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RESEARCH

# Effective simultaneous removal of 17β-estradiol and tetracycline by a novel *Alkalibacterium* strain: characteristics, mechanisms, and application in livestock wastewater treatment

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## Abstract

The environmental risk posed by 17β-estradiol (E2) and tetracycline (TC) contamination within livestock wastewater has been widely concerned. Especially, the co-occurrence of these pollutants poses a tremendous challenge to their efficient bioremediation, highlighting the need for strains capable of simultaneous E2 and TC removal. In this study, a novel strain of *Alkalibacterium* sp. AEPI-S25 was successfully isolated from the sediments of Qinghai Lake, demonstrating the ability to remove 89.91% of 20 mg $L^{-1}$ E2 and nearly 100% of 20 mg $L^{-1}$ TC simultaneously within 5 days. AEPI-S25 exhibited remarkable environmental adaptability and maintained high simultaneous removal efficiency under various stress conditions and the presence of two typical livestock wastewater samples. Based on Ultra-Performance Liquid Chromatography coupled with Orbitrap High-Resolution Mass Spectrometry (UPLC-Orbitrap-HRMS) analysis, dehydrogenation and monooxygenation were identified as the key steps in the removal pathways of E2 and TC, respectively. Whole-genome sequencing further identified the potential E2/TC removal genes, and the detected potential E2-dehydrogenation (*S25_gene0393*) and TC-monooxygenation (*S25_gene0878*) genes were subsequently validated through transcription analysis and heterologous expression. Notably, *S25_gene0878* exhibited significant differences from the well-characterized TC removal gene *TetX*, extending a new understanding of the bacterial TC removal mechanism. Overall, this study provides the first report of a single microbial strain capable of simultaneously removing E2 and TC, offering valuable insights for the application of microbial technologies in addressing typical E2-TC combined pollution in livestock wastewater.

### Key points

- *AEPI-S25 can remove nearly 90% and 100% of 20 mg L−1 E2 and TC within 5 days*
- *Key E2 (S25_gene0393) and TC (S25_gene0878) removal genes were cloned and expressed*
- *A novel TC-monooxygenase gene distinct from TetX was discovered within the strain*

**Keywords** 17β-estradiol · Tetracycline · Simultaneous removal · Biotransformation pathway · Genomic analysis · Gene expression

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## Introduction

In recent years, the presence and persistence of 17β-estradiol (E2) in livestock wastewater have received increased attention due to its potential spread to the environment and further endocrine-disrupting effects on humans and aquatic life at extremely low concentrations (ng $L^{-1}$) (Burkholder et al. 2007; Adeel et al. 2017; He et al. 2020; Guo et al. 2022; Gomes et al. 2022; Ciślak et al., 2023). Previous studies revealed that livestock operations are the primary source of E2 emissions, contributing 70%—90% of their total amount detected in the environment (Johnson et al. 2006; Zhang et al. 2014). Some field studies further demonstrated that wastewater from intensive livestock farming leads to high levels of E2 contamination (typically in the range of 10—1000 ng $L^{-1}$) in nearby rivers and lakes, particularly in the absence of wastewater treatment (Chen et al. 2010; Liu et al. 2018). In addition to E2, the widespread presence of antibiotic residues in livestock wastewater has garnered significant attention (Wu et al. 2024). Especially, as one of the most commonly used veterinary antibiotics, tetracycline (TC) was ubiquitously detected in livestock wastewater (typically in the range of 100—10,000 ng $L^{-1}$) and was reported to largely interfere with environmental health (Islam and Gilbride 2019; Jia et al. 2017; Kim et al. 2013; Xu et al. 2021; Murray et al. 2021). Notably, TC has also been linked to endocrine disruption in aquatic species by modulating the expression of genes related to steroid metabolism (Gracia et al. 2007). To eliminate the environmental endocrine-disrupting effects of livestock wastewater, it is necessary to minimize both E2 and TC concentrations in it.

Various physical and chemical treatment methods (e.g. membrane filtration and advanced oxidation) have been explored for the simultaneous removal of E2 and TC (Koyuncu et al. 2008; Wang et al. 2019). However, the high cost of these methods significantly limited their widespread application in livestock wastewater treatment. In contrast, microorganism-based bioremediation was emerged as a promising alternative due to its cost-effectiveness, high efficiency, and minimal generation of secondary pollutants. Previous studies have isolated and identified numerous strains from soil, sediment, and aquatic environments that were capable of removing E2 or TC at mg $L^{-1}$ level within 3–7 days (Li et al. 2020a; Hao et al. 2023; Ye et al. 2023). Nevertheless, the co-existence of E2 and TC in livestock wastewater presents a tremendous challenge for effective bioremediation. On one hand, as a broad-spectrum antibiotic, TC was expected to inhibit a variety of E2-degrading bacteria (Li et al. 2020a). For example, Li et al. demonstrated that the biodegradation of E2 by *Novosphingobium* sp. ES2-1 was significantly inhibited when TC content exceeded 10 mg $L^{-1}$ (Li et al. 2020a). On the other hand, the potential dissemination of antibiotic resistance genes (ARGs) poses a critical limitation to the application of TC-resistant E2-degrading strains. Consequently, compared to the combined use of individual degradation-functional microorganisms, E2-TC simultaneous biodegrading strains are better suited for the bioremediation of their combined pollution. Although some E2-degrading strains (e.g. *Flavobacterium longum* sp. L7, *Pseudomonas aeruginosa* sp. M10, and *Stenotrophomonas maltophilia* sp. MN08) have demonstrated resistance to TC (Alahadeb 2022; Farraj et al., 2024), their capacity to remove TC has not yet been determined. Moreover, *Novosphingobium* sp. ES2-1 was found to remove E2 and TC simultaneously, but its mechanism for reducing TC was proposed as hydrolysis rather than biodegradation (Li et al. 2020a). To date, no strains have been confirmed to biodegrade both E2 and TC simultaneously, and the underlying mechanisms of their biodegradation processes remain poorly understood. Moreover, due to a variety of typical environmental stress factors (e.g. high salinity, $Cd^{2+}$, and $Cu^{2+}$) in natural livestock wastewater will also largely affect the degradation effects of the strains (Kim et al. 2016; Hejna et al. 2021; Wu et al. 2024), highly adaptable strains were required in practical treatment.

Qinghai Lake is the largest alkaline (pH 8.8–9.3) saltwater (~ 16 g $L^{-1}$) lake in China, serving as a natural library and treasure house for high environmental adaptability pollutant degradation microorganisms (Huang et al. 2022; Wang et al. 2024a, b). Since the Qinghai Lake basin is a closed watershed with no river outflow, the sediment of the lake is prone to becoming a sink for antibiotics and estrogens with the expansion of surrounding animal agriculture (Jia et al. 2024; Li et al. 2020b). For example, one study reported that the mean concentration of tetracyclines (TCs) in the sediment of Qinghai Lake was about 1.5 ng/g, which was nearly 2 times higher than that in its input rivers (Li et al. 2020b). Although the estrogen data in Qinghai Lake are still lacking, widespread grazing around the Qinghai Lake (Sun et al. 2024) has long been considered one of the main contributors to elevated estrogen concentrations in the surrounding environment (Kolodziej and Sedlak., 2007). Thus, these unique ecological characteristics render it an ideal source for isolating environmentally resilient microbial strains capable of simultaneous E2 and TC biodegradation.

In this study, we describe the enrichment and adaptive evolution of microbial consortia from Qinghai Lake sediments for the simultaneous removal of E2 and TC. A novel bacterial strain, *Alkalibacterium* sp. AEPI-S25, was successfully isolated and its simultaneous pollutant removal capacity was checked under various environmental stress conditions, including exposure to two distinct types of livestock wastewater samples. HPLC-Orbitrap-HRMS and

whole-genome analysis were then employed to identify potential pathways and mechanisms underlying E2 and TC removal by the strain, and the discovered potential key genes for the two pollutants' removal were further validated using transcription analysis and heterologous expression.

## Materials and methods

### Chemicals and culture media

The 17β-estradiol (E2, purity > 98%) and tetracycline hydrochloride (TC, purity > 97%) used in this study were purchased from Macklin (Shanghai, China) and prepared as 10 mg $mL^{-1}$ stock solutions in DMSO and sterile water, respectively. HPLC-grade acetonitrile and ethyl acetate were obtained from Fisher Scientific (Pittsburgh, USA). The enrichment medium (EM, 1 g $L^{-1}$ tryptone, 0.5 g $L^{-1}$ yeast extract, 20 g $L^{-1}$ NaCl, pH = 9.0) was prepared referencing the natural conditions of the Qinghai Lake. Mineral salt medium (MSM, 3.815 g $L^{-1}$ $K_2HPO_4$, 0.5 g $L^{-1}$ $KH_2PO_4$, 0.825 g $L^{-1}$ $(NH_4)_2HPO_4$, 1.5 g $L^{-1}$ $KNO_3$, 0.2 g $L^{-1}$ $Na_2SO_4$, 0.02 g $L^{-1}$ $CaCl_2$, 0.002 g $L^{-1}$ $FeCl_3$, 0.02 g $L^{-1}$ $MgCl_2$, 20 g $L^{-1}$ NaCl, pH = 9.0) and Biodegradation medium (BM, 10 g $L^{-1}$ tryptone, 0.5 g $L^{-1}$ yeast extract, 20 g $L^{-1}$ NaCl, pH = 9.0) were used to assess the removal efficiency of the isolated strains. *Escherichia coli* BL21 (DE3) was employed for heterologous gene expression to functionally validate identified genes. Luria–Bertani medium (LB, 10 g $L^{-1}$ tryptone, 5 g $L^{-1}$ yeast extract, 10 g $L^{-1}$ NaCl, pH = 7.0) was used for the cultivation of *E. coli* BL21 (DE3).

### Strain enrichment and isolation

For strain enrichment, 5 g of surface sediment from Qinghai Lake was inoculated into a 250 mL flask containing 100 mL of EM medium supplemented with 5 mg $L^{-1}$ E2 and 5 mg $L^{-1}$ TC. Referring to previous studies (Leng et al. 2016; Alahadeb 2022; Xiong et al. 2020, 2023; Ye et al. 2023), the endpoint of enrichment culture was selected as 20 mg $L^{-1}$ for both E2 and TC in order to isolate strains with potential strong degradation ability. The mixed cultures were incubated at 30 ℃ in the dark with continuous shaking at 180 rpm. After 7 days of incubation, 20 mL of the culture was transferred into a fresh EM medium containing 10 mg $L^{-1}$ E2 and TC. The enrichment procedure was repeated three times, with E2 and TC concentrations increasing by 5 mg $L^{-1}$ each time until they reached a final concentration of 20 mg $L^{-1}$ for both compounds. After enrichment, the bacterial solution was serially diluted and spread onto EM agar plates containing 20 mg $L^{-1}$ E2 and TC. Single colonies were chosen based on their morphological characteristics on EM agar plates.

### Analysis of E2 and TC removal properties

The biodegradation potentials of the isolated strains were examined after a 5-day culture in BM or MSM medium supplemented with 20 mg $L^{-1}$ E2 and/or TC. The concentrations of E2 and TC residues were quantified using ultraperformance liquid chromatography (UPLC) equipped with an ACQUITY UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm, Waters). For E2 analysis, the supernatant was centrifuged (5000 rpm, 5 min, 4℃), filtered through a 0.22-µm filter membrane, and extracted with ethyl acetate. Then, the extraction was concentrated by solvent evaporation and re-dissolved in acetonitrile. The column temperature was maintained at 30 ℃. The mobile phase for E2 detection consisted of an equal-volume mixture of acetonitrile and 0.1% phosphoric acid solution, operated at a flow rate of 0.2 mL $min^{-1}$. The UV detection wavelength was 200 nm. For TC analysis, the culture supernatant was centrifuged (5000 rpm, 5 min, 4℃) and filtered through a 0.22-µm filter membrane. The column temperature for TC detection was set to 30 ℃, with a UV wavelength of 350 nm. The mobile phase for TC detection consisted of acetonitrile and 0.1% phosphoric acid solution (30/70, v/v) with a flow rate of 0.3 mL $min^{-1}$. The removal efficiencies of E2 and TC were calculated based on concentration reductions quantified using calibration curves. Among the tested strains, AEPI-S25 demonstrated superior simultaneous removal capabilities for both E2 and TC, and was subsequently selected for further application and mechanistic analysis.

### The molecular identification of strain AEPI-S25

A single colony of strain AEPI-S25 was inoculated into EM medium and cultured at 30 ℃ for 2 days. The total genomic DNA of the strain was extracted using the E.Z.N.A.® Bacterial DNA Kit (Omega, USA) following the manufacturer's protocol. The 16S rRNA gene of the strain was amplified by polymerase chain reaction (PCR) using universal primers 27 F and 1492R (Table S1) in an S1000™ thermal cycler (Bio-Rad, USA). Approximately 5 ng of genomic DNA was used as a template in a 25 µL reaction volume containing 12.5 µL of Taq PCR mix (Takara Bio, Japan) and 10 µM of each primer. The PCR program included 95 °C for 5 min, 35 cycles of 30 s at 94 °C, 60 s at 52 °C, and 90 s at 72 °C, with a final extension of 72 °C for 10 min. The resulting PCR fragment was checked by agarose gel electrophoresis and sequenced by Beijing Genomics Institute (BGI), China. The obtained 16S rRNA gene sequence of the strain was submitted to GenBank under the accession number PP692191. Phylogenies analysis was conducted using MEGA 11 software, and a Maximum-likelihood phylogenetic tree was built with 1000 bootstrap replicates.

## Evaluation of removal efficiency under different cultural conditions

To investigate the application potential of the strain AEPI-S25, its removal efficiency under various environmental stress conditions (pH, salinity, $Cd^{2+}$, and $Cu^{2+}$) was examined at a constant E2 and TC concentration of 20 mg $L^{-1}$. The strain was grown to the mid-log phase in BM medium, harvested by centrifugation (5000 rpm, 5 min, 4℃), washed twice with sterilized 2% NaCl solution, and then inoculated into BM medium containing gradient concentrations of NaCl (1, 10, 20, 40, 80, 160 g $L^{-1}$), $Cd^{2+}$ (1, 10 mg $L^{-1}$), $Cu^{2+}$ (1, 10 mg $L^{-1}$) or designated pH values (6, 7, 8, 9,10). The inoculum of the strain with an initial $OD_{600}$ (optical density at 600 nm) at 0.03, and all treatments were conducted in triplicates for 5 days (30 ℃, 180 rpm) in the dark. The mixtures were collected on days 0, 1, 2, 3, and 5 for E2 and TC concentration analysis. Comparisons of the removal efficiency between different treatments were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's tests at a significance level of $p < 0.05$.

Remediation experiments were conducted using two typical livestock wastewater matrices collected from a swine farm in JiLin Province and a cattle farm in QingHai Province, China. The physical and chemical properties of the wastewater samples are listed in Table S2. Suspended solids in the wastewater were removed through a 1 mm sieve. Partial wastewater samples were sterilized by the autoclave at 121 °C to remove indigenous microorganisms. Then, 20 mg $L^{-1}$ E2 and TC were added to the wastewater samples. The methods for strain preparation, inoculation, and incubation were identical to those used in the preceding experiment. E2 and TC residues in the mixtures were also monitored on days 0, 1, 2, 3, and 5.

## Identification of E2 and TC transformation/degradation products

During the removal process of E2 and TC by the strain AEPI-S25, the extracted transformation/degradation products of the pollutants were purified by the PEP-2 cartridge (500 mg/6 mL). The cartridge was preconditioned with 10 mL methanol and 10 mL ultrapure water, respectively. Then, the sample was loaded on the cartridge at a rate of 1 mL $min^{-1}$. The cartridge was then vacuum-dried for 20 min after being rinsed with 10 mL ultrapure water. The elution on the cartridge was performed with organic solvent (4.5 mL ethyl acetate and 8 mL methanol for the purification of E2 and TC transformation products, respectively) at a rate of 0.7 mL $min^{-1}$. Finally, the analytes were concentrated to near dryness under a gentle flow of nitrogen gas at 25 °C, then 1:1 (v/v) acetonitrile-deionized water was used for dissolving the final extracts to 1 mL and vortexed for 30 s (2500 r·$min^{-1}$). The final purified extracts were filtered through nylon filters (0.22 μm) and analyzed using an Orbitrap Fusion HRMS (UPLC-Orbitrap-HRMS) (Ultimate 3000/Orbitrap Fusion, Thermo Fisher, Waltham, MA) equipped with an ACQUITY UPLC BEH C18 column (50 mm × 2.1 mm, 1.7 μm, Waters). The column temperature was set to 40 °C, and the injection volume was 10 μL. The mobile phases were UPLC water with 0.1% (v/v) formic acid (A) and acetonitrile (B) operated with a gradient as follows: 5% phase B was held for 0.5 min, increased to 90% at 4 min and kept for 2 min, returned to 5% in 0.1 min, and kept until 9 min for an equilibrium. The flow rate was set to 0.4 mL $min^{-1}$. The mass spectrometer for E2 and TC metabolites analysis was operated in the negative and positive electrospray ionization modes, respectively, with a m/z scan range of 70–700.

## Whole genome sequencing and annotation

The genomic DNA of the strain AEPI-S25 was extracted using the E.Z.N.A.® Bacterial DNA Kit (Omega, USA), quantified with Nanodrop, and sequenced using both PacBio and Illumina Novaseq platforms at the Meiji Biopharmaceutical Technology, China. For Illumina NovaSeq sequencing data, paired-end reads were assembled by SOAPdenovo2 (http://soap.genomics.org.cn/) to construct scaffolds and contigs. Pacbio sequencing data were assembled with Unicycler software, then integrated with NovaSeq results using Pilon (version 1.22) to generate a complete genome. The complete genome sequence of AEPI-S25 has been deposited in the NCBI GenBank under the accession number PRJNA1109601. Coding sequences (CDS) within the genome were predicted using Glimmer (version 3.02), Prodigal (version 2.6.3), and GeneMarkS (version 4.3). Functional annotation of the predicted genes was performed by aligning the genome sequences against six leading databases: NR, Swiss-Prot, Pfam, COG, GO, and KEGG.

## Transcription analysis of potential E2 and TC removal genes

Reverse transcription quantitative PCR (RT-qPCR) was employed to quantify the transcriptional levels of candidate genes involved in E2 and TC removal. Strain AEPI-S25 was cultured in BM medium with 20 mg $L^{-1}$ E2 or TC for 36 h. Then, the total RNA of the strains was extracted and reverse-transcribed into cDNA using the PrimeScript RT Reagent Kit (TaKaRa, Japan). Three independent experiments were conducted for each treatment and a blank control was established with AEPI-S25 cultured without E2/TC added. The quantitative PCR was performed using ChamQ SYBR qPCR Master Mix (Vazyme, China) on a QuantStudio™ 3 Real-Time PCR system (Thermo Fisher, USA). The 16S rRNA gene was used as an internal control, and relative

gene transcription levels were calculated using the 2-ΔΔCt algorithm. The primers used for target gene amplification were listed in Table S1.

### Heterologous expression of potential E2 and TC removal genes

The candidate genes associated with E2 (*S25_gene0393*) and TC (*S25_gene0878*) removal in strain AEPI-S25 were amplified from genomic DNA using gene-specific primers, as detailed in Table S1. The PCR products were cloned into the pET-28a expression vector (ZOMANBIO, China) and transformed into *E. coli* BL21 (DE3) by heat shock, generating recombinant strains pET-gene0393 and pET-gene0878. The transformants were cultured in LB medium containing 50 mg $L^{-1}$ kanamycin at 37 °C. When the OD600 reached approximately 0.6, 0.5 mM isopropyl β-D-1-thiogalactopyranoside was added, followed by overnight incubation at 16 °C. The expression of the target proteins was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and further validated by Western blotting.

To evaluate the functional activity of the expressed proteins, pET-gene0393 and pET-gene0878 cultures were supplemented with 20 mg $L^{-1}$ E2 or TC and incubated at 37 °C on a shaking platform (180 rpm) for 4 h or 24 h, respectively. Meanwhile, *E. coli* BL21(DE3) cells carrying an empty pET-28a vector served as the control group. The pH of the culture was adjusted to 9, consistent with previously optimized conditions. Each experimental group was conducted in triplicate to ensure reproducibility. Residual concentrations of E2 and TC were quantified using UPLC, and Orbitrap Fusion HRMS was used to identify and confirm the corresponding biotransformation products catalysed by the functional genes.

## Results

### Isolation and identification of simultaneous removal strains

A total of five strains were obtained through the isolation process, while only one of them (AEPI-S25) exhibited effective removal capabilities for both E2 and TC. Under scanning electron microscopy (SEM), the cells of the strain were observed as rod-shaped, without spores or flagellum formation. The dimensions of the cells were typically 1—2 μm in length and 0.4—0.6 μm in width (Fig. 1c). Additional biochemical tests demonstrated that strain AEPI-S25 is Gram-negative, contains oxidase activity, but does not produce catalase. The phylogenetic analysis of AEPI-S25 was conducted based on the 16S rRNA gene sequencing. As shown in Fig. 1d, the 16S rRNA gene sequence of AEPI-S25 indicated a close phylogenetic relationship with the genus *Alkalibacterium*. Based on these characteristics, the strain was named as *Alkalibacterium* sp. AEPI-S25 and deposited into the China General Microbiological Culture Collection Center (CGMCC) under accession number CGMCC30529.

Under single-pollutant conditions, AEPI-S25 was capable of removing more than 94.77% of 20 mg $L^{-1}$ E2 and approximately 100% of 20 mg $L^{-1}$ TC in the BM medium within 3 days (Fig. 1a, b). The degradation kinetics of E2 and TC by the strain were both fitted to the pseudo-first-order dynamics model, with determination coefficient ($R^2$) values of 0.999 and 0.979, respectively. In the presence of AEPI-S25, the degradation rate constant ($k_d$) of E2 and TC were 2.201 and 0.586 $d^{-1}$, respectively. Comparatively, when E2 and TC were added simultaneously to the BM medium, the strain's efficiency of E2 removal decreased significantly (the $k_d$ of E2 decreased to 0.614 $d^{-1}$), while TC removal remained largely unaffected (the $k_d$ of TC remained at 0.575 $d^{-1}$). After 5 days of cultivation, 89.91% of 20 mg $L^{-1}$ E2 and nearly 100% of 20 mg $L^{-1}$ TC were simultaneously removed in the inoculated cultures. In contrast, the E2 and TC removal efficiency observed in the control group (without strain inoculation) was only about 1.5% and 40%, respectively (Fig. 1a, b). These results clearly demonstrated that AEPI-S25 possesses exceptional capability for the simultaneous removal of E2 and TC. Interestingly, AEPI-S25 also exhibited outstanding E2 removal capacity in the MSM medium (the $k_d$ of E2 was 1.684 $d^{-1}$). However, the strain's TC removal efficiency was very limited in the MSM medium, suggesting that it may remove TC through co-metabolism (Fig. S1).

### Removal characteristics of strain AEPI-S25 under different conditions

To investigate the environmental suitability of AEPI-S25, we examined its E2-TC simultaneous removal efficiency under various conditions. AEPI-S25 presented broad adaptability to changes in salinity (Fig. 2a, e), maintaining stable removal efficiency for both E2 and TC even at an extremely high salinity of 80 g $L^{-1}$. In contrast, changes in pH significantly ($p < 0.05$) impacted the removal efficiency of AEPI-S25 for E2 and TC. The simultaneous removal efficiency of the strain performed well at pH 9 and 10, but its removal efficiency was significantly inhibited at pH levels below 8 (Fig. 2b, f). The addition of $Cu^{2+}$ and a low concentration of $Cd^{2+}$ (1 mg $L^{-1}$) only initially reduced the removal efficiency of both E2 and TC (Fig. 2c, g). However, the addition of a higher concentration of $Cd^{2+}$ (10 mg $L^{-1}$) largely reduced E2 and TC removal efficiencies to 26.10% and 54.98%, respectively (Fig. 2c, g). Furthermore, AEPI-S25 demonstrated exceptional simultaneous removal efficiency in both swine and cattle wastewater samples, which

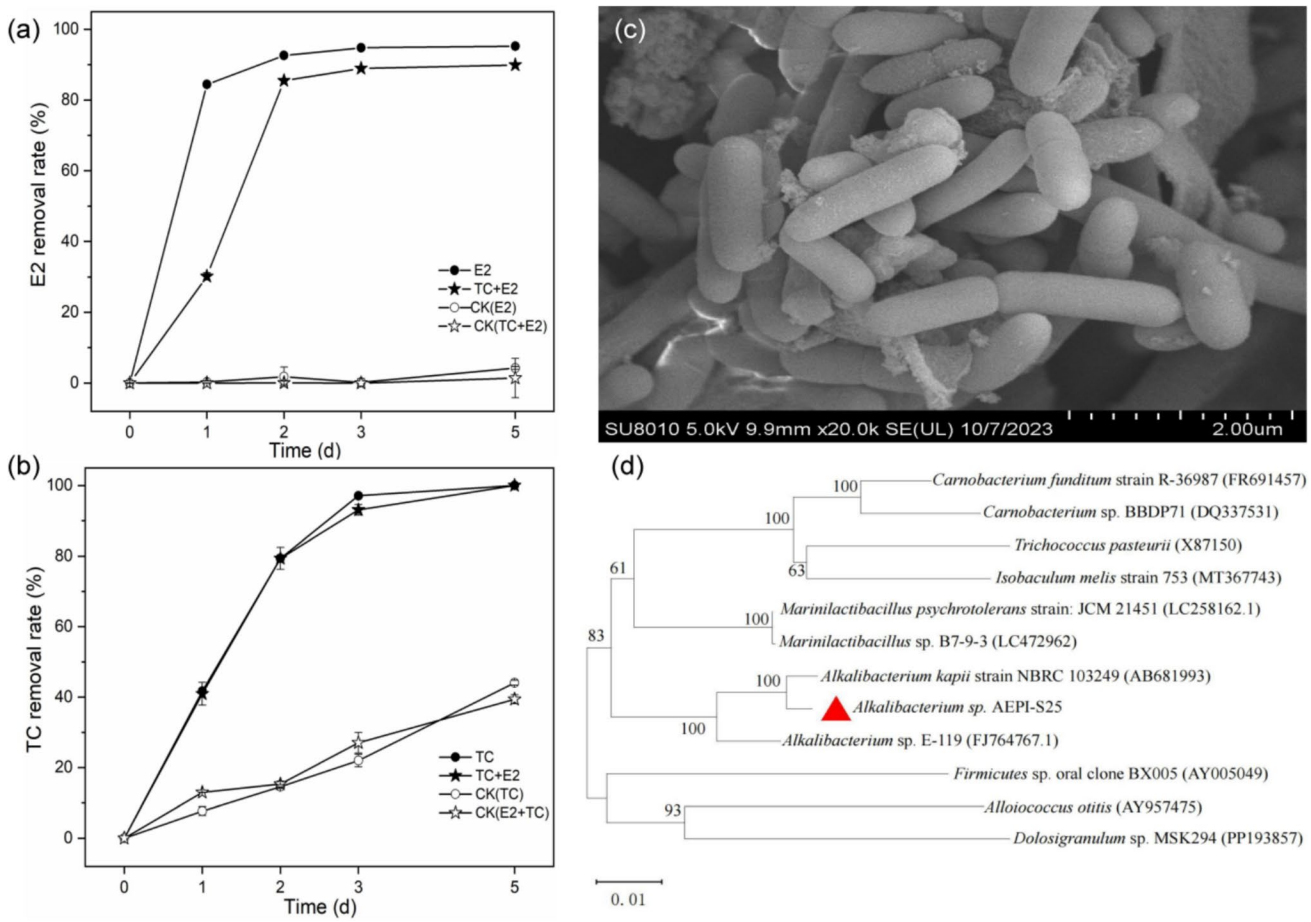


**Fig. 1** Removal curves (a, b), Morphological characters (c) and Maximum-likelihood phylogenetic tree (d) of the isolated strain AEPI-S25. CK in (a) and (b) represents the control treatments without AEPI-S25 inoculum. Maximum-likelihood phylogenetic tree in (d) was built with 1000 bootstrap replicates. Error bars represent standard deviation from triplicate experiments

were characterized by high salinity and nutrient concentrations (Table S2). With strain inoculation, the 5-day removal efficiencies of the added E2 and TC in sterilized wastewater samples reached 60.33%—69.15% and 51.34%—66.91%, respectively. In unsterilized livestock wastewater samples from Qinghai Province (QH), the 5-day removal efficiencies of E2 and TC by the strain were 51.92% and 42.31%, respectively (Fig. S2). Although these rates were lower than those observed in sterilized samples, they were still significantly higher than those of the control group without inoculation of the activated strain (0.32%—2.11% and 25.41%—32.72% for E2 and TC removal) (Fig. 2d, h; Fig. S2), highlighting the strain's potential for practical applications in livestock wastewater treatment.

## Detection of transformation/degradation products and pathways

The potential transformation/degradation products and pathways of E2 and TC removal were listed in Fig. 3. As shown in Fig. S3a, TP269 ($m/z$ = 269.15, $[M\text{-}H]^-$) was significantly accumulated with the E2 removal by the strain. Based on the standard substance (purity > 98%, purchased from Macklin) test, TP269 was confirmed as estrone (E1), which was most likely derived from the dehydrogenation of E2 (Fig. 3a, Fig. S3). TP285 ($m/z$ = 285.15, $[M\text{-}H]^-$) was identified as another potential E2 biotransformation product during E2 removal process. It was proposed to be 4-hydroxyestrone, resulting from hydroxylation at the C-4 position of the produced E1 (Fig. 3a) (Chen et al. 2017).

Four major probable TC transformation products (TP427, TP431, TP461, and TP413) were identified throughout the pollutant removal period by the strain AEPI-S25 (Fig. 3, Fig. S4). TP427 (m/z = 427.15, $[M + H]^+$) exhibited a decrease of 18 Da compared to TC (m/z = 445.15, $[M + H]^+$), suggesting it was a dehydration product of TC (Fig. 3b). TP431 (m/z = 431.15, $[M + H]^+$) was likely derived from TC demethylation and could subsequently undergo dehydration to form TP413 (m/z = 413.13, $[M + H]^+$) (Fig. 3b). TP461 (m/z = 461.15, $[M + H]^+$) was likely generated through monooxygenation of TC and may further

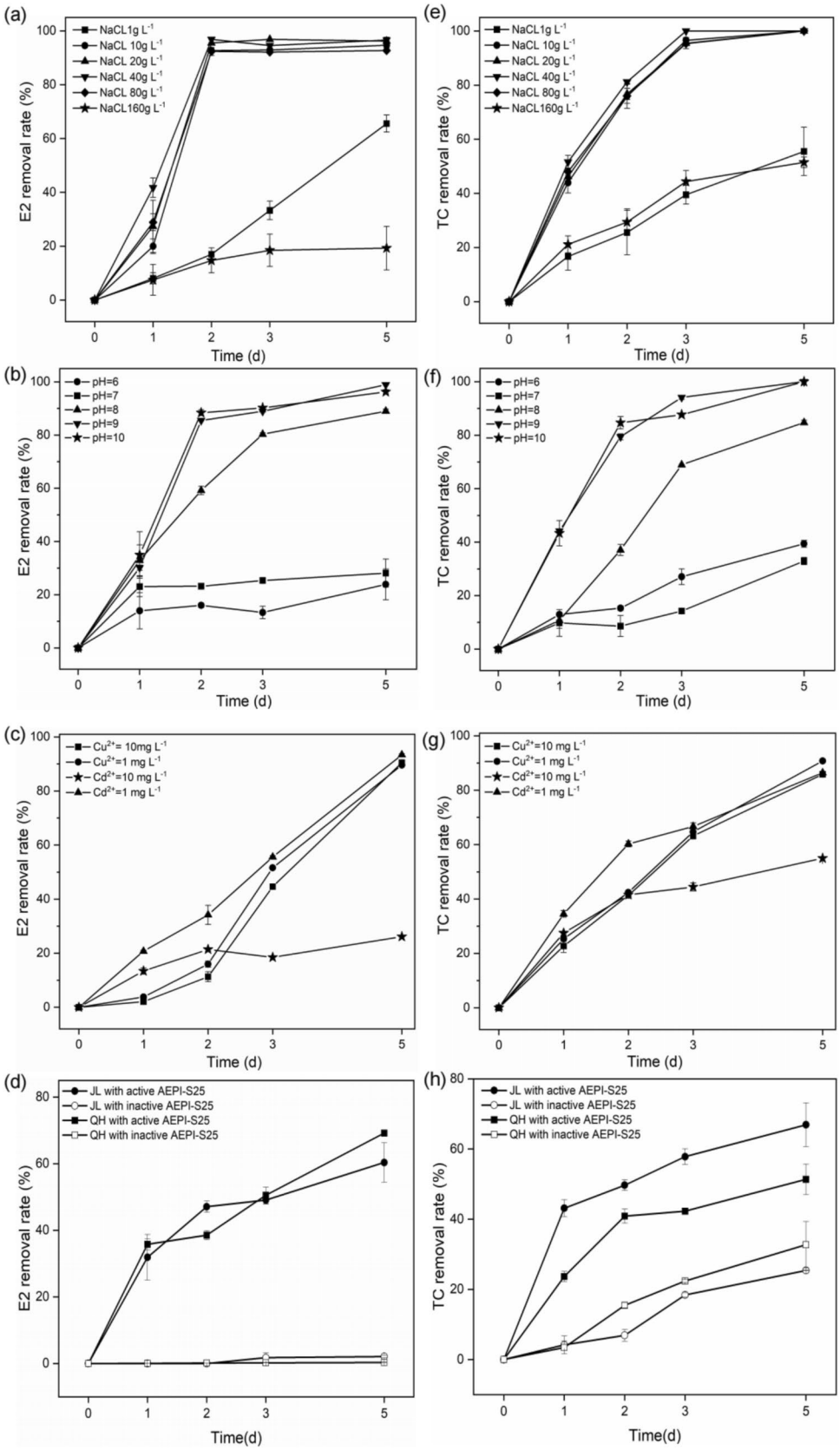


**Fig. 2** The impact of pH (a, e), NaCl (b, f), heavy metal (c, g), and livestock wastewater matrices (d, h) on the E2 and TC removal efficiency of AEPI-S25. JL and QH in (d) and (h) refer to sterilized wastewater samples from a swine farm in JiLin (JL) and a cattle farm in QingHai (QH) province, China. Error bars represent standard deviation from triplicate experiments

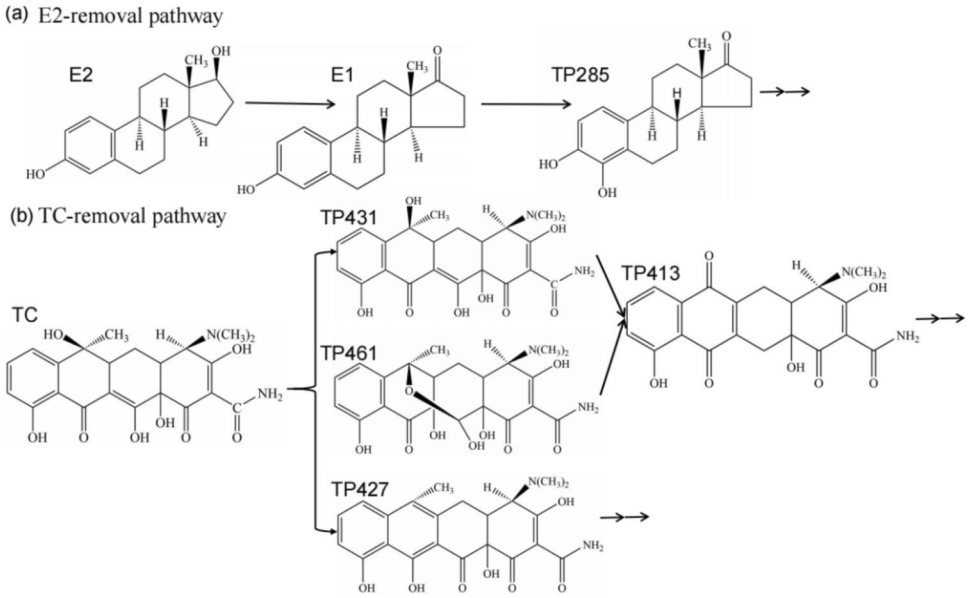


**Fig. 3** The potential transformation/degradation products and pathways of E2(a) and TC(b) removal

transform into TP413 via multiple further reactions. Notably, TP461 and TP413 corresponded to previously reported TC biotransformation products in *TetX* gene-dependent pathways (Ghosh et al. 2015; Chen et al. 2023), implying that AEPI-S25 may remove TC through a similar mechanism. On the other hand, AEPI-S25 may also promote the removal of antibiotics by secreting hydrolytic enzymes. For example, Ye et al. investigated changes in gene levels of *Serratia marcescens* sp. AEPI 0–0 during the TC removal process by transcriptomic studies and found that genes related to hydrolases were significantly upregulated (Ye et al. 2023). Moreover, all of the potential transformation products observed in the AEPI-S25 treatment were also found in the abiotic treatment (Fig. S4b), which may be related to the complex natural hydrolysis of TC in aqueous solution and has also been described in some previous studies (Leng et al. 2016; Zhong et al. 2022; Chen et al. 2023).

## Identification and characterization of the genome of AEPI-S25

The whole genome of the strain was sequenced to reveal the genetic basis for its E2-TC simultaneous removal ability and environmental adaptability. The AEPI-S25 complete genome contained a single chromosome of 2,657,884 bp with a GC content of 43.58%, without the presence of any plasmids. A total of 2,492 CDS (coding sequences) were predicted and presented on the genomic circle plot (Fig. S5).

Based on the annotation of the AEPIS-25 genome, 51 genes were identified potentially referring to the tolerance of environmental stresses (Table S3). Among them, 2 sodium-dependent transporter encoding genes, 1 proton antiporter encoding gene, and 3 $Na^+/H^+$ antiporter encoding genes were probably involved in resistance to saline and alkaline stresses. Additionally, 25 genes related to oxidative stress response were identified, including those encoding catalase, superoxide dismutase, and thioredoxins (Table S3). Furthermore, there were 20 genes encoding proteins responsible for heavy metal resistance, such as the arsenite efflux pump, copper chaperone, manganese transport protein, cadmium resistance transporter, $Zn^{2+}$-exporting ATPase, and chromate transporter (Table S3).

A total of 14 potential E2/TC removal genes were identified in the genome (Table S4). These genes contained short-chain dehydrogenase/reductases (SDRs), 3-hydroxyacyl-CoA dehydrogenase, short-chain alcohol dehydrogenase, monooxygenase, and dioxygenase encoding genes, which were likely involved in dehydrogenation, hydroxylation, and ring-opening reactions of the pollutants. Due to previous results revealing that E2 was mainly dehydrogenated to E1 by the strain, we hypothesize a potential 17β-hydroxysteroid dehydrogenase (*17β-HSD*) encoding gene (*S25_gene0393*) may play a dominant role in E2 removal. Further sequence alignment revealed that the amino acid sequences of *S25_gene0393* were closely related to some previously reported *17β-HSD* genes (Fig. 4a), and shared similar conserved substrate catalytic motifs ('Y-X-X-X-K') and SDR characteristic sequences ('G-X-X-X-G-X-G') with these E2 removal genes (Fig. 4a) (Ye et al. 2017).

For the TC removal, *TetX* genes were surprisingly absent in the AEPI-S25 genome. Interestingly, a potential flavin adenine dinucleotide (FAD)-dependent monooxygenase gene (*S25_gene0878*) was found to be similar to a *TetX* homologous gene (*MabTetX*) (Fig. 5), implying its potential role in TC removal. However, sequence analysis revealed that *S25_gene0878* shared only 28.6% similar amino acid

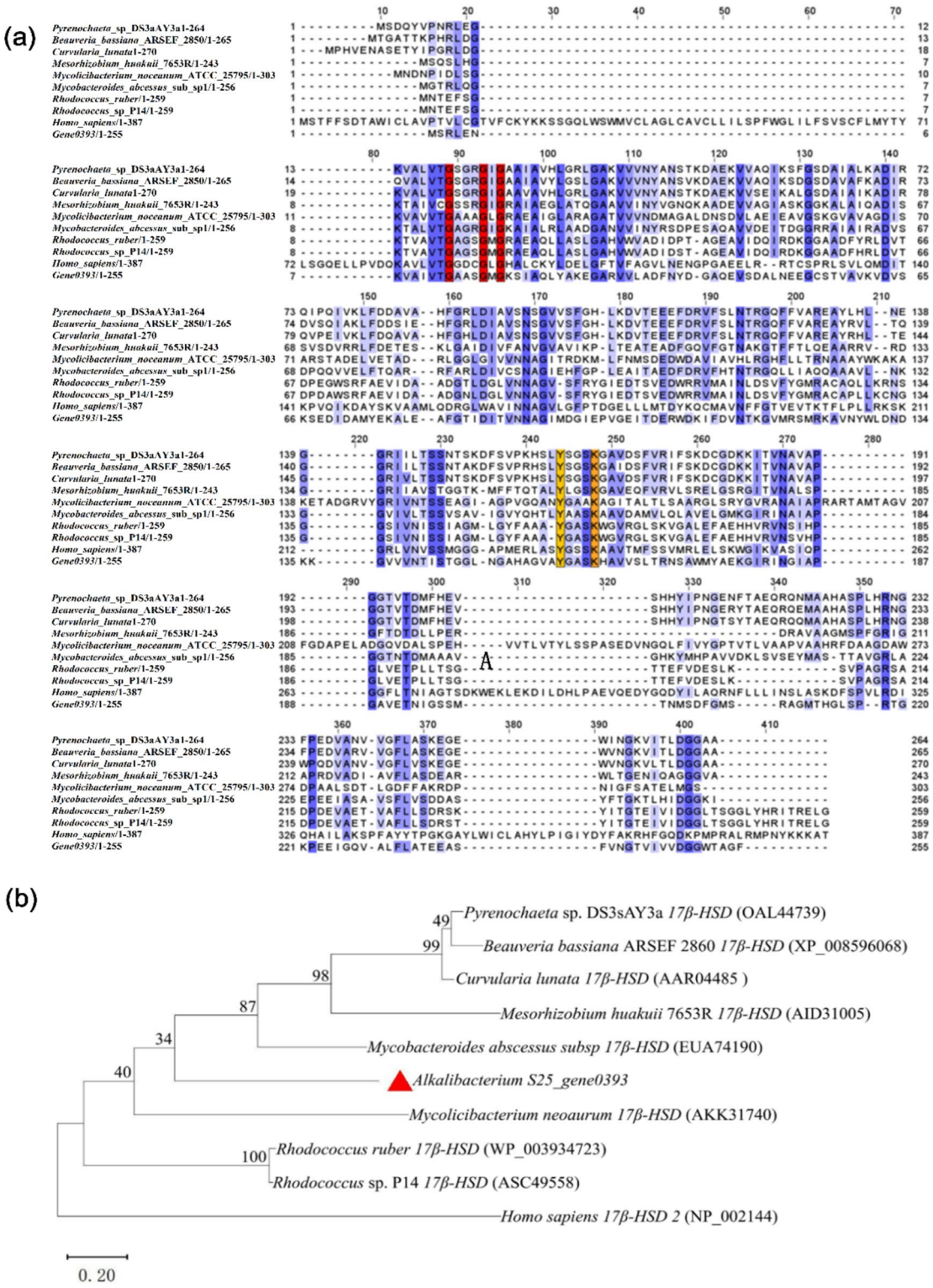


**Fig. 4** Multiple sequence alignment (a) and Maximum-likelihood tree (b) of *S25_gene0393* and typical *17β-HSD* genes. The Maximum-likelihood phylogenetic tree was built with 1000 bootstrap replicates. The conserved substrate catalytic motifs of *17β-HSD* and the characteristic sequences of short-chain dehydrogenase/reductase (SDR) were labelled in yellow and red, respectively

sequence with *MabTetX* (Fig. 5a), implying it may be a novel TC-removal gene. The similarity proportions between *S25_gene0878* and *TetX* genes were even lower (typically 10%—20%), and the *TetX* conserved active sites (e.g., Q221, R242, H263, G265) were also missing in *S25_gene0878* (Volkers et al. 2011; Blake et al. 2024).

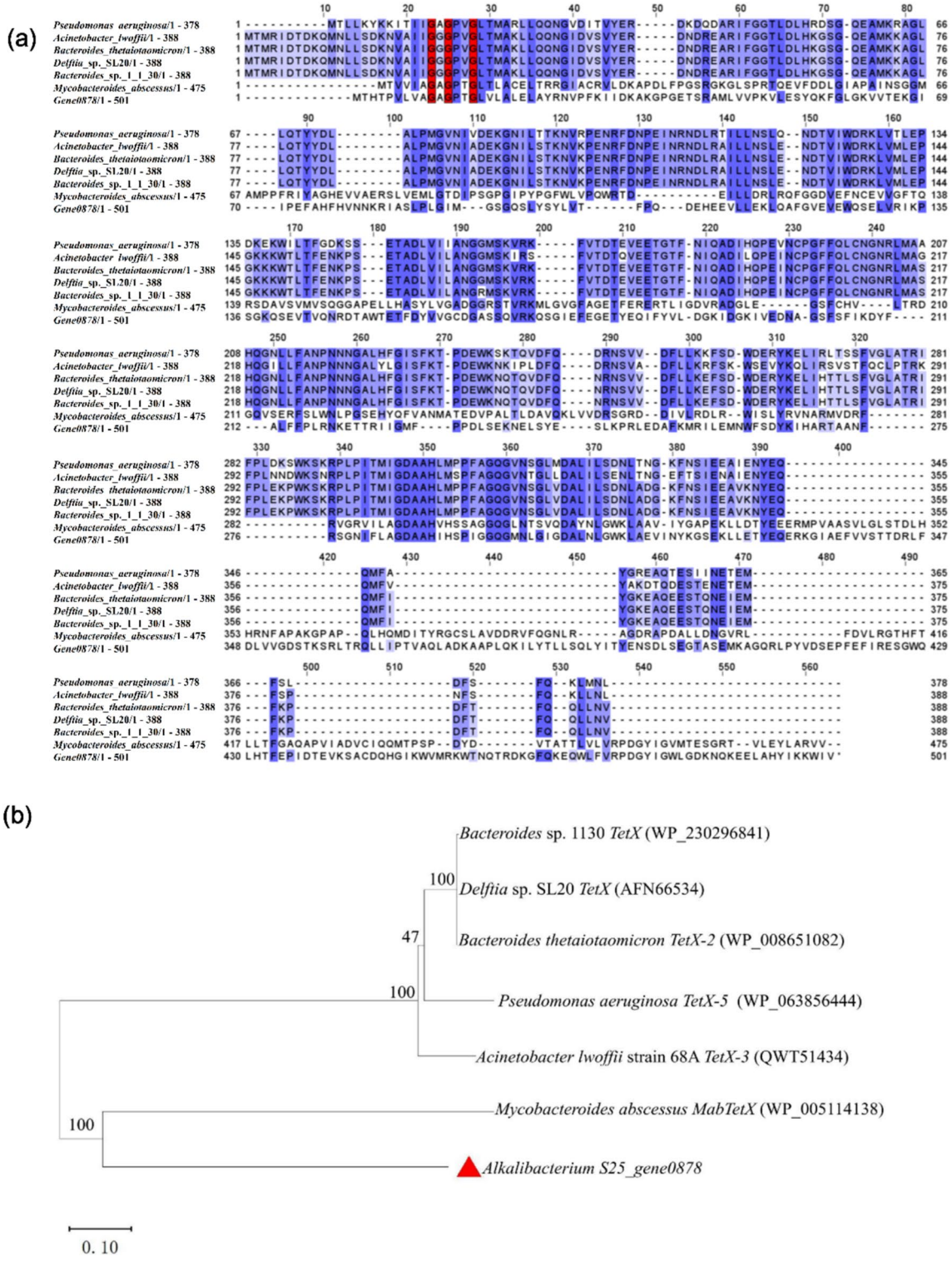


**Fig. 5** Multiple sequence alignment (a) and Maximum-likelihood tree (b) of *S25_gene0878* and typical *TetX* genes. The Maximum-likelihood phylogenetic tree was built with 1000 bootstrap replicates. Flavin adenine dinucleotide (FAD) binding consensus sequences were marked in red

## Verification of potential E2 and TC removal genes within the strain

RT-qPCR was performed to test the expression of potential E2 (*S25_gene0393*) and TC (*S25_gene0878*) removal genes during their removal progress. Following a 36-h incubation period, the expression of *S25_gene0393* in the E2-added treatment was over 20 times higher than that in the TC-added treatment or the blank control, indicating its specific induction by E2 (Fig. 6a). Similarly, *S25_gene0878* expression increased significantly only under the TC-added treatment, showing approximately a 12-fold upregulation compared to other groups (Fig. 6c).

To further verify their roles in pollutant removal, *S25_gene0393* and *S25_gene0878* were successfully ligated to an expression vector and transferred into *E. coli* BL21 (DE3). The purified proteins of *S25_gene0393* and *S25_gene0878* appeared as single bands on SDS-PAGE, and their molecular weights (approximately 35 kDa and 63 kDa, respectively) determined by SDS-PAGE/Western blot were consistent with their theoretical calculated values (Fig. S6). As we expected, the recombinant strain pET-gene0393 rapidly transformed E2 to E1, achieving about 80% E2 removal within 1 h (Fig. 6b & Table S5). Similarly, pET-gene0878 significantly enhanced TC removal efficiencies by 3.4-fold within 24 h, and the speculated biotransformation product TP461 was found to accumulate significantly along with TC removal (Fig. 6d & Table S6).

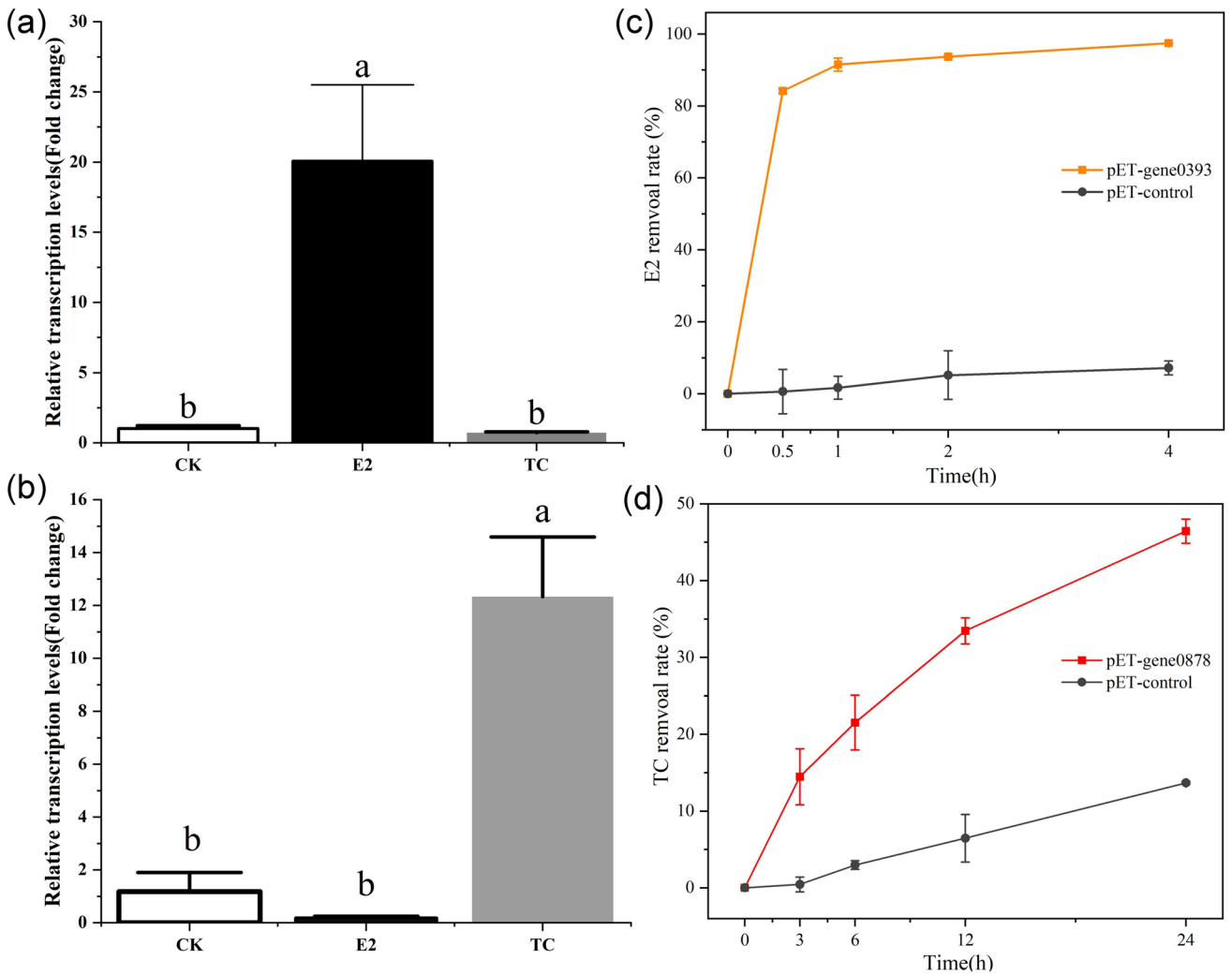


**Fig. 6** Transcription analysis and heterologous expression of *S25_gene0393* (a, c) and *S25_gene0878* (b, d). The transcription analysis contained three conditions: E2-added (E2), TC-added (TC), and blank control (CK). Statistical analysis of transcription analysis was performed using one-way ANOVA. The pET-control in heterologous expression means the transfer of an empty plasmid. Error bars represent standard deviation from triplicate experiments

## Discussion

### Application potential and advantages of AEPI-S25 used in wastewater treatment

Previous studies have indicated the significant potential of microbial remediation in reducing the risks of 17β-estradiol (E2) and tetracycline (TC) in wastewater treatment (Almazrouei et al. 2023; Shao and Wu 2020). For example, *Stenotrophomonas maltophilia* sp. SJTL3 and *Stenotrophomonas maltophilia* sp.DT1 were reported to completely degrade 10 mg $L^{-1}$ E2 and 50 mg $L^{-1}$ TC within 10 and 7 days, respectively (Leng et al. 2016; Xiong et al. 2020). *Sphingobacterium* sp. GEMB-CSS-01 and *Sphingobacterium* sp. S121 achieved 80.0% removal of E2 (10 mg $L^{-1}$) and complete removal of TC (20 mg $L^{-1}$) within 10 and 5 days, respectively (Cao et al. 2024; Tan et al. 2022). Nevertheless, most of these studies primarily focused on the removal of individual pollutants, overlooking the potential of these strains for simultaneous E2 and TC removal. To the best of our knowledge, only one strain *Novosphingobium* sp. ES2-1 has been reported to simultaneously remove 85% E2 (20 mg $L^{-1}$) and 50% TC (0.5 mg $L^{-1}$) within 7 days (Li et al. 2020a). But the removal curves of TC within *Novosphingobium* sp. ES2-1 showed no significant difference from that of the blank control, indicating TC decrease was unrelated to the strain (Li et al. 2020a). In this study, *Alkalibacterium* sp. AEPI-S25 demonstrated the ability to remove 20 mg $L^{-1}$ TC and E2 within 3–5 days, providing the first solid evidence for the simultaneous removal of E2 and TC by a single strain.

Based on the analysis of removal pathways and mechanisms, AEPI-S25 was discovered to transform E2 and TC through initial dehydrogenation and monooxygenation. Notably, although the dehydrogenation of E2 has been reported to reduce its estrogenic activity by 70–90%, the resulting E1 still poses potential environmental risks (Van den Belt et al. 2004; Chen et al. 2017). To better evaluate the reduction in endocrine-disrupting effects by AEPI-S25, we quantified estrogenic compounds (E2 and E1) in the EM medium (initially containing 20 mg $L^{-1}$ E2) after 5 days of incubation. The extraction and UPLC analysis methods for E1 were the same as those used for E2 (described in the "Analysis of E2 and TC removal properties" section), with differentiation based on retention time. The median values of the estimated 17β-estradiol equivalencies ($\Sigma EEQm = 0.2 \times C_{E1} + C_{E2}$) were then calculated to reflect the endocrine-disrupting effects in different treatments (Yu et al. 2020). The results indicated that the ΣEEQm in the medium decreased to 4.08 mg $L^{-1}$ after 5-day culture with the strain, which was significantly lower than in the abiotic control (ΣEEQm = 19.54 mg $L^{-1}$) (Fig. S7). However, the remaining E1 still contributed a considerable ΣEEQm (3.31 mg $L^{-1}$) in the medium, suggesting that AEPI-S25 mainly removed E2 through transformation rather than complete mineralization, and further elimination of residues E1 is necessary in the future. The monooxygenation of TC has been shown to inactivate its antibiotic properties, and the removal of TC was expected to decrease the relative abundance of antibiotic-resistant bacteria in the environment (Ghosh et al. 2015; Wen et al. 2020). Using *Escherichia coli* ATCC25922 as an indicator, we further evaluated the impact of the strain AEPI-S25 on the antibacterial activities of the medium (initially containing 20 mg $L^{-1}$ TC) after 5 days of cultivation. *E.coli* cells were first cultured in LB liquid medium to an $OD_{600}$ of 1.0 and then spread onto LB agar plates. Oxford cups were then placed on the plates and filled with 200 µL of the test medium solutions (pre-filtered through a 0.22-µm sterile membrane). The plates were incubated at 30 °C for 16 h, after which the diameters of the inhibition zones (including the 7.8 mm diameter of the cup) were measured. As expected, the diameter of the inhibition zones decreased from 22.6 mm (on Day 0) to 10.4 mm (on Day 5) following inoculation with AEPI-S25, indicating that the TC bioremediation using the strain was desirable (Fig. S8). Comparatively, the inhibition zone's diameters in the abiotic control also declined to 18.1 mm, likely related to the previously observed reduction in TC concentration (Fig. 1; Fig. 2). Indeed, in aqueous solution, a 20%−50% loss of TC in the abiotic treatment usually occurs within 5–7 days, which was commonly attributed to photodegradation, hydrolytic transformation, and adsorption (Li et al. 2020a; Tan et al. 2022; Wang et al. 2024a, b). Considering that our experiments were conducted in the dark, hydrolytic transformation and adsorption likely dominated the TC loss in the abiotic treatment observed in this study. Especially, as shown in Fig. S4, the significant accumulation of representative hydrolytic products (e.g. TP427) (Leng et al. 2016; Zhong et al. 2022; Chen et al. 2023) provides additional evidence that hydrolysis is an important pathway for TC removal under abiotic conditions. Notably, previous researches have shown that the hydrolytic transformation products of TC exhibit reduced toxicity compared to the parent TC, which also aligns with our findings (Chen et al. 2022a; Wang et al. 2024a, b). However, it is worth noting that after 5 days of abiotic removal, the solution still exhibited considerable antibacterial activities (Fig. S8). Moreover, the efficiency of abiotic TC removal in the environment is often unstable and susceptible to external factors such as temperature and pH (Loftin et al. 2008; Leng et al. 2016). In contrast, the application of AEPI-S25 not only rapidly reduces the risks associated with antibiotic contamination but also helps alleviate endocrine disruption, demonstrating significant potential for future remediation practices in livestock wastewater.

## Broad application range of AEPI-S25 in pollution treatments

Livestock effluents were often characterized by high salinity levels (Kim et al. 2016; Rivas Lucero et al. 2018), which inhibited microbial growth and resulted in low treatability of the wastewater (Pronk et al. 2014). In livestock farms near coastal areas or salt lakes, the use of high-salinity water from nearby sources should make the situation even worse. For example, the salinity of wastewater (11.73 g $L^{-1}$) collected from the cattle farm near Qinghai Lake in this study was about 3.5 times higher than that of ordinary livestock wastewater (3.33 g $L^{-1}$) collected from Jilin Province (Table S1). The discovery of the high salinity adaptability (within the range of 1—80 g $L^{-1}$) strain AEPI-S25 offered a promising solution for effectively removing E2 and TC from high-salinity livestock wastewater, addressing a critical limitation in current wastewater treatment practices. Moreover, previous studies have identified substantial contamination of E2 and/or TC in various high-salinity aquatic environments, including marine aquaculture and antibiotic industrial wastewaters (Akhil et al., 2021; Bennett et al., 2018; Han et al. 2018; Song et al. 2020). The exceptional salinity adaptability of AEPI-S25 also suggested its potential for addressing E2 and TC contamination in these environments.

Cadmium (Cd) and copper (Cu) are two common heavy metals found in livestock wastewater that have a great influence on cell growth and metabolism (Hejna et al. 2021). Although high concentrations of $Cd^{2+}$ (10 mg $L^{-1}$) were found to inhibit the strain's removal efficiency, the actual $Cd^{2+}$ concentration in real wastewater typically ranges at μg $L^{-1}$ level, which is even lower than the low concentration (1 mg $L^{-1}$) used in this study (Table S2; Dias et al. 2020; Li et al. 2022). Furthermore, genomic analysis revealed that AEPI-S25 contains a number of heavy-metal resistant genes (e.g. arsenite efflux pump, chromate transporter, and $Zn^{2+}$-exporting ATPase), implying that AEPI-S25 may be resistant to other heavy-metal ions that were not tested in this study. Overall, these findings suggested that the impact of heavy-metal ions in wastewater on the remediation function of AEPI-S25 was very limited.

Compared to salinity and heavy metals, pH was identified as the primary environmental factor influencing the simultaneous removal of E2 and TC by AEPI-S25. Notably, most of the known *Alkalibacterium* strains were also isolated from alkaline environments, with optimal living pH ranges between 8.5 and 10 (Yomoto et al., 2008; Ishikawa et al. 2009). Consistent with these strains, the growth of AEPI-S25 was also inhibited in acidic environments, which may be one of the important reasons for its reduced ability to remove E2 and TC (Fig. S9). Furthermore, during heterologous expression pre-experiments, the recombinant strain pET-gene0878 exhibited limited TC removal capacity until the medium pH was adjusted from neutral to alkaline (data was not shown). It suggested that the TC biotransformation enzyme produced by AEPI-S25 was alkaline-dependent and thus inhibited under acidic conditions. Fortunately, the pH value of livestock wastewater generally falls between 7.5—8.5 (Tak et al. 2015), which is acceptable for the application of the strain. In this study, the pH values of the wastewater collected from Qinghai (pH = 7.56) and Jilin (pH = 8.50) provinces were also within this range, which may be one of the important reasons for ensuring the successful removal of E2 and TC by the strain AEPI-S25. However, it was worth noting that the pollutant removal efficiency in sterilized wastewater samples was still lower than that in the laboratory media, likely due to the pH of wastewater not reaching the optimal level (> 9) for remediation (Fig. 2). In future practical bioremediation applications of the strain, maintaining a higher pH level may be necessary for achieving efficient simultaneous removal of the pollutants. Moreover, the bioremediation efficiencies of AEPI-S25 in non-sterilized samples were significantly lower than those observed in sterilized samples (Fig. 2; Fig. S2), suggesting that indigenous microorganisms may have exerted inhibitory or competitive effects on the bioremediation process. Previous studies have also reported that the added bioremediation strains were easily inhibited by indigenous microorganisms, particularly when free cells were applied. For example, Yasir et al. found that the competition from indigenous microorganisms reduced the removal efficient of 2-chlorobiphenyl by *Pseudomonas pseudoalcaligene*s sp. K7 from 81 to 57% after 120 h of incubation (Yasir et al. 2021). To address this issue, microbial immobilization techniques have been commonly employed to enhance the colonization and competitiveness of inoculated strains. For instance, after immobilizing *Bacillus cereus* sp. WL08 on bamboo charcoal and sodium alginate, the removal efficiency of dimethomorph by the strain increased from 44.23% to 86.07% within 3 days (Zhang et al. 2020). For the application of AEPI-S25 in real environments, appropriate immobilization strategies may also enhance its bioremediation performance and warrant further investigation.

## Novel mechanisms for E2 and TC removal within the strain

The dehydrogenation of E2 was considered to be the restricted step in E2 removal (Ibero et al. 2020). Previous studies have reported that various hydroxysteroid dehydrogenases (e.g., *OecA* and *17β-HSDs*) are responsible for the biotransformation of E2 to E1, as observed in E2 removal strains within *Sphingomonas*, *Pseudomonas putida,* and *Microbacterium himinis* genera/species (Chen et al. 2017, 2018; Wang et al. 2018; Xiong et al. 2023). Our results indicated that the E2-removal strain within the *Alkalibacterium*

genus employed a similar mechanism, highlighting the universality of this pathway in microbial E2 removal. Under the catalysis of some strains (e.g. *Rhodococcus equi* sp. DSSKP-R-001 and *Sphingomonas* sp. KC8), the produced E1 can be subsequently converted to 4-hydroxyestrone, followed by A-ring cleavage between positions C-4 and C-5 (Chen et al. 2017, 2018; Li et al. 2020c; Tian et al. 2022). This pathway is known as the 4,5-seco pathway, and it is one of the most common bacterial E2 degradation routes. In this study, the potential metabolite 4-hydroxyestrone (TP285) was also discovered during E2 removal by the strain AEPI-S25, suggesting the possible presence of the 4,5-seco pathway in the strain. However, other typical downstream products in the 4,5-seco pathway (e.g. pyridinesterone acid) were not found, implying that the strain might only contain part of the entire 4,5-seco pathway. In addition, it is worth noting that although many previous studies have characterized E2 transformation/degradation products using ethyl acetate extraction (Chen et al. 2017; Wu et al. 2019; Tian et al. 2022), a recent study suggested that some of these metabolites may be easily lost during the extraction process (Xiong et al. 2023). In the future, some measures such as shortening extraction time and controlling extraction temperature should be performed during the extraction process, which may reduce the evaporation and thus minimize the loss of extracted metabolites. Moreover, alternative organic extraction solvents (e.g. acetonitrile and ethyl acetate-dichloromethane) should be considered for use (Nakai et al. 2011; Liu et al. 2020) to eliminate the possible loss of E2 transformation/degradation products due to ethyl acetate extraction.

Currently, the majority of verified TC removal pathways have been linked to *TetX* genes (Chen et al. 2022b). The *TetX*-associated removal mechanism involves epoxidation, leading to TC hydroxylation at the C11a position and forming TP461 (Yang et al. 2004). The resulting ketone at C12 will be rapidly converted to 6,12-hemiketal and can undergo non-enzymatic reactions to produce various downstream compounds, such as TP413 (Yang et al. 2004). Notably, TP461 and TP413 have been identified as characteristic *TetX*-related biotransformation products in the TC removal processes of several strains, including *Sphingobacterium* sp. WM1 and *Sphingobacterium* sp. PM2-P1-29 (Chen et al. 2023; Ghosh et al. 2015). In this study, a novel flavin-dependent monooxygenase gene (*S25_gene0878*) rather than the *TetX* genes was identified as responsible for transforming TC to TP461, highlighting the genetic diversity of microbial tetracycline biotransformation mechanisms. Compared to *TetX* genes, *S25_gene0878* was more closely related to *MabTetX* (Fig. 5). *MabTetX* was first identified in a *Mycobacterium tuberculosis* strain and has also been reported to convert TC to TP461 through monooxygenation (Rudra et al. 2018). Importantly, both *TetX* and *MabTetX* have been demonstrated the ability to degrade TC and doxycycline, suggesting that bacteria harboring these genes can simultaneously remove multiple tetracycline antibiotics (Rudra et al. 2018; Wen et al. 2020). Considering the widespread use and high concentrations of various tetracycline antibiotics in livestock wastewater, further investigation is needed to determine whether AEPI-S25 can also remove other tetracycline antibiotics, such as doxycycline, oxytetracycline, and chlortetracycline.

In addition to the two key mechanisms discussed above, some potential E2 and TC transformation/degradation products and associated genes were predicted through UPLC-Orbitrap-HRMS and genomic analysis. However, confirming the complete E2/TC removal pathways of the strain still needs substantial work, particularly through quantitative studies using standard substances of the potential transformation/degradation products. Notably, several potential key genes (e.g. those from the SDR family) were believed to play significant roles in the removal of both E2 and TC, implying a potential coupling relationship in their downstream biotransformation processes (Hao et al. 2023; Ye et al. 2023). Future studies should incorporate further analyses (e.g. multi-omic analysis) to elucidate the strain's complete catabolic pathways, ultimately explaining the regulation mechanisms underlying the E2-TC simultaneous removal and improving simultaneous removal efficiency at the molecular level.

In conclusion, this study isolated a novel strain, *Alkalibacterium* sp. AEPI-S25, which exhibited an efficient ability to remove 17β-estradiol (E2) and tetracycline (TC) simultaneously. AEPI-S25 demonstrated excellent environmental adaptability and maintained high simultaneous removal efficiency under high concentrations of salinity, $Cu^{2+}$, and $Cd^{2+}$. However, its removal efficiency in the real wastewater still requires improvement in the future, particularly under acidic conditions and in the presence of indigenous microbial communities. Further analysis of removal pathways and mechanisms suggested AEPI-S25 primarily removes E2 and TC through initial dehydrogenation and monooxygenation, respectively. The functional genes associated with E2-dehydrogenation (*S25_gene0393*) and TC-monooxygenation (*S25_gene0878*) were then discovered and validated through transcription analysis and heterologous expression. In addition, based on HRMS and genomic analyses, several potential downstream E2 and TC transformation/degradation products and associated genes were predicted in this study. Nevertheless, confirming the complete E2/TC removal pathways of the strain still requires further investigation, particularly the quantitative analyses of the identified intermediates using standard substances.

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**Author contribution** M.B., Y.W., and Y.H. designed the experiments; W.L., S.Y., H.J., and X.M. performed the experiments; M.B., H.Y. and W.L. prepared the figures; M.B., Y.W., and H.Y. wrote the manuscript; H.L., L.Z., Y.S., and X.L. gave support on the experiments. All the authors discussed the results and commented on the manuscript.

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

## Declarations

**Ethical approval** Not applicable.

**Consent to participate** Not applicable.

**Consent for publication** All authors approved the final version of the manuscript.

**Competing interests** The authors declare no competing interests.



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