal efficiency in the control groups inoculated with boiled cells, only 4% of EE2 and lower than 1% of E1 (Fig. 3A, Fig. 3B, Fig. S3A). Besides, the number of strain SJTE-3 cells in the lake water samples changed indistinctively, probably due to the unsupported cell growth in this limited energy source environment (Fig. S4A).

3.4. Strain SJTE-3 removed EE2 efficiently in simulated severe-polluted lake water

The water system can be an important estrogenic contamination reservoir. To investigate EE2-removing ability of strain SJTE-3 from severe-contaminated water system, the filtered lake water samples were supplied with EE2 of 1 mg L^{-1} to simulate the lake water with serious pollution of estrogen. The results show that bioaugmentation of strain SJTE-3 cells diminished about 90% of the amended EE2 within 2 days and remained stably till 14 days (Fig. 3C, Fig. S3B). The removal rate and biodegradation period of EE2 by strain SJTE-3 in simulated water samples was similar to those in laboratory culture medium (92% removal of EE2 of 1 mg L^{-1} in one week) (Peng et al., 2022). Also the biomass of strain SJTE-3 accumulated quickly in the first 2 days synchronized to the EE2 removal process, while it decreased significantly over time (Fig. S4B). It may be the result of restricted energy source and

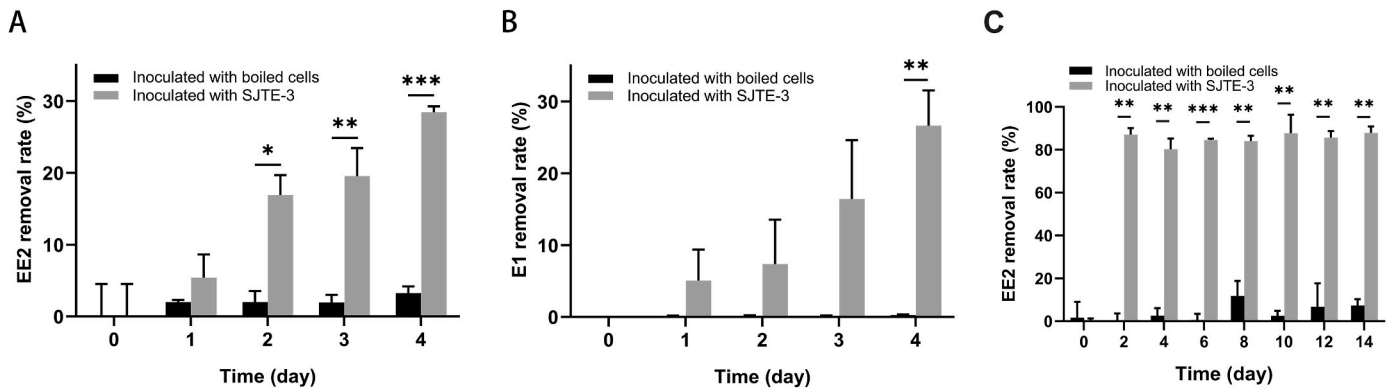

Fig. 3. Degradation of estrogens by strain SJTE-3 in natural and simulated lake water. The environmental water samples were inoculated with strain SJTE-3 cells or the boiled cells with the initial cell density of 0.05 OD_{600}, and cultivated for 7 days. The residues of EE2 (A) and E1 (B) in the natural untreated lake water were detected every 2 days with GC-MS system and were plotted. For the simulated lake water with amended EE2 (1 mg L^{-1}), the strain SJTE-3 cells or the boiled cells were cultured for two weeks. The residual EE2 were detected every 2 days with HPLC system and were plotted. The mean values were calculated and the error bars represented the standard errors of five independent experiments. Statistical analysis was performed and the *p* values were calculated, marked with star (*, *p* value < 0.05; **, *p* value < 0.01; ***, *p* value < 0.001).

unfitted buffer system of environmental lake water after exhausting the supplied ethanol and EE2. These results indicate that even in nutrition-deficient natural aqueous system, strain SJTE-3 still efficiently diminished intrinsic and extrinsic natural and synthetic estrogenic pollutants, implying its potential as an exogenous biodegrader to remedy estrogen-polluted natural water.

3.5. Strain SJTE-3 removed EE2 contamination in the simulated solid soil effectively

The soil system is one of the main estrogen-contaminated environments for the reuse of manure as organic fertilizer (Abdellah et al., 2020; Adeel et al., 2017). To investigate the EE2-removing ability of strain SJTE-3 from soil environment, solid soil was collected, sieved, and supplied with EE2 to simulate the estrogen-polluted soil samples. The properties of the solid soil samples were determined, which is clay loam with 52.28% clay and 28.90% silt, 27.26% WHC, 21.9 mg of total C, and 0.17 mg of total N per gram soil samples (Table S2). With the bioaugmentation of strain SJTE-3, the removal rate of EE2 of 1 mg kg^{-1} in the simulated soil samples was more than 40% in a week and over 55% after two weeks (Fig. 4A, Fig. S3C). It was significantly higher than the removal rate of 10% in control groups inoculated with the boiled cells, probably due to absorption of solid soil particles. Compared to its EE2-degrading rate in laboratory conditions (92% removal of EE2 of 1 mg L^{-1} in 1 week), the EE2-degrading period in solid soil was much longer (Peng et al., 2022). It may be caused by limited dissolved oxygen, restricted energy amount, and confined substrate accessibility.

Meanwhile, the growth of strain SJTE-3 in solid soil samples was also monitored. The result showed that the biomass of strain SJTE-3 increased quickly in the first 2 days of cultivation and reached the peak of growth at day 6; then decreased a little and kept stable cell quantity (Fig. S4C). The EE2 removal rate of strain SJTE-3 was in consistent with its cell growth profile. It meant that most of the amended EE2 in solid soil samples was utilized by strain SJTE-3 for biomass accumulation, implying this strain can fit the soil environment and maintain normal growth. Compared with other EE2-degrading strains, strain SJTE-3 shows efficient and stable EE2-degrading ability in solid soils with practical application potential for soil bioremediation (Table S3).

3.6. Strain SJTE-3 degraded EE2 amended in the pig manure with high efficiency

Animal manure is the most important source of environmental estrogenic pollutants, and its treatment efficiency directly affects the estrogen discharge into environment (Abdellah et al., 2020; Casey et al., 2020). To study whether strain SJTE-3 could maintain its EE2 degradation in a complex manure environment, fresh pig manure waste was sampled and EE2 of 1 mg L^{-1} was amended to simulate a serious EE2-polluted manure environment; the properties of the fresh manure samples were determined. In one gram fresh pig manure samples, the total C of was 314.7 mg, the total organic carbon (TOC) was 127.6 mg,

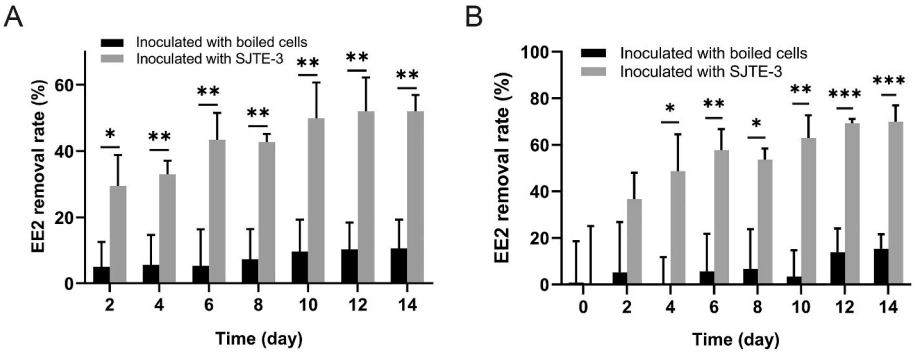

Fig. 4. Degradation of EE2 by strain SJTE-3 in the simulated solid soil and pig manure samples. (A) The removal rate of EE2 by strain SJTE-3 in simulated solid soil. The solid soil supplied with EE2 (1 mg kg^{-1}) were inoculated with strain SJTE-3 cells (the initial cell density of 0.05 OD_{600}) or the boiled cells, and cultivated for two weeks. The residues of EE2 were determined every 2 days with HPLC system and the EE2 removal rate was plotted. (B) The removal rate of EE2 by strain SJTE-3 in simulated animal manure. The pig manure samples supplied with EE2 (1 mg kg^{-1}) were inoculated with strain SJTE-3 cells (the initial cell density of 0.05 OD_{600}) or the boiled cells, and cultivated for two weeks. The residues of EE2 were determined every 2 days with HPLC system and the EE2 removal rate was plotted. The mean values were calculated and the error bars represented the standard errors of five independent experiments. Statistical analysis was performed and the *p* values were calculated, marked with star (*, *p* value < 0.05; **, *p* value < 0.01; ***, *p* value < 0.001).

and the total N was 23.6 mg; there also existed about 2.91 mg of zinc and 0.29 mg of copper (Table S4). It meant the pig manure waste is a nutrient-rich and complex environment with heavy-metal-combined pollutants. The bioaugmentation results showed that approximately 70% of EE2 was removed with the inoculation of strain SJTE-3 and cultivation for two weeks (Fig. 4B, Fig. S3D). The removal of EE2 was only about 15% in control groups inoculated with the boiled cells, probably due to physical absorption or intrinsic biodegradation. It implies that strain SJTE-3 can fit for the actual complex combined-polluted manure environment and can remove EE2 effectively. Compared with the EE2 removal rates by strain SJTE-3 in various conditions or natural environments, dissolved oxygen, energy sources, and indigenous microorganism all affect the biodegradation efficiency (Fischer and Majewsky, 2014; He et al., 2018; Peng et al., 2022; Zhang et al., 2019).

3.7. Bioaugmentation of strain SJTE-3 slightly affected intrinsic bacterial community in pig manure samples

To investigate the influence of strain SJTE-3 inoculation on the inherent bacterial community in pig manure, the microbial composition and relative abundance in all simulated pig manure samples were analyzed. The control groups were added with EE2 without strain SJTE-3, used to study the effect of this chemical. The community diversity (Shannon, Simpson, and Faith's PD), community richness (observed species and Chao 1), community evenness (Pielou's evenness), and sequencing depth (Good's coverage) were described in Table 1. On day 14, the observed species and Chao 1 were approximately 10% and 20% higher in control groups than those in experiment groups, respectively. The observed species in control and experiment groups showed similar descent degrees, although the variation in Chao 1 in the former was much greater (Table 1). It meant that both chemical addition and cell inoculation decreased the richness of bacterial community in manure samples and the effect of EE2 may dominate, by enriching EE2 utilization-associated species and facilitating metabolic process. However, after 14 days of cultivation, the values of Shannon, Simpson, and Pielou's evenness showed little difference between the control and experiment groups (Table 1). It suggested that the diversity and evenness of bacterial community were not significantly affected by cell inoculation, meaning the disturbance of strain SJTE-3 to the bacterial community was subtle.

Further the bacterial community changes during cultivation in the manure samples with different additions were analyzed (Fig. 5). *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria* were the most abundant phyla among all of the manure samples; *Firmicutes* (41.35%) and *Bacteroidetes* (33.3%) were the dominant phyla in the untreated fresh manure samples (Fig. 5A). In control groups, with cultivation continued, the relative abundance of *Proteobacteria* (from 9.04% to 34.13%) and *Actinobacteria* (from 3.86% to 29.66%) increased, and the relative abundance of *Firmicutes* (from 41.35% to 20.72%) and *Bacteroidetes* (from 33.3% to 7.14%) declined. In experiment groups, inoculation of strain SJTE-3 elevated the abundance of *Proteobacteria* (from 9.04% to 52.22%). The relative abundance of *Proteobacteria* (from 52.22% to 46.51%), *Firmicutes* (from 31% to 24.47%), *Bacteroidetes* (from 12.52% to 22.67%), and *Actinobacteria* (from 2.31% to 5.62%) all varied within a relatively narrow range compared to their wide variation in control groups (Fig. 5A).

Moreover, the variations in bacterial community structures were also analyzed to explore the chemical and biological interference with indigenous microorganisms (Fig. 5B). The most abundant genera in the raw manure samples belonged to *Clostridium* (5.73%), *Sphaerochaeta* (4.47%), and *Streptococcus* (4.22%). With the cultivation continued, the relative abundance of *Brevundimonas* (from 0.1% to 19.41%) and *Corynebacterium* (from 1.83% to 25.41%) increased greatly in control groups. The inoculation of strain SJTE-3 elevated the relative abundance of *Pseudomonas* from 0.1% to 22.38%. The most abundant genus in experiment groups after cultivation were *Brevundimonas* (from 0.07% to 17.18%) and *Advenella* (from 0.01% to 15.15%). In all pig manure samples, the abundance of *Stenotrophomonas* and *Pusillimonas* increased first and then decreased; the most significant changes on genus all occurred within 4 days (Fig. 5B).

On this basis, the community structures of different groups were analyzed using principal component analysis (PCA). The PCA results show that the first principal component explains 46.8% of variation, and the second principal component explains 20.7%, accounting for 67.5% of total variation in the dataset (Fig. S5A). The samples of EXP_4 d, EXP_8 d, and EXP_14 d were grouped, meaning the bacterial community in experiment groups changed significantly in the first 4 days and remained stable after 4 days of cultivation. The samples of control groups (CON_4 d, CON_8 d and CON_14 d) were more separated than those in experiment groups, implying that the bioaugmentation of strain SJTE-3 cells can probably amend the influence of EE2 by removing this chemical efficiently (Fig. S5A). The holistic composition of the control and experiment groups were similar, indicating inoculation of strain SJTE-3 slightly changed the bacterial community structure. Furthermore, the similarities and dissimilarities in bacterial community structure were analyzed using principal coordinates analysis (PCoA) based on the Bray–Curtis dissimilarity. The first principal co-ordinate explains 28.2% of variation, and the second explains 21.7%, which accounts for 49.9% of total variation in the dataset (Fig. S5B). All samples on day 0 (CON_0 d and EXP_0 d) were clustered closely; on days 4, 8, and 14, the control and experiment groups were separated but close on PCo1, indicating their similarity in the composition of bacterial community. Therefore, inoculation of strain SJTE-3 affects indigenous bacterial community of pig manure slightly. It can dissipate from manure ecological system with little disturbance after fulfilling the EE2 degradation.

4. Discussion

The typical synthetic estrogen EE2 is an important environmental contaminant of EDCs and human pharmaceuticals and personal care products (PPCPs) of great emerging concern. Its usage is increasing gradually with the increase in human populations and the universality of industrial farming (Gaston et al., 2019; Tang et al., 2021). The physical and chemical methods can remove estrogens with fast speed and indiscriminate selectivity. Physical adsorption strategies remove

Table 1
Analysis of alpha diversity metrics for the experiment groups (EXP) and the control groups (CON).

Group	Shannon	Simpson	Faith's PD	Observed species	Chao 1	Pielou's evenness	Good's coverage
CON_0 d	7.37 ± 0.06	0.97 ± 0.00	120.86 ± 5.21	1625.26 ± 126.82	1852.22 ± 194.20	0.69 ± 0.01	0.99 ± 0.00
CON_4 d	6.03 ± 0.17	0.95 ± 0.01	61.10 ± 2.67	834.00 ± 44.96	923.73 ± 61.00	0.62 ± 0.01	0.99 ± 0.00
CON_8 d	5.65 ± 0.31	0.94 ± 0.01	88.01 ± 6.33	999.53 ± 108.65	1163.53 ± 102.93	0.56 ± 0.02	0.99 ± 0.00
CON_14 d	5.08 ± 0.22	0.90 ± 0.02	78.83 ± 9.59	723.83 ± 92.65	834.29 ± 79.22	0.53 ± 0.03	0.99 ± 0.00
EXP_0 d	5.83 ± 0.16	0.93 ± 0.01	82.47 ± 4.88	974.70 ± 84.20	1008.53 ± 85.19	0.58 ± 0.01	0.99 ± 0.00
EXP_4 d	4.87 ± 0.19	0.90 ± 0.01	59.41 ± 12.74	614.16 ± 151.35	684.93 ± 172.63	0.52 ± 0.03	0.99 ± 0.00
EXP_8 d	4.72 ± 0.27	0.90 ± 0.01	49.54 ± 7.22	531.60 ± 122.81	620.80 ± 157.65	0.52 ± 0.01	0.99 ± 0.00
EXP_14 d	4.96 ± 0.24	0.91 ± 0.01	54.79 ± 16.26	542.20 ± 176.01	615.78 ± 220.75	0.55 ± 0.00	0.99 ± 0.00

The values were the mean values of three independent experiments with standard deviation.

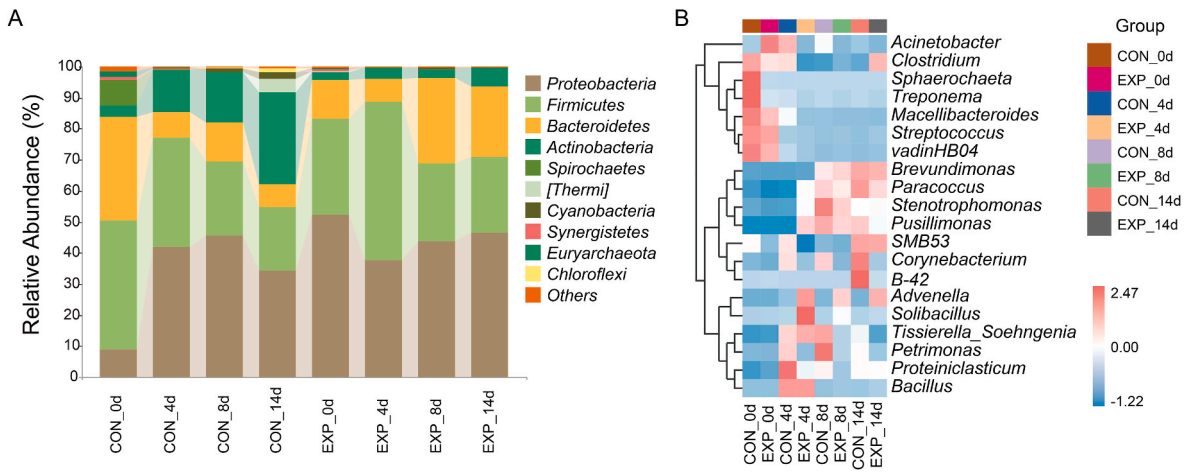

Fig. 5. Composition of bacterial communities in pig manure samples. (A) Relative abundances of the top 10 bacterial taxa at the phyla level in the pig manure samples during cultivation. (B) The heatmap representing the cluster pattern of top 20 bacterial communities at genus level during cultivation. Unweighted pair-group method with arithmetic means (UPGMA) clustering was performed according to the Pearson correlation coefficient matrix of microbial composition data (Euclidean distance-based) and arranged according to sample clustering results. Relative abundance of a bacterial taxon was calculated with z-scores and indicated with different color.

estrogens through absorption by soils, water matrices, or activated carbon (Han et al., 2012). Chemical oxidation methods using strong oxidizers can scarcely achieve the complete oxidation of estrogens and possibly result in secondary pollution (Liu et al., 2009). However, their high energy consumption makes them hard to meet the needs of green and friendly environmental remediation.

Biodegradation is considered efficient to diminish estrogen pollution, especially synthetic estrogen, which has high chemical stability and low solubility (Chiang et al., 2019). Earlier studies have indicated that EE2 can be transformed and degraded by microbes under aerobic and anaerobic environments, normally co-metabolized with other sources (Pollard and Morra, 2017) (Table S3). For example, *Pseudomonas* sp. removes 95% of EE2 of 1 mg L^{-1} in 5 days co-metabolizing with E2, and *Nitrosomonas europaea* ATCC 19718 removes 76% of EE2 of 0.2 mg L^{-1} in 100 h together with 500 mg N/L ammonia. *P. putida* LMG2321 can co-metabolize 0.5 μM EE2 in 2 days with 75 μM Mn^{2+}. *S. oneidensis* MR-1 degrades 41.6% of EE2 of 0.5 mg L^{-1} after 132-h cultivation with an electrochemically modified dissolved organic matter under anoxic conditions (He et al., 2018). *Rhodococcus equi* can remove 39% of EE2 of 1.4 mg L^{-1} in 65 h, and *Rhodococcus zopfii* can degrade EE2 together with E3 (Yoshimoto et al., 2004). Our previous study reported that strain SJTE-3 can remove approximately 92% EE2 (1 mg/L) in one week co-metabolizing with 0.1% ethanol or other energy sources (Peng et al., 2022). It shows higher efficiency and shorter period of EE2 degradation than other EE2-degrading bacteria, with potential for practical use (Table S3).

It is worth noting that bacteria can change their metabolic properties to adapt harsh or complex conditions, which may alter their removal rate of pollutants (Li et al., 2012; Xu et al., 2017). The dissolved oxygen, limited energy, combined pollutants and unwanted adsorption in real environments all affect cell growth and impede estrogen degradation (He et al., 2018; Zhang et al., 2019). Among them, combined pollution commonly occurs in natural environments. Heavy metals frequently coexist with estrogenic chemicals in actual estrogen-polluted environments (Kyakuwaire et al., 2019; Zhang et al., 2021). Zn^{2+}, Cu^{2+}, and Cr^{3+} are the widely-existed metal pollutants in water and soil; livestock manures and sewage sludge are important sources for the total Zn and Cu inputs to ecological systems (Nicholson et al., 2003). And chromate is recognized as one of the most hazardous pollutants in soil, threatening the qualities of soil and underground water (Zhou et al., 2018). According to the Heavy metal pollution index (HPI) for various heavy metals in surface water bodies based on WHO (2017) guidelines for drinking water, the highest permitted value for drinking water is 5 μg L^{-1} for Cr, 3000 μg L^{-1} for Cu, and 5000 μg L^{-1} for Zn. According to the concentration of various heavy metals in surface water bodies, the average concentration of Cr is 413.27 μg L^{-1}, the concentration of Cu is 537.87 μg L^{-1}, and the concentration of Zn is 723.11 μg L^{-1} (World Health Organization, 2017; Kuma et al., 2019).

Metal ions normally form metal-organic complexes or chelates, affecting cell growth and degradation efficiency greatly. Except for certain strains containing genes able to inactivate or pump-out metal ions, most microorganisms are quite sensitive to heavy metal ions. There are a series of metal-resistant genes in the big plasmid pRBL16 of strain SJTE-3, implying its tolerating potential to heavy metals. This study showed that strain SJTE-3 could grow in culture with high concentration of Zn^{2+} or Cr^{3+} (hundreds of concentrations in actual polluted sites), while its EE2 removal rate was inhibited at concentrations higher than 0.25 mg L^{-1}. The inhibition of Zn^{2+} and Cr^{3+} to EE2 degradation by strain SJTE-3 was probably associated with the metal-ion sensitivity of enzymes involved in estrogen metabolism. Although the metabolic mechanisms of EE2 in bacteria are not clarified yet, some enzymes like 17β-hydroxysteroid dehydrogenase (17β-HSD), short-chain dehydrogenase (SDR) and cytochrome P450 were found to function in the biodegradation pathway of natural estrogen (Chiang et al., 2019). 17β-HSD, the key enzyme for estrogen biodegradation in bacteria, is reported to be sensitive to Cu^{2+} and Zn^{2+}; cytochrome P450 was also found to be sensitive to Cr^{3+} (Kostaropoulos et al., 2005; Wang et al., 2019). Very limited enzymes were identified to be involved in the EE2 degradation. In strain SJTE-3, the short chain dehydrogenase SDR3, transporter YjcH and cytochrome P450 enzyme CYP2 were found to participate in the EE2 degradation process of this strain, which may be sensitive to heavy metals (Peng et al., 2022). Inhibition of these vital enzymes by heavy metals results in the degeneration of EE2 degradation efficiency. In addition, Cu^{2+} can restrain cell growth by generation of reactive oxygen species (ROS), displacement of iron from Fe–S clusters and inactivation of metallo-proteins (Li et al., 2021). Genome analysis of strain SJTE-3 shows that there are the zinc resistant genes (*fieF*), copper resistant genes (*copC*, *copD*) and chromate resistant genes (*chrB*) in genome, accounting for resistance to zinc and chromate. However, the complete copper resistant operon (*cop* operon) normally contains *copA* (periplasmic copper binding), *copB* (outer membrane copper binding), *copC* (periplasmic copper binding/transport), *copD* (inner membrane copper transport), *copR* (cytoplasmic activator) and *copS* (inner membrane copper sensor) (Cooksey, 1994). As our results shown in this work,

strain SJTE-3 can tolerate heavy metals at the environmental concentration and grow normally, while its degrading efficiency to high concentration of EE2 (10 mg L^{-1}) is influenced. However, the bioaugmentation results of strain SJTE-3 into pig manure showed that strain SJTE-3 can remove over 70% of EE2 of 1 mg L^{-1} in such a heavy-metal-coexisting conditions, implying its fitness and potential in actual combined polluted environments.

Nonionic surfactants are widely applied in organic pollutants bioremediation. The weaker repulsion among headgroups enables them to form larger micelles and exhibit higher solubilization power towards organic pollutants than ionic surfactants. Surfactant-enhanced remediation (SER) is considered a promising and efficient remediation approach for insoluble pollutants (Paria, 2008). Currently, Tween-80 and Triton X-100 are the commonly used nonionic surfactants in SER (Liang et al., 2017). As the physical properties like surface tension, interfacial tension, adsorption, and detergence change below the CMC with the concentrations, but there is no change in these properties above the CMC (Paria, 2008). Therefore, we supplied two surfactants into the culture medium with the concentration above and below CMC and the cultures were selected for analysis in this work. The findings in this study show that supplementation of Tween-80 (0.1 CMC) in culture medium significantly promoted EE2 degradation by strain SJTE-3, although high concentration of Tween-80 (higher than 1 CMC) prolonged the lagging period of EE2-removing process. Tween-80 is a polyethylene sorbitol ester assuming 20 ethylene oxide units, sorbitol, and one oleic acid as the primary fatty acid, suggesting it may be used as a carbon source. Although Tween-80 could not be used by strain SJTE-3 as a sole carbon source, it improved cell growth of strain SJTE-3 when ethanol used as the carbon source, with a short growth lag phase and fast biomass accumulation. It meant that Tween-80 may be co-metabolized by strain SJTE-3 before utilizing EE2. Therefore, the improved final EE2-degrading efficiency of strain SJTE-3 in culture supplied with Tween 80 can be attributed to elevated bacteria biomass and enhanced substrate accessibility. In contrast, Tween series surfactants can modify cell surface hydrophobicity and affect biodegradation process. Tween 80 of high concentration can trap substrates into its micellar structure and diminish bioavailability of nonpolar compounds, limiting EE2 acquisition and prolonging the EE2 removal period by strain SJTE-3. Regarding Triton X-100, it can increase cell membrane permeability, commonly used for solubilizing proteins, permeabilizing the cell membrane and lysing cells (Schnaitman, 1971). It also cannot be used by strain SJTE-3 solely or together with ethanol, thereby inhibiting cell growth and EE2 removal at low a concentration. Triton X-100 at a high concentration (5 CMC) may disrupt the membrane integrity of strain SJTE-3 cells and release the cellular enzymes, thus suppressing cell growth and promoting EE2 removal. Therefore, two surfactants function in various ways, referable for different purposes or at different stages. Besides, the affect of two surfactants to the growth and metabolism of the estrogen-degrading strains are great and similar. Compared with other reported estrogen-degrading strains, strain SJTE3 showed great tolerance to two surfactants (Table S3). For example, no significant affect of Tween 80 on cell growth and E2-degrading efficiency of strain SJTH1 was observed; while Triton X-100 had no promotion on cell growth at all and significantly inhibited the estrogen-degrading efficiency at high concentration (5 CMC) (Xiong et al., 2020).

In the meantime, biosurfactants are amphiphilic compounds conferring organism ability to accumulate between fluid phases, reducing surface and interfacial tension. Some microorganisms like *Acinetobacter* sp., *Bacillus* sp., *Candida* sp. and *Pseudomonas* sp. can produce biosurfactant. The produced biosurfactants can improve the bioavailability of insoluble organic pollutants and enhance the bioremediation efficiency (Souza et al., 2014). *Pseudomonas* sp. can produce rhamnolipid and glycolipids surfactants to influence adsorption and desorption process and reduce interfacial tension (Guo et al., 2018). The biosurfactants produced by strain SJTE-3 may also play an important role in its EE2 removal process.

Although the biodegradation of EE2 by active sludges, oxidation ditches, and batch reactors in wastewater treatment plants have been studied, the removal property of EE2 by a bioaugmented EE2-degrading strain from simulated or actual environments has rarely been reported, probably because of the scarcity of EE2-degrading strains fit for natural environments (Clouzot et al., 2010; Lust et al., 2015; Ziels et al., 2014). This study shows that strain SJTE-3 efficiently removes the intrinsic EE2 and natural estrogen, and the extrinsic EE2 in soil, lake water, and pig manure, which is more robust and more suitable for practical use than other strains (Forrez et al., 2009; Gaulke et al., 2008; Haiyan et al., 2007; Hofmann and Schlosser, 2016; Khunjar et al., 2011; Pauwels et al., 2008; Sedighi et al., 2019; Sheng et al., 2021; Skotnicka-Pitak et al., 2009; Yi and Harper, 2007). On the hand, the metabolite intermediates of EE2 in environmental samples are also of concern. However, the previously reported intermediate metabolites of EE2 degraded by strain SJTE-3 are undetectable in all the environmental samples, probably due to a relatively lower EE2 concentration and interference from complex environments.

Natural water and solid soil are usually oligotrophic environments and are also the main estrogen-polluted ecological systems (Casey et al., 2020; Castellanos et al., 2021; Klaic and Jirsa, 2022; Zorzal-Almeida et al., 2020). Taihu Lake is close to cities and dwelling districts, serving as an indispensable water source and urban landscape water and facing serious eutrophication and distinct risk of estrogenic pollution. The amounts of EE2 and E1 in the Taihu Lake water detected in this study are consistent with previous reports, implying that Taihu Lake is an estrogen-polluted water environment (Klaic and Jirsa, 2022; Yan et al., 2014). Based on this, we designed two assays for environmental lake water samples. One ex-situ bioaugmentation assay is designed to study the suitability of strain SJTE-3 in oligotrophic and assess the possibility of ex-situ remediation by this strain. Thus the cell inocula of strain SJTE-3 was added directly at 0.05 OD_{600} per mL lake water samples without extra chemicals. Another simulated biodegradation assay is performed to analyze the adaptability and EE2 removal rate of strain SJTE-3 in a simulated severe EE2-polluted lake water. Therefore, 1 g of the lake water samples were supplied with 1 μg EE2, 1 μL ethanol and the cell inocula of strain SJTE-3 at 0.05 OD_{600}. Bioaugmentation of strain SJTE-3 can diminish the intrinsic natural estrogen and synthetic estrogen efficiently. Trace soluble carbon sources promoted the co-metabolizing efficiency of strain SJTE-3 to inherent estrogens and extrinsic high-concentration EE2. In simulated lake water samples of severe EE2 pollution, about 90% of EE2 was removed by bioaugmented strain SJTE-3 in 2 days, similar to its removal rate in laboratory culture medium. Although the environmental water is normally quite diverse from laboratory medium in nutrient content, buffer system, ion concentration and trace elements, strain SJTE-3 is still adaptive and vibrant, implying its good environment suitability and wide application potential. Meanwhile, it is worth noting that solid soil is often affected by estrogen contamination as livestock discharge and organic farming (Casey et al., 2020; Pollard and Morra, 2017). Biodegradation is vital for removal of estrogenic pollutants from soil (Chiang et al., 2019; Kyakuwaire et al., 2019). In this study, bioaugmentation of strain SJTE-3 eliminated EE2 in simulated EE2-polluted solid soil effectively and quickly (Table S3). According to previous studies, the soil particles can adsorb estrogens molecules, and high adsorption affinity with correlations to mineral particle size and organic matter content. The adsorption affinity appeared to be associated with the surface area and the cation exchange capacity of the soil. Approximately 10% removal of EE2 was achieved with inoculation of the boiled cells, very likely due to the irreversible adsorption of EE2 onto soil matrix (Muller et al., 2008). Therefore, strain SJTE-3 can fit into oligotrophic environments and play a role in practical bioremediation of EE2.

Generally, wastewater and excrement of poultry and livestock husbandry are the chief sources of estrogenic pollutants discharged into soil and aqueous environments. Animal manure on swine farms is regularly processed by aerobic composting before application to crop fields as

organic fertilizer (Abdellah et al., 2020; Casey et al., 2020; Pollard and Morra, 2017). The biodegrading efficiency in the composting is crucial for preventing estrogenic chemicals from actual environments. Estrogen pollutants from fertilizer dissemination are detectable in compost-amended soils, compost samples, and even nearby agricultural soils (Xu et al., 2018). Consequently, the manure microbial community and their estrogen-degrading activities are pivotal factors for manure estrogen management. In fact, EE2 cannot be effectively used by indigenous bacterial community; EE2 was not degraded even after over one year of incubation or long-term batch experiments (de Mes et al., 2008). It is probably due to the low abundance or inferior activities of EE2-degrading microorganisms in such environments. Bioaugmentation or biostimulation is a frequently-used and valuable approach to improve estrogen removal efficiency for manure management (Roh and Chu, 2010). However, inoculation of alien microorganisms may disturb indigenous community to some extent (Abdellah et al., 2020). Thus, the extrinsic microorganisms removing estrogenic pollutants efficiently with mild effect on microbial community are still being researched. In this study, the inoculated strain SJTE-3 showed great EE2 removal efficiency in simulated EE2-contaminated pig manure, with a subtle disturbance to intrinsic microbial community. The intrinsic energy source, organic matter, and food residues in eutrophic manure samples facilitate the EE2 metabolism by strain SJTE-3. And indigenous bacteria in manure samples may be simulated by EE2 or strain SJTE-3, contribute to basal EE2 removal in non-bioaugmented groups.

PCoA is a visualization method used to study the similarity or difference in data based on the Bray-Cutis dissimilarity. The microbial analysis of the manure samples in this study showed that the control groups were less dispersed than the experiment groups, suggesting higher parallelism in the control groups. The samples in the control and experimental groups showed clustering at 0.2 on PCo1. This may due to the fact that strain inoculation and EE2 supplementation result in the enrichment of certain communities associated with EE2 metabolism. The separation of the control and experiment groups on PCo2 indicated that the inoculation of strain SJTE-3 amended the variation in bacterial community structure caused by EE2. From bacterial community composition analysis, the inoculation of strain SJTE-3 did not significantly change the community structure of manure samples. The EE2-supplied control groups and the strain-inoculated experiment groups showed a similar change. The addition of EE2 enhanced the abundance of certain genera in fresh pig manure samples, including *Pseudomonas*, *Clostridium*, and *Acinetobacter*; the abundance of *Pseudomonas* in control groups increased from 0.10% (CON_0 d) to 1.98% (CON_14 d) after 14 days of cultivation. *Pseudomonas* genus showed great degradation capabilities to organic chemicals, and several strains have been reported with estrogen-degrading abilities (Wang et al., 2019; Chiang et al., 2019). The increase in the abundance of *Pseudomonas* genus implied that there may exist potential EE2-degrading strains. In the bioaugmented groups, inoculation of strain SJTE-3 increased the proportion of *Pseudomonas* genus on day 0; therefore, it probably inhibited growth of intrinsic *Pseudomonas* species and caused a decrease and restoration in the abundance of *Pseudomonas* genus after day 14. The decrease in strain SJTE-3 after EE2 removal may be related to growth properties coming from the habitat difference, making it more ecologically safe for practical use.

Similar to previous reports, the predominant phyla in fresh pig manure samples in this work were Proteobacteria (9.04%), Actinobacteria (3.86%), Firmicutes (41.35%), and Bacteroidetes (33.30%) on day 0 (Liu et al., 2020; Zhang et al., 2018). The most abundant bacterial genera were *Clostridium*, *Sphaerochaeta*, *Streptococcus*, and *Acinetobacter*. After 14-day cultivation, the most abundant bacterial phyla in the EE2-supplied groups were Proteobacteria (34.13%), Actinobacteria (29.66%), Firmicutes (31.00%), and Bacteroidetes (12.52%). The abundance of these phyla in the strain-inoculated groups was Proteobacteria (46.51%), Actinobacteria (5.62%), Firmicutes (24.47%), and Bacteroidetes (22.67%). It indicates that EE2 significantly enhanced the abundance of Proteobacteria and Actinobacteria in pig manure, similar to those in manure composting (Liu et al., 2020). Proteobacteria and Actinobacteria have been identified as estrogen degraders using a culture-dependent approach (Chiang et al., 2019). In most aerobic studies in manure management, Proteobacteria are typically the most abundant phylum (Liu et al., 2020). Actinobacteria have been found at high abundances in substrates rich in cellulose, lignose, and other organic substrates (Zhang et al., 2018). Various species of estrogen-degrading Actinobacteria have been reported. For example, *Rhodococcus* sp., *Agromyces* sp., and *Mirobacteria* sp. were reported to be versatile in degrading natural and synthetic estrogens (Ke et al., 2007; Menashe et al., 2020; Yu et al., 2007). In the strain-inoculated groups of this study, the abundance of Actinobacteria was significantly lower than those in the EE2-added groups, implying that bioaugmentation of strain SJTE-3 may restrain the induction of intrinsic Actinobacteria strains with EE2 utilizing potentials in manure samples. Amending EE2 may support the growth of Actinobacteria strains and enrich some strains associated with EE2 metabolism, which likely accounted for the basic removal of EE2 in control groups.

In contrast, abundance of Bacteroidetes in experiment groups was significantly higher than that in control groups. The Bacteroidetes phylum is important for host cholesterol homeostasis and dietary lipid consumption (Le et al., 2022). The degradation pathway of cholesterol was expected to be similar with that of estrogens, as they share the same "6-6-6-5" main structure (Chen et al., 2017). The change in the abundance of Bacteroidetes in the strain-inoculated groups may be because the EE2 metabolism by strain SJTE-3 increased the available carbon sources for certain strains in Bacteroidetes with the degrading potentials to the chemicals of this skeleton.

In addition, the abundance of some genera reduced along with the cultivation, including *Acinetobacter*, *Clostridium*, *Streptococcus*, *VadinHB04*, and *Macellibacteroides*. Most of these genera are anaerobic bacteria, and this reduction may be due to their unfitness in the aerobic culture. Among them, the Firmicutes often exhibited relatively high enrichment throughout the composting process due to their great temperature tolerance (Subirats et al., 2020). *Clostridium* was the most abundant genus in the Firmicutes, and its relative abundances in all the groups were similar and reduced gradually. As mentioned above, cell growth of *Clostridium* may be inhibited by anerobic culture and its abundance in manure is restricted (Yi et al., 2012). Besides, our work showed that the enriched genera in the strain-inoculated groups were mainly *Brevundimonas*, *Paracoccus*, *Stenotrophomonas*, and *Pusillimonas*, implying their estrogen utilizing potentials in aerobic environment.

5. Conclusion

In this study, the stress tolerance, environmental suitability, and ecological impact of *P. citronellolis* strain SJTE-3 were studied. This strain shows good tolerance to heavy metals and surfactants, and maintains stable EE2 removal efficiency in simulated EE2-contaminated solid soil, lake water, and pig manure samples. Its bioaugmentation slightly affects the bacterial community diversity and evenness in the pig manure. The addition of EE2 and strain SJTE-3 to pig manure can partly enrich the abundance of potential estrogen-degrading strains in certain genera. This study can deepen the understanding of EE2 removal by strain SJTE-3 and provide insights to explore its applicable remediation potential in natural environments.

Credit author statement

W.P. Designed, performed and analyzed most of the experiments. W. P. And R.L. Prepared and revised the manuscript. Z.D., S.L. And R.L. Contributed to the funding acquisition and project administration, R.L. Conceived the project and supervised the study.

Compliance with ethical standards

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Ethical approval

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

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.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.137893.

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