Microbial degradation of estrogens using activated sludge and night soil-composting microorganisms

J.H. Shi*, Y. Suzuki*, S. Nakai* and M. Hosomi*

* Department of Applied Chemistry, Graduate School of Technology, Tokyo University of Agriculture and Technology, 2-24-16 Naka, Koganei, Tokyo 184-8588, Japan (E-mail: *shijiang@cc.tuat.ac.jp*)

Abstract In order to investigate the potential for microbial degradation of estrogens, and the products formed, activated sludge collected from Korea (ASK) and night soil-composting microorganisms (NSCM) were used to degrade estrogens. Results showed that both ASK and NSCM degraded almost 100% of the natural estrogens estrone (E1), 17β-estradiol (E2), and estriol (E3) from initial concentrations of 20–25 mg/L, while synthetic estrogen, ethynylestradiol (EE2), was not degraded. Analysis of degradation products of E2 by using HPLC-ECD and a consecutive first-order reaction calculation confirmed that E2 was sequentially degraded to E1, which was further degraded to other unknown compounds by ASK and NSCM. We then used the yeast two-hybrid assay to show that the unknown degradation products did not appear to possess estrogenic activity when E1, E2 or E3 were degraded to below the detection limit after 14 days of incubation, indicating that ASK and NSCM not only degrade natural estrogens, but also remove their estrogenic activities.

Keywords Activated sludge; degradation; estrogens; night soil-composting microorganisms

Introduction

Natural estrogens, i.e. estrone (E1), 17β-estradiol (E2), and estriol (E3), are excreted into wastewater by humans and animals through their urine or night soil (Ternes *et al*., 1999). The synthetic estrogen, ethynylestradiol (EE2), is a component of oral contraceptives, and is also discharged into wastewater by women. In an investigation of seven effluents from sewage treatment plants in Britain, E2 and E1 were detected in the ranges of 1–50 ng/L and 1–80 ng/L, respectively, while in three of the effluents, EE2 was detected in the range of 0.2–7.0 ng/L (Desbrow *et al*., 1998). Recently, it has been suspected that natural and synthetic estrogens are endocrine-disrupting substances that disrupt reproductive and developmental systems of aquatic organisms by mimicking endogenous hormones (Routledge *et al*., 1998). Since most endocrine disrupters such as bisphenol A and nonylphenol are weakly estrogenic, often with potencies three or more orders of magnitude less than those of E2, E1, or EE2, it has been suggested that in sewage wastewater, estrogens are the major compounds responsible for endocrine disruption (Desbrow *et al*., 1998; Onda *et al*., 2001).

Because the excretion of estrogens by humans and animals is unavoidable, there has been increasing interest in the potential for microorganisms to degrade these compounds in sewage treatment plants. Ternes *et al*. (1999) showed that activated sludge from a sewage treatment plant oxidized E2 to E1, and E1 was then degraded further ; however, EE2 appeared to be largely stable in contact with activated sludge. Lee *et al*. (2002) showed that E2 and E1 are not persistent compounds, and they can be degraded by sewage bacteria. The biodegradation of E2 appeared to initiate at the hydroxy group at C-17 (ring D) of the molecule, leading to the formation of the major metabolite E1. Michael *et al*. (2001) demonstrated that E2, E1, and EE2 rapidly dissipated in agricultural soils, and that the decline in E2, E1, and EE2 concentrations was closely accompanied by a decline in total estrogenic activity, as determined by the yeast estrogenic activity screen assay. They suggested that this estrogen removal was microbially mediated.

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In order to eliminate estrogens from sewage wastewater, it is essential to confirm the fate of estrogens during biodegradation. Therefore, the aims of this work were (i) to find microorganisms with excellent estrogen-degrading abilities, (ii) to reveal how estrogens are degraded, and (iii) to determine whether or not the estrogen degradation products are endocrine disruptors.

Materials and methods

Estrogens, activated sludge, and night soil-composting microorganisms

Natural and synthetic estrogens E1, E2, E3, and EE2 were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Activated sludge (ASK) was obtained from a wastewater reuse system in Grand Hotel in Pusan City, Korea. It was reported that ASK could reduce the BOD very well, and remove the stench (NastNews, 2001a). Night soil-composting microorganisms (NSCM) were obtained from Ina Sanitation Center in Nagano, Japan, which contained priority bacteria (*Bacillus* genus), and was probably able to degrade endocrine-disrupting substances such as NP and BPA (NastNews, 2001b).

Estrogen degradation experiments using ASK or NSCM

In the estrogen degradation experiments, solutions of each estrogen (E1, E2, E3 or EE2) as the sole carbon source in ethanol were added to 500-mL culture flasks in order to achieve final concentrations of 20 to 25 mg/L. Following solvent evaporation, about 1 g of ASK or NSCM and 100 mL of sterilized mineral salts medium (K_2HPO_4, 1 gL^{-1}; $(NH_4)_2SO_4$, 1 gL^{-1}; NaCl, 0.1 gL^{-1}; $MgSO_4 \cdot 7H_2O$, 0.2 gL^{-1}; $FeCl_3$, 0.01 gL^{-1}; $CaCl_2$, 0.1 gL^{-1}) were added to the sterilized 500-mL culture flasks. The culture flasks were incubated on a rotary shaker at 120 rpm and 30°C. During the incubation periods, estrogen concentrations and estrogenic activities were measured.

Analyses of estrogen concentrations

Samples for estrogen concentration measurements were prepared by adding CH_3CN to the remaining culture medium at a volume ratio of 1:1, in order to uniformly dissolve the estrogen. Following filtration (Whatman, 13 mm disposable filter device, 0.22-μm pore size), the sample (100 μL) was subjected to reverse-phase high-performance liquid chromatography (RP-HPLC) using an ODS column (TSK-gel ODS 80Ts, Tosoh, Japan) and an electrochemical detector (1049 A, Hewlett Packard) that detects estrogen by oxidizing its phenolic group. In the RP-HPLC analysis, elution was carried out by using 45% (E3 35%) v/v acetonitrile/water at a flow rate of 1.0 mL/min. In a recovery experiment conducted beforehand (data not shown), we confirmed that more than 95% of estrogens adsorbed on sludge could be collected by using acetonitrile extraction.

Preparation for estrogenic activity screening

We used two pretreatment procedures before measuring the estrogenic activity of the estrogen and its products: solid-phase extraction (SPE) and membrane-filter filtration (MFF). The scheme for the SPE procedure developed in this study is as follows. The C_{18} cartridge (Waters) was conditioned with 10 mL of MeOH and 10 mL of Millipore water prior to use. About 20 mL of sample was filtered through a GF/F glass-fiber filter (1.0-μm pore size) and the filtrate was then adjusted to pH 5 with 1 M acetic acid. The pH-adjusted filtrate was passed through the C_{18} cartridge, and the cartridge was then eluted with 5 mL of MeOH. Suspended solids (SS) collected on the GF/F were washed with 5 mL of MeOH (twice) in an ultrasonic bath to extract the fraction adsorbed on the SS, and this SS extract was then mixed with the MeOH sample eluted from the cartridge. The mixed MeOH sample was dried under a gentle stream of nitrogen gas and was then dissolved in 1 mL of dimethyl

sulfoxide (DMSO). This DMSO sample was subjected to the yeast two-hybrid assay. It should be noted that some portions of the degradation products could possibly pass through the C_{18} cartridge. In order to overcome this problem, we developed a MFF procedure in this study, as follows. Firstly, about 20 mL of sample was filtered through a GF/F (Whatman 1.0-μm pore size), and yeast extract and glucose were then added to the filtrate. Sterilized SD medium (without yeast extract and glucose) was then mixed with the filtrate. This mixed filtrate was passed through a sterilized membrane filter (Toyo Roshi kaisha Ltd., 0.2-μm pore size). The MFF filtrate was subjected to the yeast two-hybrid assay.

Estrogenicity screening using the yeast two-hybrid assay

In order to evaluate the degradation effects of ASK and NSCM on estrogens, we investigated the estrogenic activity of samples using the yeast two-hybrid assay as described by Nishikawa *et al*. (1999), with detection wavelengths of 420 nm, 550 nm, and 620 nm. The two-hybrid yeast strain (Y190 *Saccharomyces cerevisiae*) was obtained from Professor Nishihara at Osaka University, Japan. The yeast two-hybrid assay system contains the estrogen receptor (ER) and the coactivator (TIF2), and the protein interactions between ER and TIF2 are strictly dependent on the presence of estrogens or some other endocrine-disrupting substances. β-galactosidase is then synthesized and secreted into the medium (Nishikawa *et al*., 1999). We can use this bioassay to investigate variations in estrogenic activity during estrogen degradation periods, regardless of the identity of the products.

Results and discussion

Estrogen degradation by ASK

Figure 1 shows changes in E1, E2, E3, and EE2 concentrations over time during the ASK cultivation period, where ASK degraded almost 100% of E1 in eight days, 100% of E2 in five days, and 100% of E3 in ten days, but only about 25% of EE2 in ten days, starting from initial concentrations of 20 to 25 mg/L. These results confirm that ASK degrades natural estrogens significantly, and degrades synthetic estrogen slightly. According to the degradation rates, as shown in Figure 2, the decreases in estrogen concentrations obey first-order reaction kinetics with degradation rate constants of 0.31 d^{-1} for E1, 1.14 d^{-1} for E2, and 0.44 d^{-1} for E3. Among the three natural estrogens, E2 was most easily degraded.

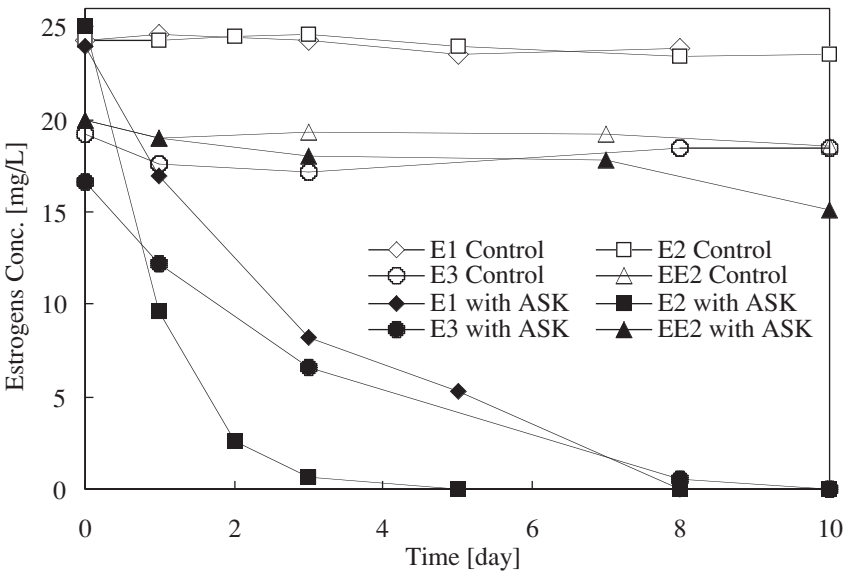

Figure 1 Changes in EE2, E1, E2, and E3 concentrations over the ASK cultivation period

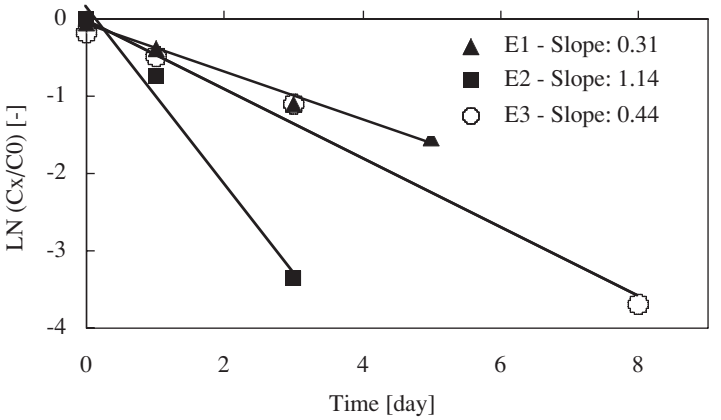

Figure 2 Degradation rates of E1, E2, and E3 by ASK

Estrogen degradation by NSCM

Figure 3 shows the changes in E1, E2, E3, and EE2 concentrations over time during the NSCM cultivation period, where NSCM degraded 97% of E1 in ten days, 100% of E2 in eight days, and 98% of E3 in 14 days, but only about 10% of EE2, starting from initial concentrations of 20 to 25 mg/L. These results confirmed that NSCM could degrade the three natural estrogens, but not EE2. As with the results obtained for degradation by ASK, the decreases in estrogen concentrations by NSCM degradation obeyed first-order reaction kinetics. Table 1 summarizes the degradation rate constants.

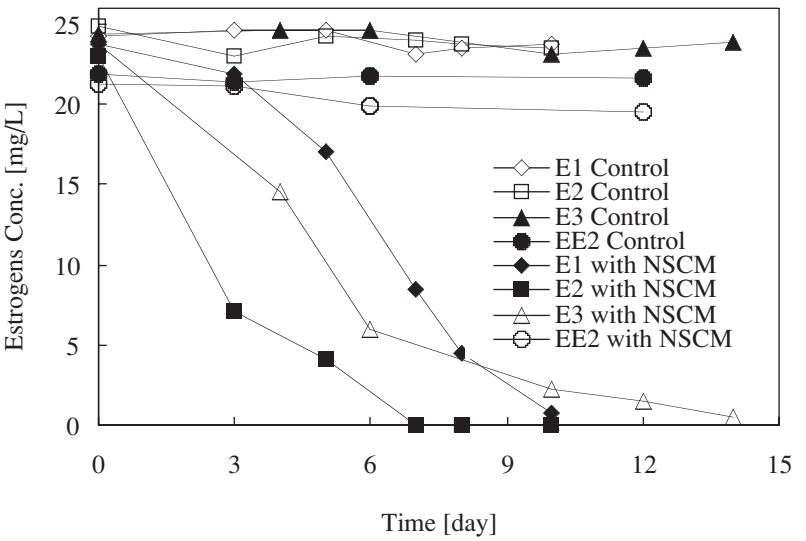

Figure 3 Changes in EE2, E1, E2, and E3 concentrations over the NSCM cultivation period

Table 1 Estrogen degradation rate constants by ASK and NSCM [d^{-1}]

Estrogen	With ASK	With NSCM
E1	0.31	0.20
E2	1.14	0.35
E3	0.44	0.27

Estrogen-degrading pathways using ASK and NSCM

As reported in the previous studies (Ternes *et al.*, 1999; Shi *et al.*, 2002), E1 was produced by ASK and NSCM during E2 degradation periods. Figure 4 shows changes in E1 and E2 concentrations during E2 degradation by ASK, where E1 was detected and then degraded further. E1 production from E2 degradation was confirmed using spiked tests in which E1 was added to the culture samples. Considering the amount of E1 produced from E2, we surmised whether or not E2 was consecutively degraded via E1, and then calculated concentrations of E1 using a consecutive first-order reaction equation. Since the calculated E1 values show a good agreement with the actual measured E1 values, these results indicate that E2 was consecutively degraded to other compounds via E1 by ASK and NSCM.

The RP-HPLC analysis showed unknown intermediate products of E1 and E3 that were eluted faster than the estrogens. Due to the elution characteristics of RP-HPLC and the detection mode of the electrochemical detector, it was surmised that these products are more polar compounds that contain a phenolic group. Furthermore, peak areas of these products decreased with increasing incubation time, indicating that their phenolic frames were cleaved as degradation proceeded.

Estrogenic activity screening

Table 2 shows the relative estrogenic activities of estrogen degradation products, as well as relative estrogenic activities of 10^{-5} mol L^{-1} of E1, E2, and E3. When E1, E2, and E3 were degraded completely and not detected after 14 days of incubation, the relative estrogenic activities of the sample were below the detection limit and the products derived from degradation did not appear to possess estrogenic activity, except that E2 by NSCM still retained 1% activities. These results confirm that ASK and NSCM not only significantly degrade natural estrogens, but also decrease estrogenic activities of the samples.

Comparison between two pretreatment procedures: SPE and MFF

When we prepared the sample using the SPE pretreatment procedure, suspended solids (SS) collected on GF/F were subjected to the yeast two-hybrid assay, while with the MFF pretreatment procedure, SS collected on GF/F were not subjected to the yeast two-hybrid assay. Due to possible adsorption of analytes that appear to possess estrogenic activity onto

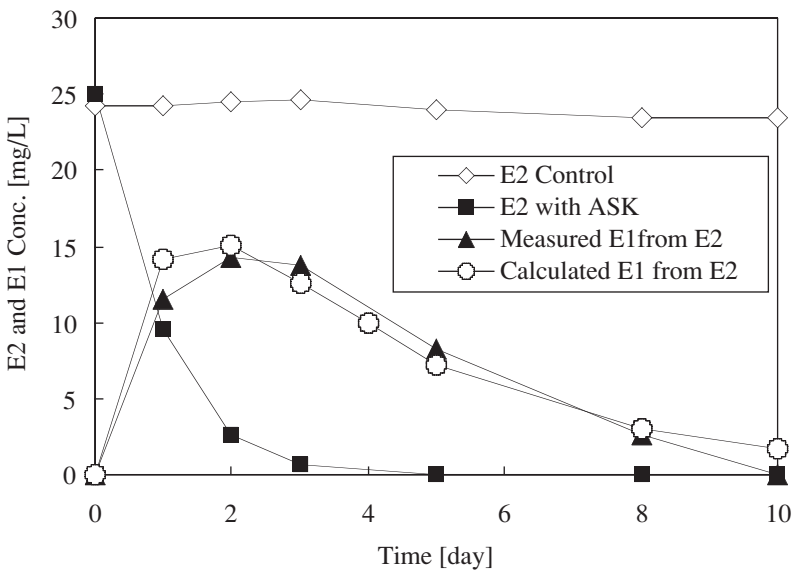

Figure 4 Prediction of E1 concentration during the E2-degradation period using ASK

Table 2 Relative estrogenic activities over ASK and NSCM degradation periods

Parent compounds	Pretreatment procedure	ASK control	Degradation by ASK (14 d)	NSCM Control	Degradation by NSCM (14 d)
E1	SPE	84 (±7)	–	78 ± 7	–
E2		109 (±10)	–	113 ± 11	1.1
E3		2.3 (±2)	–	2.6 ± 0.3	–
E1	MFF	73 (±5)	–	59 ± 6	–
E2		96 (±6)	–	90 ± 8	0.6
E3		1.5 (±0.5)	–	2.1 ± 1.0	–
E1 (10^{-5} molL^{-1})		80			
E2 (10^{-5} molL^{-1})		100			
E3 (10^{-5} molL^{-1})		2			

–: Negative activity

SS, we washed SS collected on a GF/F with 5 mL of MeOH and ultrasound in order to remove analytes adsorbed on the SS, and then analyzed the SS extract by HPLC. This confirmed that the extract contained 20%–40% of the total estrogens for those samples (data not shown). From this test, it was possible to assess the total estrogenic activity of estrogens in the samples prepared by the SPE procedure described here. This is why the relative estrogenic activities of controls processed by SPE are bigger than those of controls processed by MFF, as shown in Table 2.

With MFF, the estrogenic activities were low because of estrogens adsorbed on GF/F that were not subjected to the assay. With SPE, some estrogen products passed through the C_{18} cartridge that were not subjected to the assay. It is, therefore, recommended to do a hybrid MFF-SPE sample pretreatment to recover all estrogens and their products.

Conclusions

In this study, we demonstrated that ASK and NSCM not only significantly degrade natural estrogens, but also remove their estrogenic activity. In further work we would like to isolate the estrogen-degrading microorganisms from ASK and NSCM, and identify the estrogen degradation products.

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