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Original Research Paper

Isolation and Characteristics of 17β-Estradiol-Degrading *Dyella* spp. Strains From Activated Sludge

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ABSTRACT

A 17β-estradiol (E2) degrading strain was isolated by using enrichment culture of activated sludge from wastewater treatment plants and identified as the genus *Dyella*. The estradiol degradation by this strain fitted in a first-order reaction. The degradation rate constant K was 0.025 h^{-1}. Methanol and sodium acetate could be utilized by the bacteria. In contrast, ethanol and glucose were not completely utilized within 10 days. Estradiol was converted to estrone first and then used for further degradation. Estrone degradation by the bacteria also fitted in a first-order reaction (K=0.0133 h^{-1}). The relationship between the specific degradation rate and substrate concentration fitted in the Monod equation.

INTRODUCTION

Endocrine disrupting compounds (EDCs) such as estrogens are chemicals produced by human and animal metabolism. EDCs are discharged into wastewater with urine and feces, and finally discharged into natural water bodies after sewage treatment. Natural (estrone (E1) and 17β-estradiol (E2) and synthetic (17α-ethynyl estradiol (EE2) estrogens in treated wastewater are considered to contribute the most estrogenic activity (Desbrow et al. 1998, Nakada et al. 2004). However, these estrogens are only partially removed by domestic wastewater treatment and are continuously discharged into receiving water bodies (Kolodiej et al. 2003, Ternes et al. 1999). Although the amount of estrogens detected in treated wastewater is at nanogram levels or even lower, the presence of estrogens in treated wastewater is reportedly responsible for male fish feminization and sexual disruption in aquatic wildlife (IUPAC 2003).

Estrogen removal by wastewater treatment has been extensively investigated, which is mainly attributed to adsorption and degradation by activated sludge (Estrada-Arriaga & Mijaylova 2010, Ren et al. 2007, Taro & Tomoya 2005). The adsorption behaviour of estrogens has been frequently reported; however, little is known about the microorganisms responsible for the environment. Several human intestinal bacteria and oral microorganisms are capable of converting E2 to E1 and vice versa (Jarvenpaa et al. 1980, Kornman & Loesche 1982, Ojanotko-Harri et al. 1991). Fujii et al. (2002) isolated the first E2-degrading bacterium *Novosphingobium* species (ARI-1) from activated sludge. Six estrogen-degrading isolates (four *Rhodococcus* strains, an *Achromobacter* strain, and a *Ralstonia* strain) from activated sludge were reported by (Yoshimoto et al. 2004, Weber et al. 2005).

Among the estrogen variations, 17β-estradiol (E2) is the primary metabolite with the highest estrogenic activity. Despite this fact, studies on 17β-estradiol-degrading bacteria are limited. This paper reports isolation of an E2-degrading strain, and its growth curve and degradation characteristics were studied to gain better insight into the E2 degradation mechanism.

MATERIALS AND METHODS

Chemicals: The estrogens used in this experiment were of analytical grade. E1, E2 were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). The E2-d4 isotope was purchased from Sigma-Aldrich Inc. (USA). All the organic solvents used were of HPLC grade. Stock solutions (100 μg/L) of each estrogen were prepared in methanol in glass tubes and kept at 4°C until use.

Isolation of 17β-estradiol-degrading strain and strain identification: Activated sludge collected from the aeration tank of a municipal wastewater treatment plant in Beijing, China was used as inoculum. A volume ratio of 2.5% was inoculated to 500 mL of mixing liquid with E2 as the sole carbon source. Estrogen dissolved in methanol was added

to a sterilized flask. After the methanol evaporated, a sterilized medium was added to the flask. The other reagents used were 0.1 g of K_2HPO_4, 0.1 g of $(NH_4)_2HPO_4$, 0.02 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeCl_3$, 0.1 g of $CaCl_2$ $2H_2O$, 0.1 g of NaCl and 1000 mL of distilled water. The pH was adjusted to 7.0~7.2 using (1+3) HCl. For plate preparation, 1.5% of agar was added. The flasks were placed on a rotary shaker at 150 rpm at 35°C in the dark. Every 3 days, 100 mL of medium was discharged and an equal volume medium was added to the flasks. Strain identification was performed by Sangon Biotech (Shanghai, China) Co., Ltd based on 16S RNA gene sequencing.

Estrogen degradation tests: The degradation tests were conducted in a series of 250 mL flasks containing 50 mL of isolates culture medium with an initial concentration of 10 mg/L E2 or E1. The flasks were incubated in a rotary shaker at 150 rpm and 35°C. The liquid samples collected over time were analysed for estrogen concentrations by GC/MS analysis.

Single colonies that developed on the plates were collected and inoculated into 250-mL flasks containing 10 mg/L estrogen medium after sterilization. The flasks were incubated on a rotary shaker at 150 rpm and 35°C for 6 days. The liquid samples were collected over time to analyse estrogen, and OD_{600} was used to determine the biomass.

Estrogen analysis: The collected liquid samples were first filtered through 0.45 μm glass fibre filters, and subsequently filtered to Oasis HLB SPE RP-C18cartridges (Waters, USA). The analytes were eluted with 5 mL of ethyl acetate. 50 ng E2-d4 was added into the elution as the internal standard substance. The ethyl acetate extract was completely evaporated by a gentle nitrogen stream (45°C). The analytes were derivatized with 50 μL of BSTFA (1% TMCS) and 100 μL of pyridine at 70°C for 30 min. Finally, the analytes were examined by GC-MS/MS (Agilent 6890N GC connected to an Agilent 5973 inert mass selective detector). A splitless injection mode was used to inject 2 μL of the sample at an injection port temperature of 250°C. The carrier gas was helium at a constant flow rate of 1.0 mL/min. For the analysis of all compounds, the oven program was as follows: 100°C for 1 min, 10°C/min to 200°C, and 3°C/min to 300°C for 10 min. The detector was used predominantly in the selected ion monitoring mode. The electron impact source temperature was 230°C with an electron energy of 70 eV, an excitation amplitude of 0.2 volts, an emission current of 100 microamperes, and a window for MS/MS of 3 *m/z*.

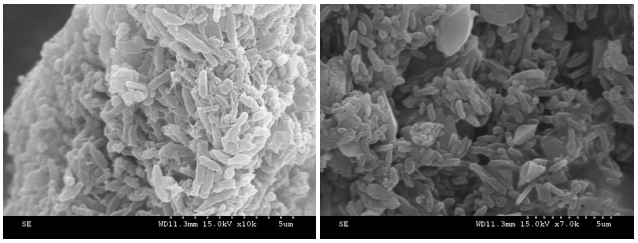


Fig. 1. The micrograph of 17β-estradiol-degrading bacteria flocs observed by SEM.

Image analysis: The pretreatment of flocs for scanning electron microscopy (SEM) was carried out using a method described by Massol-Deya et al. (1995) with some modifications. The flocs at the bottom of the flask were collected after 30 min of sedimentation and washed three times with 1×PBS. The samples were fixed for 2 h with 2.5% vol/vol glutaraldehyde (pH 6.8) at 4°C. After dehydrating through a graded series of ethanol solutions (25%, 50%, 70%, 80%, 90%, 100% ethanol), the samples were replaced by isoamyl acetate and ethanol mixed solution (1:1 volume ratio), as well as isoamyl acetate for 15 min. The samples were dried and coated with gold. Floc morphology was examined using a high-resolution SEM system (Hitachi S-4300, Japan).

RESULTS AND DISCUSSION

Isolation and identification of 17β-estradiol-degrading bacteria: Two weeks after inoculation, single 17β-estradiol-degrading bacteria was isolated from the activated sludge using the plate-streaking method. The colonies were 1.0-3.0 mm in diameter, milky pigmented, smooth, circular, entire, and transparent with clear edges. The plates containing single colonies were mailed to Sangon Biotech for strain identification. Genomic DNA was extracted and the DNA sequence was analysed and identified by the sequences in GenBank using the Basic Local Alignment Search Tool (GenBank accession number: JX173884). The 17β-estradiol-degrading strain was identified as the genus *Dyella*, Family Xanthomonadaceae, Order Xanthomonadales, Class Gammaproteobacteria. Many *Dyella* species such as *Dyella ginsengisoli* sp. nov., *Dyella japonica* gen. nov., sp. nov., *Dyella marensis* sp. nov., *Dyella soli* sp. nov., and *Dyella terrae* sp. nov. were mainly isolated from soil (Jung et al. 2009, Lee & Lee 2009, Weon et al. 2009, Xie & Akira 2005). The 17β-estradiol-degrading ability of *Dyella* has not been reported before. However, some studies showed that *Dyella* has a highly efficient biphenyl-degrading ability (Li et al. 2010, 2011, Qu et al. 2011, Zhao et al. 2010). The molecular structures of E2 and biphenyl are similar but the molecular structure of E2 is more complicated.

During the growth tests, some flocs formed and were separated at the end of the tests. The flocs were pretreated and observed with SEM. The floc micrographs are shown in Fig. 1. The bacteria were rod-shaped cells, 2μm long and aggregated as flocs.

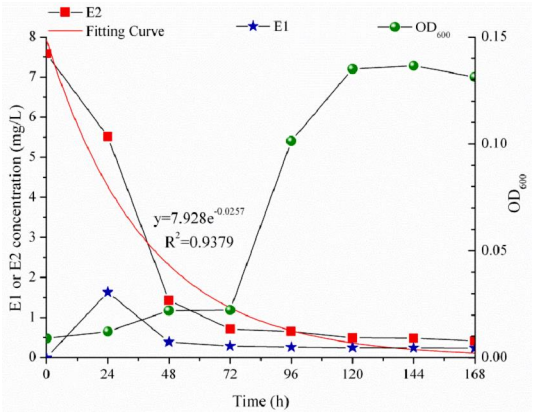


Fig. 2: Growth of 17β-estradiol-degrading bacteria with E2 as sole energy source.

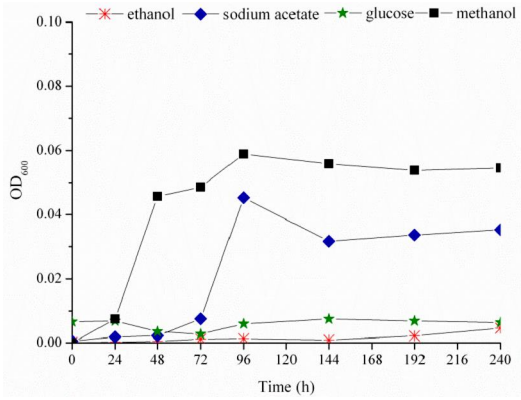
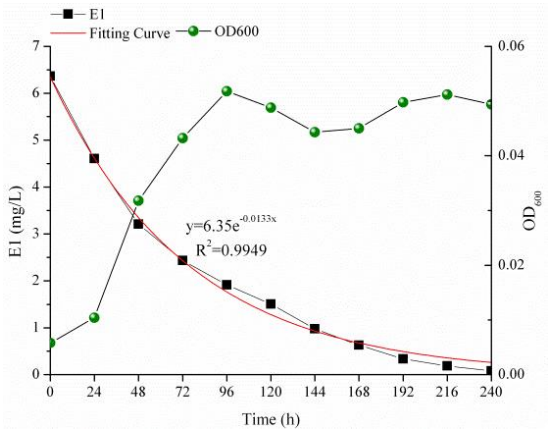
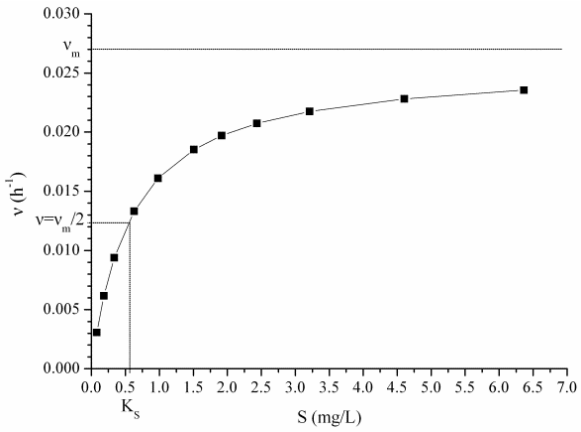


Fig. 3: Growth of 17β-estradiol-degrading bacteria with simple organic carbon sources.



(a)



(b)

Fig. 4: Degradation of estrone(E1) by 17β-estradiol-degrading bacteria (a) and relation between specific degradation rate (v) and substrate concentration (S) fitted to Monod equation (b).

Growth tests and 17β-estradiol degradation characteristics: As shown in Fig. 2, OD_{600} was used to determine the biomass. The growth curve had an apparent pattern of adaptation, logarithmic growth and stability stages. During the procedure, E2 was degraded rapidly within the first 48h. Then, the E2 concentration gradually decreased. The procedure fitted a first-order reaction ($R^2 = 0.906$). The degradation rate constant K was 0.025 h^{-1}.

However, E1 appeared and reached a concentration peak at 24h, which indicated that E2 was first converted to E1 and then gone for for further degradation. The 17β-estradiol-degrading bacteria *Dyella* genus can also degrade E1. These results are consistent with previous reports that E2 is highly susceptible to biodegradation, with the transformation to E1 being the first step (Pauwels et al. 2008, Yoshimoto et al. 2004, Yu et al. 2007). Within 6 days, 93.5% of the added E2 with an initial concentration of 7.58 mg/L was degraded.

Role of simple organic carbon sources in 17β-estradiol-degrading bacterial growth: After isolation, the 17β-estradiol-degrading bacteria were cultured in the liquid medium with other simple organic carbon sources. Ethanol, sodium acetate, glucose and methanol were selected as the organic carbon sources, and the added amount was calculated as 100 mg/L COD. The growth curves are shown in Fig. 3. The 17β-estradiol-degrading bacteria first utilized methanol and then sodium acetate. Compared with E2 as the carbon source, the beginning of the logarithmic growth stage using methanol was at 24 h and the duration was much shorter. Methanol can be used as an organic carbon source for the enrichment of 17β-estradiol-degrading bacteria. However, ethanol and glucose were not completely utilized by the 17β-estradiol-degrading bacteria within 10 days.

Estrone degradation by 17β-estradiol-degrading bacteria: The E1 degradation characteristics by 17β-estradiol-degrading bacteria were studied, and the results are shown in Fig. 4. The 17β-estradiol-degrading bacteria can degrade E1 but at a slow rate. Within 11 days, 98.7% of the added E2 with an initial concentration of 7.58 mg/L was degraded.

The kinetic studies showed that the experimental data fitted the first-order model (R^2=0.9594) well. The relationship between the specific degradation rate (v) and substrate concentration (S) fitted the Monod equation (Fig. 4b). The maximum specific degradation rate (v_m) was 0.0257 h^{-1}.

$$v = v_m \frac{S}{K_s + S} = 0.0257 \frac{S}{0.5846 + S} \quad ...(1)$$

CONCLUSIONS

A 17 β-estradiol-degrading strain was isolated from activated sludge and was identified as the genus *Dyella*. E2 was first converted to E1 before being degraded by the strain. The degradation processes fitted first-order reaction kinetics. The degradation rate constants for E2 and E1 were 0.025 and 0.017 h^{-1}, respectively. Methanol and sodium acetate could be utilized by the strain. However, ethanol and glucose were not completely utilized by the 17β-estradiol-degrading bacteria within 10 days. Estrone degradation by the bacteria also fitted a first-order reaction. The relationship between the specific degradation rate and substrate concentration fitted the Monod equation.

ACKNOWLEDGEMENTS

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