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ORIGINAL ARTICLE

Biodegradation of 17 β-estradiol by *Serratia marcescens* and *Stenotrophomonas tumulicola* co-culture isolated from a sewage treatment plant in Upper Egypt

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ABSTRACT

Background and Objectives: 17 β- estradiol (E2) is an important pollutant of the aquatic system. It is responsible for sexual disruptions in the majority of aquatic organisms. This study aimed to search for bacteria with high potential degradation of E2 as an important method for bioremediation.

Materials and Methods: Sewage water samples were collected and treated to isolate bacterial strains which were identified by conventional methods and 16S ribosomal RNA gene sequence analysis. The biodegradation of E2 by the isolated strains was evaluated under different environmental conditions.

Results: Two bacterial strains were recovered from sewage water samples and identified as *Stenotrophomonas tumulicola* and *Serratia marcescens*, (named ASc2 and ASc5 respectively). Co-culture of the two strains showed biodegradation of approximately 93.6 % of E2 (50 mg. L^{-1}) within 48 hours. However, the biodegradation capacity of the same E2 concentration was 69.4% and 71.2% for ASc2 and ASc5 each alone, respectively. The optimum cultivation conditions for efficient E2 biodegradation by co-culture were 5% (v/v) inoculation volume with 50 mg. L^{-1} of E2 as the initial concentration at pH 7 and 30°C within 48 hours inoculation period.

Conclusion: This study detected new bacterial strains that are capable of rapid degradation of estrogen as an environmental pollutant.

Keywords: 17 β-estradiol (E2); Biodegradation; *Serratia marcescens*; Sewage; *Stenotrophomonas tumulicola*

INTRODUCTION

The two main categories of estrogens, which are significant endocrine disruptors, are natural estrogens like estrone, 17 β-estradiol (E2), and estriol and synthetic estrogens like ethinyloestradiol and diethylstilbestrol, which may be detrimental to aquatic creatures (1). In aquatic habitats, exposure to E2 at doses of 1-10 ng/L causes feminization in male fish (2).

E2 is considered an atypical estrogen (3) that has a great impact on the growth, upkeep, and contraception of female reproductive organs (4). Furthermore, at extremely low quantities (≥ 1 ng L^{-1}) it can have a

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feministic effect on males (5). As a consequence, it gained increasing public concern in recent years (6).

The main sources of estrogen leakage into the environment are human and animal wastewater (7). According to the study of 30 pregnant women, 133 premenopausal women, and two adult men excreted 347, 4.71, and 1.5 µg/ day of E2 per person through urine respectively (8).

Estrogens are eliminated as inactive polar conjugates in the urine and feces including Estrone-3-glucuronide, Estrone-3-sulfate, 17 β-estradiol-3-glucuronide, 17 β-estradiol-3-sulfate, and 17 β-estradiol-17-sulfate, Estriol-3-glucuronide, Estriol-3-sulfate, and Estriol-17-sulfate. These conjugates can be transformed into active forms (unconjugated estrogens) by bacterial enzymes in the raw wastewater streams as well as wastewater treatment plants. Due to its chemical stability and resistance to degradation during treatment practices, E2 is introduced into the environment via effluent. As a result, one of the most probable sources of environmental estrogen contamination is cleaned wastewater (9-12). As a result, the final effluent has a high concentration of estrogenic activity, which might have negative consequences (13).

Several strategies had been employed for the decontamination of E2 from wastewater treatment plants such as adsorption, degradation, and physical treatments. Physical techniques like the membrane process and activated carbon adsorption, are not cost-effective due to high operational costs and high energy exhaustion (14). Another interesting approach to E2 decontamination of wastewater is the process of photocatalysis; however, residual photodegradation products with significant estrogenic activity were released into the environment (15). Microbial biodegradation of E2 is regarded as a promising and successful approach to decontamination of E2 because of its high efficiency and economic feasibility (16).

This study was conducted to isolate and screen different E2-degrading bacteria from sewage water and to evaluate their degradation efficiency under different environmental conditions.

MATERIALS AND METHODS

Sampling and isolation of E2 degrading bacteria. Water samples were collected from the main sewage water drain (Sewage treatment plant, Arab El- Madabegh, Assiut, Upper Egypt) and prefiltered through cheesecloth to remove debris then filtered via sterile 0.22 µm pore size membrane filters. To the bacterial culture, 100 mg. L^{-1} E2 (Sigma-Aldrich, USA) was added into 100 mL of liquid methanol mineral salt medium (MSM) (Oxoid, UK) in a 250-mL Erlenmeyer flask to make an estrogen mineral salt medium (EMM). The 0.22 µm filter membranes were cultivated in (MSM) and the mixture was incubated at 30°C, 150 rpm for 48 hours. 5% (v/v) of this mixture was used to inoculate fresh EMM every 48 hours and incubated at 30°C, 150 rpm, and this process was repeated three times. On several EMM agar plates; the bacterial growth was plated (17). Continuous streaking was used to select morphologically different colonies for purification until only one pure colony remained. The isolated colonies were further purified and transferred to maintenance agar slants.

Harvesting of bacterial cells. A single pure colony was cultivated in Luria Bertani (LB) medium (Lab M, UK) at 30°C and 150 rpm for bacterial growth. The bacterial pellet was obtained by centrifuging it at 8000 rpm for 4 minutes, washing it twice with phosphate-buffer saline (PBS), and then resuspending it in sterile MSM until it had an optical density of 600 nm (OD_{600nm}) of 1.

Identification of isolated strains. Gram staining and more traditional methods of identifying, such as biochemical tests (catalase, DNase, indole synthesis, oxidase, and citrate consumption) were used to identify two isolated bacterial strains known as ASc2 and ASc5, as well as their growth on various selective (Cetrimide agar and Mannitol Salt Agar) and moderately selective (Macconkey agar) media. The genomic DNA was extracted utilizing the Gene JET Genomic DNA Purification kit, and the isolated strains' identities were determined and confirmed using 16S ribosomal RNA (16 rRNA) gene sequencing (Scientific Thermo, Lithuania) using universal oligonucleotide primer pairs (18). The 16S rRNA genes were amplified by PCR using 27 forward (F) primers (5'-AGA GTT TGA TCC TGG CTC AC-3') and 1492 reverse (R) primers (5'-GGT TAC CTT GTT ACG ACT T-3'). The following cycling conditions were used for amplification: beginning denaturation for five minutes at 95°C, followed by 30 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 57°C, ex-

tension for one minute at 72°C, and final extension for ten minutes at 72°C. Sequencing of the amplification products was done (Utilizing an ABI 3730xl DNA sequencer by GATC Company). Using the BLAST service (http://blast.ncbi.nlm.nih.gov), the 16S rRNA gene sequences of the isolated bacteria were aligned with gene sequences already in the NCBI database. A phylogenetic tree was constructed by aligning 16S rRNA sequences of the highly matched strains with database sequences searched.

Screening of E2 biodegradation by ASc2 and ASc5 isolates under different conditions. The bacterial ability for E2 biodegradation was screened by growing the bacterial isolates in an EMM medium. The biodegradation process used 5% (v/v) cell suspension in 50-mL Erlenmeyer flasks of EMM containing 50 mg L^{-1} of E2. 48 hours were spent cultivating the combination in the rotary shaker at 30°C and 150 r. min^{-1}. In this investigation, we used a 48-hour incubation period to evaluate the highest E2 degradation efficiency of the isolated strains in a short time.

The impact of varying environmental factors on the deterioration of E2 by ASc2 and ASc5 isolates was evaluated, including initial E2 concentration (10-50 mg. L^{-1}), temperature (20-40°C), pH (5.0-9.0) and inoculum volume size (5%-15% (v/v)).

The residual E2 in bacterial cultures was analyzed by HPLC/UV every 12 hours as follows; to dissolve any remaining E2, 20 mL of methanol was added to flasks containing bacterial cultures. The liquid was then ultrasonically stirred for 30 minutes and filtered using 0.22 m polytetrafluoroethylene membrane filters. For HPLC/UV finding, the subsequent parameters were used: A C18 column, acetonitrile, and water (70/30 v/v) mobile phase at a flow rate of 1 mL/minute, and a 20 µL injection volume (19). The degradation efficiency (%) of E2 in batch culture was calculated using the following formula:

$$\text{Degradation efficiency}(\%) = (C_{Initia} - C_{Residua_1} / C_{Initial}) \times 100\%.$$

where the initial and final levels of estradiol in the culture solution are represented by the letters $C_{Initial}$ and $C_{Residual}$.

Statistical analysis. Microsoft Office Excel 2016 was used for all figures and data processing. SPSS version 22.0 was used to analyse variance. The data were signified by the error bars in the figures as the means and standard deviations of three similar tests.

RESULTS

Identification of isolated strains. Potential E2-degrading bacterial strains; Asc2 and ASc5 were isolated from sewage wastewater, the two isolates were Gram-negative, straight bacilli. Analysis of the biochemical characteristics of the isolated strains showed that; Asc2 isolates could not grow on Cetrimide agar were oxidase, indole, ornithine decarboxylase, and citrate negative, but positive for lactose fermentation and catalase. Despite the ASc5 isolates could not grow on Cetrimide agar and produce indole, oxidase, or ferment lactose, it was positive for citrate, catalase, and ornithine decarboxylase.

A phylogenetic study of the 16S rRNA gene sequences showed that strains ASc2 and ASc5 were 98.66% and 98.95% identical to *Stenotrophomonas tumulicola* and *Serratia marcescens* respectively (Fig. 1).

Degradation of E2 by ASc2 and ASc5 strains. The degradation rate of E2 by ASc2 and ASc5 strains was assessed separately and in co-culture mode. ASc2 and ASc5 strains exhibited E2 biodegradation efficiency of 69.4% and 71.2%, respectively, with initial E2 values of 50 mg L^{-1} after 48 hours. In bacterial co-culture, 93.6% of E2 was degraded after 48 hours. Different environmental conditions for the degradation efficiency of E2 by bacterial co-culture were assessed. Different E2 initial values of (10 mg. L^{-1}, 20 mg. L^{-1}, 30 mg. L^{-1}, 40 mg. L^{-1}, and 50 mg. L^{-1}) were tested, of which 50 mg. L^{-1} showed the highest biodegradation rate by *Serratia marcescens* and *Stenotrophomonas tumulicola* co-culture (Fig. 2).

The effect of change in the culture medium pH on E2 degradation was investigated. 93.6% of E2 was biotransformed at pH (7.00) by *Serratia marcescens* and *Stenotrophomonas tumulicola* co-culture. The degradation efficiencies at pH (5.00), (6.00), (8.00), and (9.00) were (43.2%), (82.5%), (79.5%), and (53.7%) respectively (Fig. 3).

A temperature of 30°C was the most suitable condition for enhancing E2 removal by *Serratia marcescens* and *Stenotrophomonas tumulicola* co-culture. The E2 concentration decreased to 36.25, 15.8, and 3.25 mg. L^{-1} after 48 hours at 40, 20, and 30°C respectively (Fig. 4). To examine the impact of inoculum volume on E2 degradation behavior, experimental runs using different inoculum volumes (5%, 10%, and 15% (v/v)) were conducted under 30°C, (pH 7.00) for 48 hours of incubation. The maximum degradation

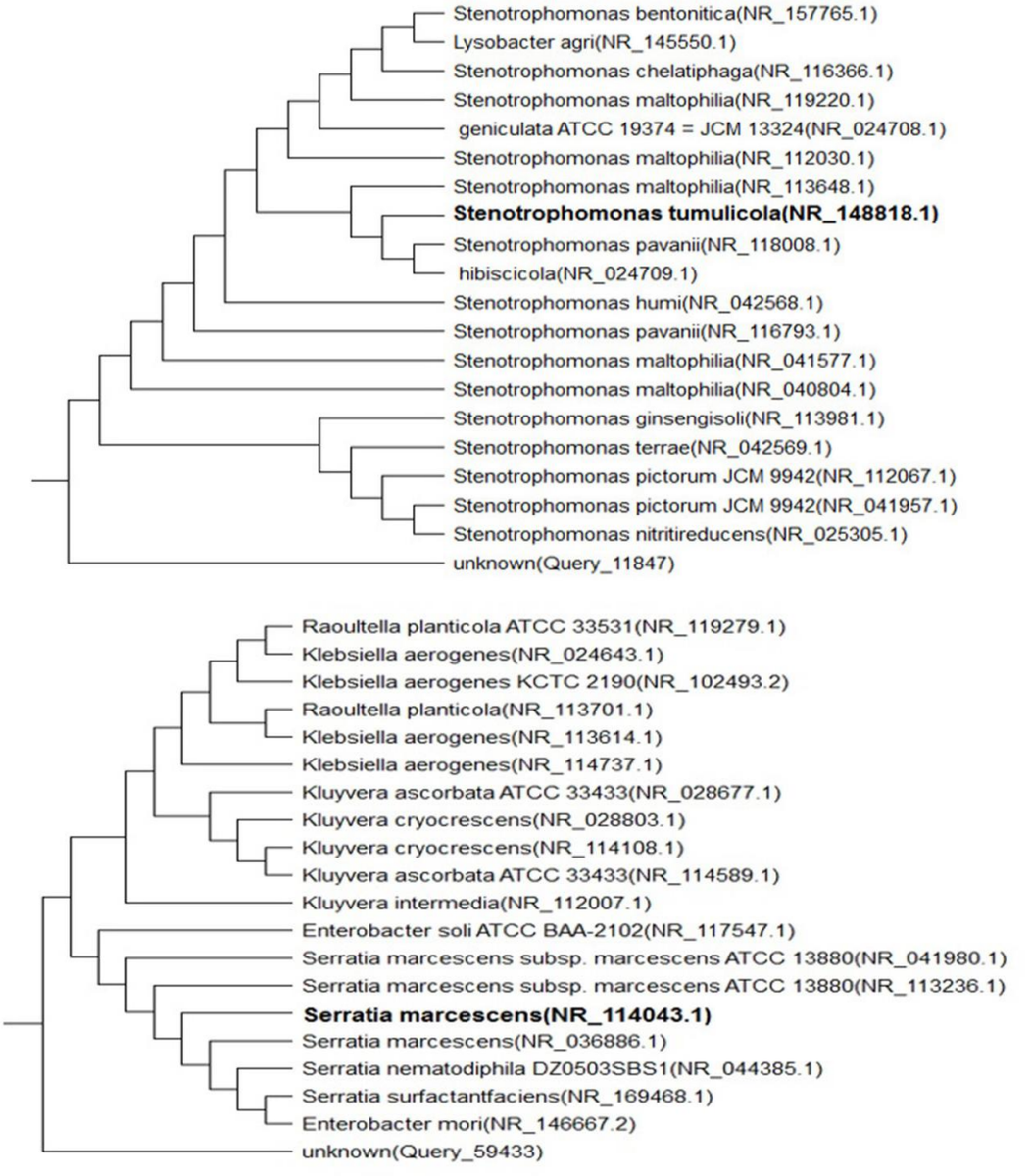

Fig. 1. Phylogenetic tree of both *Stenotrophomonas tumulicola* and *Serratia marcescens* and closely related species

rate was obtained by an inoculation volume of 5% of the reaction system (Fig. 5).

DISCUSSION

Nowadays, estrogens have become a major concern due to their detrimental effects on soil, plants, and humans. Estrogens present in the environment can have adverse effects on many organisms particularly humans and have been associated with prostate and breast cancer (20).

Over the last twenty years, there has been a dramatic increase in reports of bacteria that are actively degrading E2. According to Fujii et al. the ARI-1 strain, which closely resembles many *Novosphingobium* species, was the first E2-degrading bacterium to be identified from activated sludge in Japan (21). Jiang et al. reported that five strains of the genus Bacillus showed different degradation rates (22). Additional six communal strains (*Klebsiella* sp, *Bacillus* sp, *Enterobacter* sp, and *Enterobacter* sp II. *Aero-*

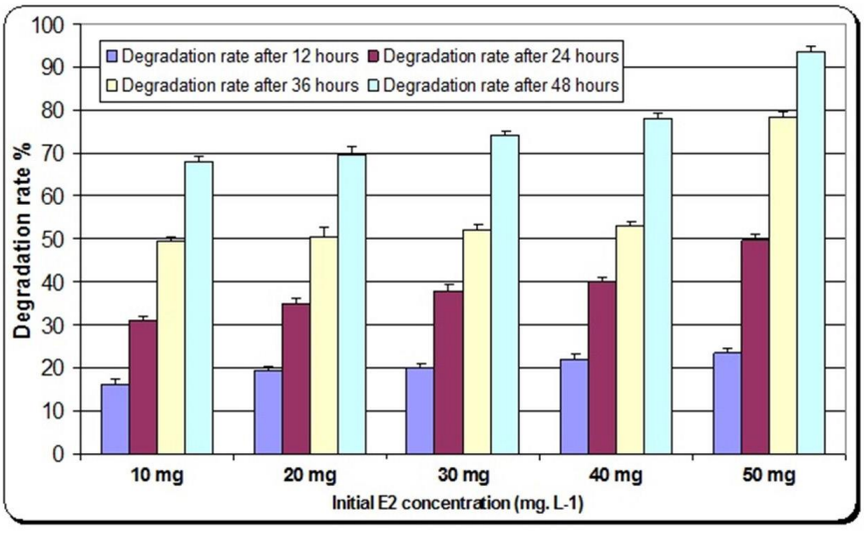

Fig. 2. Degradation of E2 by *Serratia marcescens* and *Stenotrophomonas tumulicola* co-culture in MSM with E2 at a concentration of (10 - 50 mg. L^{-1}) over 48 hours of incubation.

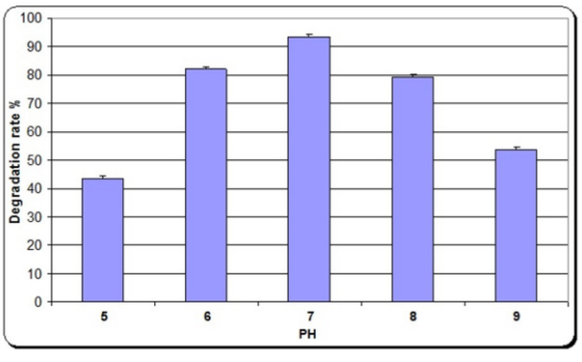

Fig. 3. Effect of pH on bacterial co-cultures ability to degrade E2. pH of MSM with E2 (50 mg. L^{-1}) was adjusted to 5.0, 6.0, 7.0, 8.0, and 9.0 using 0.1 mol L^{-1} NaOH or 0.1 mol l^{-1} HCL.

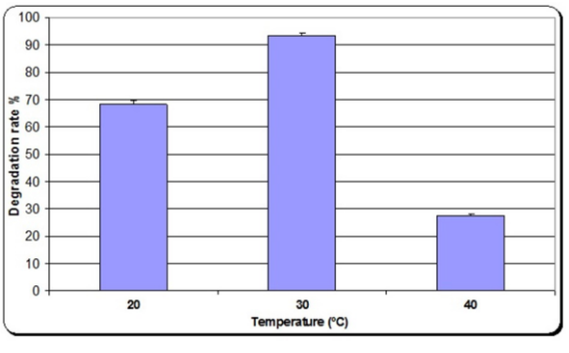

Fig. 4. Effect of temperature on E2 degradation by bacterial co-culture in MSM with E2 concentration at (50 mg/L), pH (7.00) after 48 hours of incubation

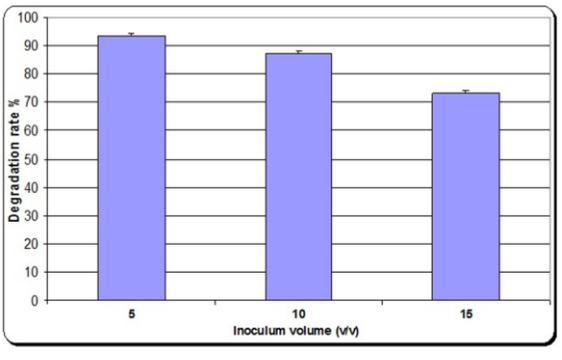

Fig. 5. Effect of inoculum volume on E2 biodegradation by bacterial co-culture.

monas veronii strain and *Aeromonas punctata* strain) were obtained from sewage discharges in Egypt (Saft El-Henna, Northern Egypt) and were able to break down E2 (23).

In the current study, we isolated strains of *Stenotrophomonas tumulicola* and *Serratia marcescens* from wastewater that could degrade E2. It was discovered that *Stenotrophomonas maltophilia* isolated from activated sludge uses several aromatic substrates as its exclusive source of carbon and energy (24). *Stenotrophomonas maltophilia* was also used as a model for 17 β- estradiol degrading bacteria (25). The genus *Stenotrophomonas* is well studied for its potential E2 degradation, with numerous species including *Stenotrophomonas maltophilia* (26, 27). For the genus *Serratia*, organic pollutants in wastewater were bio-

degraded by *Serratia* species isolated from sewage sludge (28). In China, *Serratia nematodiphila* was isolated and purified from wastewater samples and demonstrated the ability to use E2 as the sole source of carbon (29).

In the current research, the strain *Stenotrophomonas tumulicola* showed a moderate E2 biodegradation rate (69.4%) after 48 hours of enriched culture. Xiong et al. reported a higher biodegradation rate (90%) during a longer period (one week) of culture enrichment by *Stenotrophomonas maltophilia* (26).

Our study found that the *Serratia marcescens* strain exhibited a relatively high E2 degradation rate (71.2%) after 48 hours of cultivation, a higher degradation rate (93.2%) was reported by Zhao et al. in a longer duration of enrichment cultivation (96 hours) using a *Serratia nematodiphila* strain (29).

In the present study, the bacterial co-culture with *Stenotrophomonas tumulicola* (ASc1) and *Serratia marcescens* (ASc5) strains was able to rapidly degrade rates of about 93.6% E2 (50 mg. L^{-1}) as the sole carbon source after 48 hours of incubation. Other studies found different degradation rates. For example, a degradation rate of 98% from a much lower concentration of E2 (5 mg. L^{-1}) after a longer incubation period (seven days) using a bacterial co-culture containing the strains *Acinetobacter calcoaceticus* and *Pseudomonas putida* (30). Tian et al. (2020), reported an E2 degradation rate of 97.3% after 96 hours of enrichment culture with *Rhodococcus equi* DSS-KP-R-001 (31). In another study, a co-culture system of *Flavobacterium longum* and *Pseudomonas aeruginosa* was able to eliminate 91.3% of a starting quantity of 10 mg. L^{-1} E2 over a prolonged period (120 hours) (32).

Regarding the starting quantity of E2, 50 mg. L^{-1} of E2 was the ideal initial concentration at which the degradation rate was the highest among other concentrations (10, 20, 30, and 40 mg. L^{-1}). This follows the finding of Li et al. where the initial concentration showing the highest rate of degradation was 40 mg/L (17).

Regarding the role of culture medium pH in E2 removal, this study found that the pH at which the bacterial co-culture adequately removed E2 ranged from 6.00 to 8.00. This result is similar to that reported by Li et al. where the pH ranged from 7.00 to 9.00 (30). The results are also consistent with some previous investigations. We found that 93.6% of E2 was degraded at pH 7.00, while Liu et al. and AlAhadeb found that 90% and 91.3% of E2 were broken down at pH 7.00 and pH 7.50, respectively (33) and (32). In contrast, Wu et al. used pH 5.00 and 11.00 to achieve a 90% degradation rate (34).

In this study, it can be seen from the results that temperature has a major impact on E2 degradation by bacterial co-culture. When the temperature was between 20 and 30ºC, the removal of E2 from the enrichment cultures was increased. This agrees with the data reported by Ge et al. where degradation of E2 was increasing when the temperature was between 15 and 35ºC (35). Our findings were compared to those of Yu et al. (2016) and Li et al. (2020). We found that 30ºC and 5% inoculum volume were the best conditions for E2 degradation, which agrees with Li et al. who found the same circumstances (36). On the other hand, Yu et al. found that the best conditions for E2 degradation were 30ºC and 1% of the inoculum volume (37).

CONCLUSION

Two different Gram-negative bacterial strains (ASc2 and ASc5) isolated from wastewater were identified as E2-degrading bacteria. The 16S rRNA gene sequences of isolated bacteria revealed that they belonged to the genera *Stenotrophomonas* (ASc2) and *Serratia* (ASc5). Within 48 hours of incubation, a bacterial co-culture made up of the ASc2 and ASc5 bacterial strains was able to quickly break down 93.6% of E2 when it was the sole carbon source. However, when strains ASc2 and ASc5 were incubated separately, E2 was degraded by 69.4% and 71.2%, respectively. The isolated strains of ASc2 and ASc5 showed an obvious ability to degrade E2 in enrichment culture under optimal conditions of 50 mg. L^{-1} of E2 as the initial concentration of substrate, pH 7.00, a temperature of 30ºC, and an inoculation volume of 5 % of the reaction system. E2-degrading strains have elucidated their ability to reduce the potential environmental risks from E2, so the removal and biodegradation of the pollutants from the ecosystem should be further investigated.

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