Isolation and characterization of the ethynylestradiol-biodegrading microorganism *Fusarium proliferatum* strain HNS-1

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Abstract We cultivated hundreds of sediment, soil, and manure samples taken from rivers and farms in a medium containing ethynylestradiol (EE_2) as the sole source of carbon, so that microorganisms in the samples would acclimatize to the presence of EE_2. Finally, we isolated an EE_2-degrading microorganism, designated as strain HNS-1, from a cowshed sample. Based on its partial nucleotide sequence (563 bp) of the 28S rRNA gene, strain HNS-1 was identified as *Fusarium proliferatum*. Over 15 days, *F. proliferatum* strain HNS-1 removed 97% of EE_2 at an initial concentration of 25 $mg \cdot L^{-1}$, with a first-order rate constant of 0.6 d^{-1}. Unknown products of EE_2 degradation, which may be more polar compounds that have a phenolic group, remained in the culture medium.

Keywords Ethynylestradiol; *Fusarium proliferatum*; microbial degradation

Introduction

The synthetic estrogen ethynylestradiol (17α-ethynyl-1,3,5(10)-estratriene-3,17β-diol: EE_2) is not only a key ingredient in oral contraceptives used by Western women since the 1960s, but also a hormonal agent used in the stockbreeding industry. EE_2 is supposed to be metabolized as glucuronic acid conjugates in urine and released to the aquatic environment by hydrolysis of ester bonds by glucuronidase of microorganisms before or during the sewage treatment (Maggs *et al*., 1983; Tyler and Routledge, 1998).

EE_2 is considered to be incompletely removed by conventional secondary wastewater treatment processes that use aerobically activated sludge. For example, Ternes *et al*. (1999a) confirmed that an activated sludge in a batch experiment system removed only 20% of the initial amount of EE_2 (1 $ng \cdot mL^{-1}$) in 24 h. After 24 h to 48 h the concentration of the remaining EE_2 did not decrease further. In addition, EE_2 is frequently detected in effluents from municipal sewage treatment plants. In the UK, EE_2 was detected in sewage treatment effluent at 0.2 $ng \cdot L^{-1}$ (Desbrow *et al*., 1998), while EE_2 at 9 $ng \cdot L^{-1}$ was discharged from a Canadian plant (Ternes *et al*., 1999b).

Recently, as is the case with bisphenol A and nonylphenol, EE_2 has been suspected to be an endocrine-disrupting chemical that disturbs the hormonal systems of aquatic creatures. For example, a laboratory study on the endocrine-disrupting potency of EE_2 demonstrated that EE_2 at 1 to 10 $ng \cdot L^{-1}$ induced the production of vitellogenin in male rainbow trout (Purdom *et al*., 1994). Considering that vitellogenin indicates the existence of estrogenic stimulation in fish (Purdom *et al*., 1994), this study clearly showed that EE_2 could cause feminization of male fish. Therefore, it is essential that EE_2 be eliminated from the wastewater of stockbreeding enterprises.

The use of microorganisms capable of degrading EE_2 is one way of eliminating EE_2 from wastewater. Vader *et al*. (2000) showed a nitrifying activated sludge that could degrade EE_2 completely at an initial concentration of 50 $\mu g \cdot L^{-1}$ within 6 days. This study demonstrated the feasibility of using such sludge to remove EE_2 from wastewater, but no

Water Science and Technology **Vol 45 No 12 pp 175–179**

paper has yet reported the isolation of a microorganism capable of degrading EE_2. We therefore aimed to (i) isolate and identify a microorganism capable of degrading EE_2 and (ii) investigate its EE_2-degrading abilities.

Materials and methods

Isolation and identification of the EE_2-degrading microorganism

We collected hundreds of sediment, soil, and manure samples from farms and rivers throughout Japan. About 1 g of each sample was inoculated into 100 mL of medium (MEE_2) containing EE_2 as the sole source of carbon (EE_2, 25 $mg \cdot L^{-1}$; K_2HPO_4, 1 $g \cdot L^{-1}$; $(NH_4)_2SO_4$, 1 $g \cdot L^{-1}$; NaCl, 0.1 $g \cdot L^{-1}$; $MgSO_4 \cdot 7H_2O$, 0.2 $g \cdot L^{-1}$; $FeCl_3$, 0.01 $g \cdot L^{-1}$; $CaCl_2$, 0.1 $g \cdot L^{-1}$) so that the microorganisms in the samples would acclimatize to the presence of EE_2. The culture flasks were cultivated on a reciprocal shake at 120 strokes min^{-1} at 30°C. After several enrichment cultivations, the resulting acclimatized microorganisms were separated on a medium obtained by adding agar (15 $g \cdot L^{-1}$) to the MEE_2. Finally, we obtained an EE_2-degrading microorganism named as strain HNS-1. To identify strain HNS-1, analysis of the partial nucleotide sequence of the 28S rRNA gene of this bacterium was carried out by NCIMB Japan CO., LTD (Shizuoka, Japan).

Investigation of EE_2-degrading ability

Effect of pH on the EE_2 degradation was investigated by batch experiment. We inoculated 5 mL of pre-cultured solution of strain HNS-1 into MEE_2 of which the initial pH had been adjusted to 4.5–8 by using phosphoric acid salt KH_2PO_4 and Na_2HPO_4. The culture flasks of strain HNS-1 were then shaken at 120 strokes$\cdot min^{-1}$ at 30°C for a cultivation period of 20 days.

During the cultivation period, the EE_2 concentration, pH and turbidity were measured. To measure turbidity, we placed 5 mL of culture medium in a turbidity meter (MODEL T-2600D, Tokyodenshoku, Japan). Samples for measurement of EE_2 concentration were prepared by adding CH_3CN to the remaining culture medium at a volume ratio of 1:1 to dissolve EE_2 uniformly. Following filtration (filter pore size 0.22 μm), the sample (100 μL) was subjected to reverse-phase high-performance liquid chromatography (RP-HPLC) on an ODS column (TSK gel ODS 80TM, Tosoh, Japan) equipped with an electrochemical detector (1049A, Hewlett Packard) that detects EE_2 by oxidizing its phenolic group. In the RP-HPLC analysis, elution was carried out by using 45% v/v acetonitrile/water at a flow rate of 1.0 $mL \cdot min^{-1}$. The pH of cultures was measured with a pH meter.

Results and discussion

Isolation and identification of the EE_2-degrading microorganism

As the result of acclimatization, we obtained from a cowshed sample a microbial consortium that could degrade 94% of EE_2 at an initial concentration of 25 $mg \cdot L^{-1}$ in 20 days (data not shown). By separating this consortium on MEE_2–agar medium, we obtained an EE_2-degrading microorganism, strain HNS-1. This strain formed pale yellow colonies fringed with hair-like structures on the MEE_2 agar-medium plate (Figure 1). Microscopic observation showed conidiophores and mycelia, leading us to conclude that the strain was a fungus. Table 1 shows the observed characteristics of strain HNS-1, which indicate that strain HNS-1 could be *Acremonium* sp. or *Fusarium* sp. (Malloch, 1981).

Table 2 shows the partial nucleotide sequence (563 bp) of the 28S rRNA gene of strain HNS-1, which matched 100% with that of the fungus *Fusarium proliferatum* (BLAST accession nos. AJ271215 and AF0603882). Conclusively, strain HNS-1 was identified as *F. proliferatum*. As far as we know, this is the first study to have succeeded in isolating and identifying an EE_2-biodegrading microorganism.

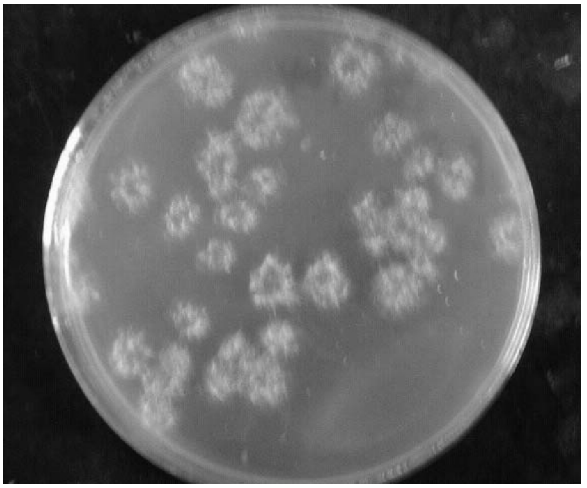

Figure 1 Colonies of strain HNS-1

Table 1 Characteristics of strain HNS-1

Mycelium color	Pale yellow
Mycelium size	hair-like shape, 3–4 μm diameter
Mycelium septum	Regular
Conidiophore	Acremonium
Conidia	Single-celled
Ramus	Negative
Chlamydospore	Negative
Colony size	5–10 mm diameter

Table 2 Partial nucleotide sequence (563 bp) of the 28S rRNA gene of strain HNS-1

AAACCAACAG	GGATTGCCCT	AGTAACGGCG	AGTGAAGCGG	CAACAGCTCA	AATTTGAAAT	60
CTGGCTCTCG	GGCCCGAGTT	GTAATTTGTA	GAGGATACTT	TTGATGCGGT	GCCTTCCGAG	120
TTCCCTGGAA	CGGGACGCCA	TAGAGGGTGA	GAGCCCCGTC	TGGTTGGATG	CCAAATCTCT	180
GTAAAGTTCC	TTCGACGAGT	CGAGTAGTTT	GGGAATGCTG	CTCTAAATGG	GAGGTATATG	240
TCTTCTAAAG	CTAAATACCG	GCCAGAGACC	GATAGCGCAC	AAGTAGAGTG	ATCGAAAGAT	300
GAAAAGCACT	TTGAAAAGAG	AGTTAAAAAG	TACGTGAAAT	TGTTGAAAGG	GAAGCGTTTA	360
TGACCAGACT	TGGGCTTGGT	TAATCATCTG	GGGTTCTCCC	CAGTGCACTT	TTCCAGTCCA	420
GGCCAGCATC	AGTTTTCCCC	GGGGGATAAA	GACTTCGGGA	ATGTGGCTCT	CTTCGGGGAG	480
TGTTATAGCC	CGTTGTGTAA	TACCCTGGGG	GGGACTGAGG	TTCGCGCATC	TGCAAGGATG	540
CTGGCGTAAT	GGTCATCAAC	GAC				563

EE_2-degrading ability of *F. proliferatum* strain HNS-1

Figure 2 shows the time variation in turbidity and EE_2 concentration when *F. proliferatum* strain HNS-1 biodegraded EE_2 to grow in the culture medium (at pH 7.2). *F. proliferatum* strain HNS-1 could remove 97% of EE_2 at an initial concentration of 25 $mg \cdot L^{-1}$ in 15 days at a first-order rate constant of 0.6 d^{-1}. It should be noted that the RP-HPLC analysis showed unknown products of EE_2-degradation that were eluted faster than EE_2 (Figure 3). Because of the elution characteristics of RP-HPLC and the detection mode of the electrochemical detector, we hypothesized that these products of EE_2 degradation may be more polar compounds that have a phenolic group. Future research is needed to determine whether these products have endocrine-disrupting activity and how they can be degraded.

The pH value of the culture medium is known to affect microbial activity. To eliminate EE_2 with *F. proliferatum* strain HNS-1, it is important to determine the optimum pH value for EE_2 biodegradation. Therefore, we decided to investigate the effect of pH on

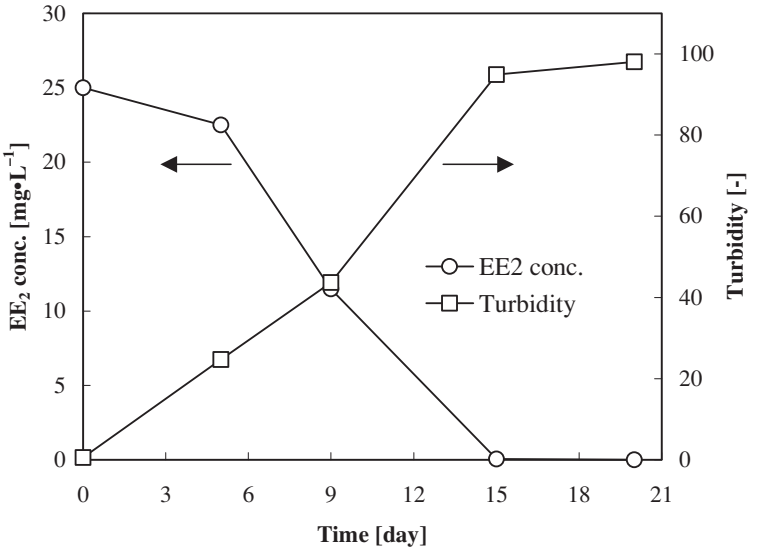

Figure 2 Degradation of EE_2 by *F. proliferatum* strain HNS-1

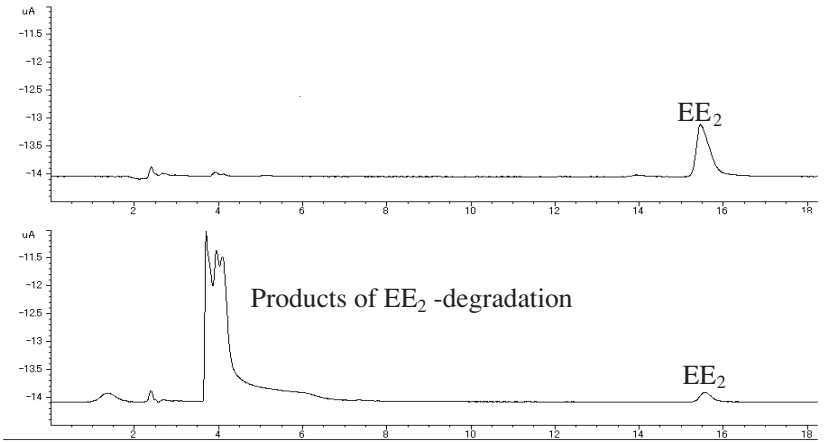

Figure 3 RP-HPLC chromatogram of the culture medium of *F. proliferatum* strain HNS-1

EE_2-biodegradation activity by using pH-adjusted media from 4.5 to 8.0. Because the decrease of EE_2 concentration in the culture medium obeyed good first-order kinetics, we calculated first-order EE_2 degradation rate based on the time variation of EE_2 concentration. The effect of the initial pH value of the culture medium on EE_2-biodegradation rate is shown in Figure 4.

Because the highest EE_2-biodegradation rate was obtained at pH 7.2, we suggest that maintaining the culture of *F. proliferatum* strain HNS-1 at pH 7.2 would maintain a high level of EE_2-biodegradation activity.

Conclusions

We isolated an EE_2-degrading fungus, strain HNS-1, from a manure sample. Based on its partial nucleotide sequence (563 bp) of the 28S rRNA gene, strain HNS-1 was identified as *Fusarium proliferatum*. *F. proliferatum* strain HNS-1 degraded 97% of EE_2 at an initial concentration of 25 $mg \cdot L^{-1}$ in 15 days and a initial pH of 7.2, with a first-order rate constant

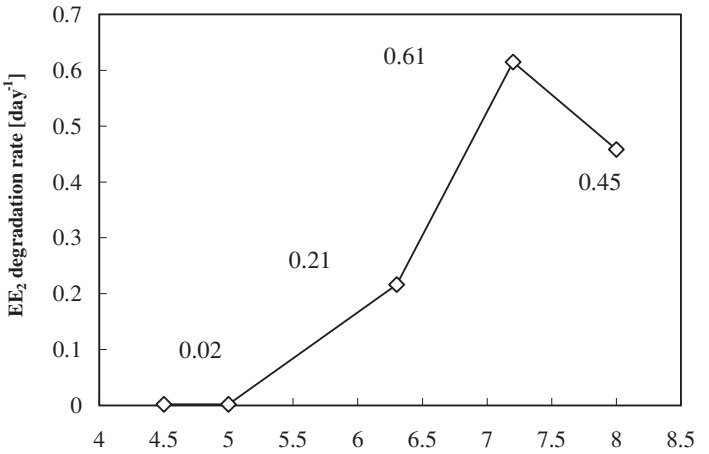

Figure 4 Effect of initial pH value on EE_2-degradation activity of strain HNS-1

0.6 d^{-1}, providing evidence that strain HNS-1 has the potential to effectively remove EE_2 from wastewater discharged from stockbreeding enterprises.

Products of EE_2 degradation, which may have been more polar compounds with a phenolic group, were still present. The identification of these products and the investigation of their endocrine-disrupting ability are critical areas for future research.

Acknowledgement

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