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ORIGINAL PAPER

17α-ethinylestradiol cometabolism by bacteria degrading estrone, 17β-estradiol and estriol

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Abstract 17α-ethinylestradiol (EE2), the active compound of the contraceptive pill, is a recalcitrant estrogen, which is encountered at ng/l levels in wastewater treatment plant (WWTP) effluents and rivers and can cause feminization of aquatic organisms. The aim of this study was to isolate microorganisms that could remove such low EE2 concentrations. In this study, six bacterial strains were isolated from compost that cometabolize EE2 when metabolizing estrone (E1), 17β-estradiol (E2) and estriol (E3). The strains belong to the α, β and γ-*Proteobacteria*. All six strains metabolize E2 over E1, at μg/l to ng/l concentrations. In 4 days, initial concentrations of 0.5 μg E2/l and 0.6 μg EE2/l were degraded to 1.8 ± 0.4 ng E2/l and 85 ± 16 ng EE2/l, respectively. No other metabolites besides E1, E2, E3 or EE2 were detected, suggesting that total degradation and cleavage of the aromatic ring occurred. This is the first study describing that bacteria able to metabolize E2, can subsequently cometabolize EE2 at low μg/l levels.

Keywords Estrogen · Ethinylestradiol · Cometabolism · *Acinetobacter* sp. · *Pseudomonas* sp.

Abbreviations

E1	Estrone
E2	Estradiol
E3	Estriol
EE2	Ethinylestradiol
HRT	Hydraulic residence time
SRT	Sludge retention time
WWTP	Wastewater treatment plant

Introduction

Natural estrogens such as estrone (E1), estradiol (E2), estriol (E3) and the synthetic ethinylestradiol (EE2) (Fig. 1), the active compound of the contraceptive pill, have been reported as the main causal factors of estrogenic activity in wastewater treatment plant (WWTP) effluents. E2 and EE2 and, to a minor extent E1, are considered to be the most important contributors (Thomas et al. 2001; Johnson and Williams 2004). These compounds can cause reproductive disorders in aquatic organisms at concentrations

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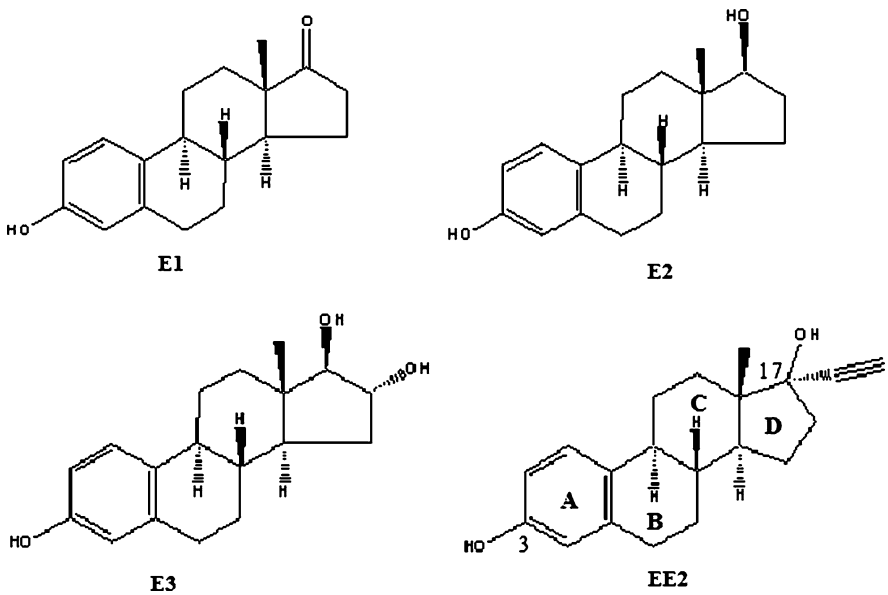

Fig. 1 Chemical structures of estrone (E1), estradiol (E2), estriol (E3) and ethinylestradiol (EE2). A, B, C and D refer to the ring nomenclature, 3 and 17 refer to the C-atom on which the functional group is placed

ranging from 0.1 to 1 ng/l. Human urine is considered as the main source of natural and synthetic estrogens in sewage. Maximum measured levels of E2 are 150 ng/l in domestic wastewater (Vethaak et al. 2002) and 64 ng/l in domestic wastewater treatment plant (WWTP) effluent (Ternes et al. 1999a). Free EE2 has been detected in domestic wastewater at maximum levels of 7 ng/l (Cargouet et al. 2003), and in WWTP effluent up to 42 ng/l (Ternes et al. 1999a). EE2 can be set free from conjugated glucuronide or sulphate complexes present in urine (Panter et al. 1999). Furthermore the high levels, above the NOEC (no observed effect concentration), in WWTP effluents indicate poor overall degradation of EE2 under normal WWTP conditions.

Several authors (Joss et al. 2004; Clara et al. 2004; Andersen et al. 2003; Johnson et al. 2005) have examined the mechanism of estrogen removal in WWTPs. For E2 the removal efficiency ranges from 30% (Adler et al. 2001) to >98% (Andersen et al. 2003). Several studies (e.g. Holbrook et al. 2004) claim that sorption is important to remove estrogens from the liquid phase. However, Andersen et al. (2003) clearly contradicted these findings. These authors state that sorption is of minor importance compared to degradation for the eventual removal of steroid estrogens in WWTPs. An increased hydraulic retention time (HRT) and sludge retention time (SRT) increases the amount of E1 removal, and enhances the removal of other biodegradable estrogens as well (Joss et al. 2004; Johnson et al. 2005). For EE2, removal efficiencies varying from 34% (Cargouet et al. 2003) to almost 100%, i.e. below the detection limit (Vethaak et al. 2002) have been reported. A relationship between SRT and EE2 removal could not be established by Clara et al. (2004).

A number of research groups identified microorganisms capable of E1, E2, E3 and EE2 transformation. Biodegradation of the natural estrogens E1, E2 and E3 by *Nitrosomonas europaea* (Vader et al. 2000; Shi et al. 2004a, Yi et al. 2006), *Ralstonia* sp., *Achromobacter xylosoxidans* (Weber et al. 2005), *Rhodococcus zopfii* and *Rhodococcus equi* isolates (Yoshimoto et al. 2004) and by an unidentified Gram negative bacterium (Fujii et al. 2002) was demonstrated. During E2 degradation, most strains formed E1 and subsequently biodegraded it. All these strains were isolated from activated sludge. Additionally, some strains were successful in biodegrading EE2. These strains comprise *Nitrosomonas europaea*, *Rhodococcus zopfii* and *Rhodococcus equi, Sphingobacterium* sp. JCR5 (Haiyan et al. 2007) and a fungus *Fusarium proliferatum* (Shi et al. 2002). Haiyan et al. (2007) proposed a degradation pathway for *Sphingobacterium* sp. JCR5 in which EE2 is directly oxidized to E1 with a subsequent cleavage of the B-ring and the A-ring to unsaturated acids which are further

mineralized to CO_2 and water. Up to now, the described organisms metabolize natural and/or synthetic estrogens at mg/l levels. Moreover, it is not clear whether the degradation of EE2 is related to that of E2.

The goals of this work are: (1) the isolation of EE2 transforming bacteria in the presence and absence of natural estrogens; (2) examine the metabolism at high (mg/l) and low (µg–ng/l) concentrations; (3) examine the cometabolic properties of isolated strains. This last goal is of interest since E1, E2, E3 and EE2 generally coincide in wastewater due to their simultaneous excretion in urine by women using the contraceptive pill.

Experimental procedures

Minimal medium composition

The minimal medium used in this study was Stanier medium (Stanier et al. 1966). After medium preparation, the estrogens (E1, E2, E3, EE2, according to the experiment), were dissolved in the medium in autoclaved 2 l Schott bottles to obtain the desired concentration. Concentrated stocks in ethanol were allowed to evaporate in the Schott bottles; afterwards the medium was added and stored in a 80°C incubator for 5 days to allow for a good dissolution. No loss of compounds was observed by the dissolution procedure, as verified by HPLC.

Sampling procedure

Samples of liquid cultures were dispersed by purging 4 ml sample (3 times) through a sterile syringe of 10 ml with a needle. Afterwards, a 0.22 µm filter (Millipore, Cork, Ireland) was put on the syringe and 3 ml of the liquid was discarded. The final 1 ml was subsequently analyzed by HPLC (High Performance Liquid Chromatography) within 24 h.

Isolation procedure

The isolation procedure was as follows: 1 l Erlenmeyer flasks containing 50 mg EE2/l were inoculated with 1 g of fresh compost, activated sludge originating from a domestic wastewater treatment plant or faeces, respectively, and incubated for 1 month at 28°C on a rotary shaker (100 revolutions per minute). Since only the compost enrichment showed EE2 removal, this culture was plated on LB (Luria Bertani) medium (5 g NaCl, 5 g yeast extract, and 10 g tryptone dissolved in one litre of ultrapure water) containing 10 mg E2/l and 10 mg EE2/l in a tenfold dilution series in order to obtain single colonies. Three plates showed different colony morphologies. Different type colonies were picked up and plated on LB medium containing 5 mg E2/l and 5 mg EE2/l yielding ten subcultures. These colonies were subsequently subcultured in liquid mineral medium containing 5 mg E2 and EE2/l to yield the isolates BP 1 to 10.

Repetitive extragenic palindromic polymerase chain reaction (rep-PCR)

DNA of the ten isolates was extracted using a wizard genomic DNA extraction kit (Promega Inc., Madison WI, USA) according to manufacturers' instructions. BOX-PCR fingerprints were obtained with the BOXA1R primer as described by to Seurinck et al. (2003).

Phylogenetic identification of isolates

The DNA of the six isolates with unique BOX-PCR patterns (BP 1, 2, 3, 7, 8 and 10) was obtained by using a wizard genomic DNA extraction kit (Promega Inc., Madison WI, USA) according to manufacturers' instructions. The 16S rRNA gene fragments were obtained by amplifying the 16S rRNA gene with primers P63f and P1378r (Øvreas et al. 1997). DNA sequencing of the PCR fragments was carried out by ITT Biotech-Bioservice (Bielefeld, Germany). Analysis of DNA sequences and homology searches were completed with standard DNA sequencing programs and the BLAST server of the National Center for Biotechnology Information (NCBI) using the BLAST algorithm (Altschul et al. 1997). 16S rRNA sequences for the isolates have been deposited in the GenBank database under the accession numbers EF198468 to EF198473.

E1, E2, E3 and EE2 degrading capacity of isolated strains

Analogous to the procedure as described above, liquid cultures of strains BP 1, 2, 3, 7, 8 and 10 were exposed to the three natural estrogens estrone (E1), estradiol (E2) and estriol (E3) and to the synthetic EE2. Therefore, 5 ml of a liquid culture in E2/EE2 containing medium (theoretical concentration 5 mg/l of each natural estrogen, no cosolvent) was inoculated in 200 ml sterile minimal medium containing 5 mg E1/l, 5 mg E2/l and 5 mg E3/l in 250 ml Erlenmeyer flasks. A flask containing 1 mg EE2/l was also inoculated with these cultures These flasks were wrapped in aluminium foil and, were incubated at 28°C on a rotary shaker (100 rpm). The E1, E2, E3 and EE2 concentrations were monitored over time (0, 0.25, 6, 24 and 48 h) with HPLC with UV and fluorescence detection.

EE2 cometabolic degrading capacity of selected strains

Liquid cultures of strains BP 1, 2, 3, 7, 8 and 10 were set up in autoclaved 250 ml Erlenmeyer flasks. The sterile medium containing 5 mg E2/l and 5 mg EE2/l was inoculated with 5 ml of the culture. The flasks were wrapped in aluminium foil, put on a rotary shaker (100 rpm) which was located in a dark room at 28°C.

The E2 and EE2 concentration were monitored over time with HPLC with fluorescence detection (method see above). After 7 days, the medium was supplemented with 100 ml fresh sterile minimal medium containing 5 mg/l of E2 in a sterile Erlenmeyer flask. After 15 and 29 days, the medium was transferred to a new sterile Erlenmeyer flask which contained E2 originating from a concentrated ethanol (100% pure, Sigma, Bornem, Belgium) stock. The latter was prepared as follows: 1.25 ml of a 1 g E2/l ethanol stock (absolute ethanol, Sigma, Bornem, Belgium) was first added to a sterile 250 ml Erlenmeyer flask and allowed for complete evaporation in a laminar flow cabinet overnight. Finally the cultures were added and the flask was filled with minimal medium to contain 200 ml of medium and culture. The amount of ethanol stock was calculated to yield a final E2 concentration of 5 mg/l in 200 ml medium.

In this way, three additions of E2 were established. After 9 days the EE2 concentration should theoretically have been halved due to the dilution with an equal volume of fresh medium, with a final E2 concentration of 2.5 mg/l. After 15 and 29 days, the final E2 concentration was 5 mg/l. Samples were taken before and after the transfer (15 min later), while the newly prepared Erlenmeyer flasks were shaken vigorously prior to sampling. E2 and EE2 concentrations were measured during 38 days at 1 day time intervals.

Induction of EE2 cometabolism

A series of BP10 cultures were set up with different E2/EE2 ratios. A 10 ml liquid BP10 culture in minimal medium from the previous experiment (OD_{610} about 1) was inoculated in 190 ml sterile minimal medium in 250 ml Erlenmeyer flasks. E2 and EE2 concentrations in the inoculum were below detection limit of 10 μg/l for each estrogen (data not shown). The initial EE2 concentration in the medium was 1 mg EE2/l. The initial E2 concentration was varied to yield E2/EE2 ratios (w/w) 0, 0.1, 0.5, 1 and 5. The E2 and EE2 concentrations were measured after 0, 2.5, 5, 17, 24, 48, 72, 120, 144 and 168 h.

Growth related degradation

In order to distinguish the kinetics of degradation in relation with bacterial growth, aliquots of 200 ml of sterile minimal medium containing 5 mg E2/l, 5 mg E2/l and 1 mg EE2/l and 5 mg E1/l and 1 mg EE2/l were inoculated with 100 μl of strain BP10 (about log 6 colony forming units (CFU)/ml). The Erlenmeyer flasks were wrapped in aluminium foil and incubated in the dark at 28°C on a rotary shaker (100 rpm). The degradation profile was monitored for 190 h.

ATP and OD measurement

In the experiment investigating the growth related degradation, the ATP concentration was measured with an ATP kit (Bac Titer Glo™, Promega,

Madison, USA) based on luminometry (Lumac Biocounter M2500, Landgraaf, the Netherlands) according to the manufacturers' instructions. Furthermore, bacterial growth was verified by measuring the OD_{610} (optical density at 610 nm) with an absorption meter (Dr Lange). A similar experiment using the same concentrations and methods was set up with E3 and EE2 (data not shown).

Degradation assay at lower concentrations

In order to evaluate the degradation of E2 and EE2 at lower, environmentally more relevant concentrations, a degradation assay with strain BP10 and an E2 concentration of 500 ng/l and EE2 concentration of 100 ng/l was set up. Therefore, strain BP10 was grown for 2 days on 5 mg E2/l and 1 mg EE2/l. This culture was centrifuged at 5,000*g*, the supernatant was decanted, and the pellet (about log 8–9 CFU) was resuspended in sterile physiological solution (8.5 g NaCl/l Milli Q water). This procedure was repeated three times, and the final pellet was suspended in 20 ml of sterile physiological solution. This suspension was inoculated in a glass bottle containing 20 l of Stanier medium with 500 ng E2/l and 100 ng EE2 /l as the sole carbon sources. The culture liquid was aerated with an air pump. Triplicate 0.5 l samples were taken on day 0, 2, 4, 7 and 10 and analyzed for estrogens according to Noppe et al. (2005). In brief, the samples were extracted with Speedisks (Baker-bond, Deventer, the Netherlands), eluted with acetone and methanol, evaporated until dryness and reconstituted in ethanol. These ethanol solutions were subjected to GC-MS-MS analysis. The cleanup step as mentioned in Noppe et al. (2005) was omitted. An uninoculated culture medium served as control and was analyzed at day 0 and day 10. No degradation of the compounds was observed in the uninoculated culture medium.

Analytical determination of estrogens

High Performance Liquid Chromatography (HPLC) analysis was performed with a Dionex HPLC (Sunnyvale, CA, USA) with an autosampler ASI-100, pump series P580, STH585 column oven, a UV–VIS detector UVD340S and a RF2000 fluorescence detector. Of the sample 50 µl was injected and eluted over a Genesis C18 column (150 × 4.6 mm, 4 µm) (Jones Chromatography, UK). The column temperature was set at 25°C and the flow rate was maintained at 1 ml/min. Solvent A was acetonitrile–acetate (100:0.1 v/v, both Sigma, Bornem, Belgium) and solvent B was water–acetate (100:0.1 v/v). Elution was as follows: At time 0 the solvent composition was 55% A and 45% B, which was linearly decreased to 38% A after 10 min. Afterwards, a linear increase to 55% A was performed until time 15 min. An isocratic elution was kept until 20 min. UV detection was applied at 205 nm. Fluorescence excitation occurred at 230 nm, emission at 290 nm. For E2, E3 and EE2 the fluorescence signal was used to detect the estrogens, for E1 UV detection was applied, since this gave a higher signal. Standard curves for individual estrogens (E1, E2, E3, EE2) were set up with concentrations of 10, 20, 50, 100, 200, 500, 1,000, 2,000 and 5,000 µg/l minimal medium. Measurements were performed in triplicate, all standard curves showed a correlation with $R^2 = 0.99$ in all cases. The detection limit for all estrogens was set at 10 µg/l since this was the lowest standard measured. Triplicate measurements of estrogens in minimal medium showed standard deviations <5% at different concentrations.

In order to reveal the presence of metabolites of E2 and EE2 degradation, filtersterilized (0.22 µm) samples of culture medium taken after 38 days were subjected to GC-MS-MS (gas chromatography tandem mass spectrometry) and LC-MS-MS (liquid chromatography tandem mass spectrometry) according to Noppe et al. (2005).

Results

Isolation and phylogenetic identification of EE2 degrading strain

The enrichment culture inoculated with compost showed more than 90% EE2 removal. This culture was plated on 1/10 LB medium containing E2 and EE2. Ten different isolates were selected for further analysis. Box-PCR analysis was performed to determine unique strains (data not shown). Six unique strains could be discriminated on their specific Box-PCR patterns. Since BP 4, 5, 6 were identical to BP 3

and since BP 9 was identical to BP 8, further tests were only performed with the specific strains BP 1, 2, 3, 7, 8 and 10. Partial 16S rRNA sequencing of the six strains showed that all strains belonged to the *Proteobacteria* (Table 1). All strains could be identified with high similarity to the genera of *Phyllobacterium*, *Ralstonia*, *Pseudomonas* and *Acinetobacter*, respectively.

E1, E2, E3 and EE2 degrading capacity of the isolated strains

The six strains were cultured in shake flasks. As a first experiment, 3.2 ± 0.2 mg/l of E1; 2.3 ± 0.2 mg/l of E2; and 3.6 ± 0.1 mg/l of E3 were added together as the only carbon and energy source. After 6 h the removal efficiencies for the six cultures ranged from 16 to 23% for E1, 12 to 15% for E2 and 1 to 2% for E3. After 24 h E1 and E3 were removed below detection limit (i.e. 10 μg/l), which corresponds to removal efficiencies of >99%. After 48 h, none of the three natural estrogens could be detected in the culture media of the respective strains. Abiotic controls showed no significant E1, E2 or E3 removal.

In a second experiment 1.0 ± 0.1 mg/l of EE2 ($n = 3$) was spiked to the cultures as the only carbon and energy source. No removal was observed for at least 500 h (= 21 days). An abiotic control showed no significant changes in EE2 concentration.

These two sets of experiments thus demonstrated that all strains only showed metabolic degradation of the natural estrogens, while the synthetic EE2 was not converted when dosed as sole carbon source.

EE2 cometabolic degrading capacity of selected strains

It was examined if the strains BP 1, 2, 3, 7, 8, and 10 could cometabolize EE2 in the presence of natural estrogens. All strains showed an EE2 removal up to 97% in the presence of 5 mg E2/l, even after three added doses of E2 (Fig. 2). This suggests that no intermediates inhibitory to the cultures were formed. The EE2 concentration at day 7 had decreased by about 50% due to the supplementation of 100 ml additional sterile medium (Fig. 2b). First order kinetic equations were fitted to the degradation patterns 0–7 days, 15–29 days and 29–38 days, and half-lives ($t_{1/2}$) ranging from 5.5 h to 14 days were calculated ($R^2 \geq 0.95$). The half-lives tended to decrease during the time course of the experiment. This was in concordance with an increase in OD_{610} in the cultures. The latter increased from 0.01 at time 0 days to 0.4–0.7 on day 15, and to 1.2–1.4 on day 29. Upon further incubation, the cells started to grow in small flocs.

E1 was qualitatively detected with HPLC. No other metabolites could be detected with the given chromatographic method. The filtered samples of culture medium taken after 38 days were analyzed with GC-MS-MS and LC-MS-MS to reveal the presence of potential metabolites of E2 and EE2. No additional peaks appeared in all samples.

Induction of EE2 cometabolism

The removal of both E2 and EE2 by a culture of strain BP10 was examined for different E2/EE2 ratios (Fig. 3).

Table 1 Overview of the isolated bacterial species that can degrade E2 and EE2

Isolate	Closest match[a] (accession nr.)	Classification[b]	Percentage similarity
BP1	*Phyllobacterium myrsinacearum* (AY785315)	Alpha-*Proteobacteria*	100 (929/929)
BP2	*Ralstonia pickettii* (AY268180)	Beta-*Proteobacteria*	99 (1247/1248)
BP3	*Pseudomonas aeruginosa* (AY738263)	Gamma-*Proteobacteria*	99 (1240/1241)
BP7	*Pseudomonas* sp. (DQ303435)	Gamma-*Proteobacteria*	99 (1238/1242)
BP8[c]	*Acinetobacter* sp. (CR543861)	Gamma-*Proteobacteria*	99 (1232/1244)
BP10[c]	*Acinetobacter* sp. (CR543861)	Gamma-*Proteobacteria*	99 (1222/1229)

[a] Based on nearest BLAST homology results

[b] Based on RDP-II query results

[c] Similarity between BP8 and BP10 was 99% (1224/1229)

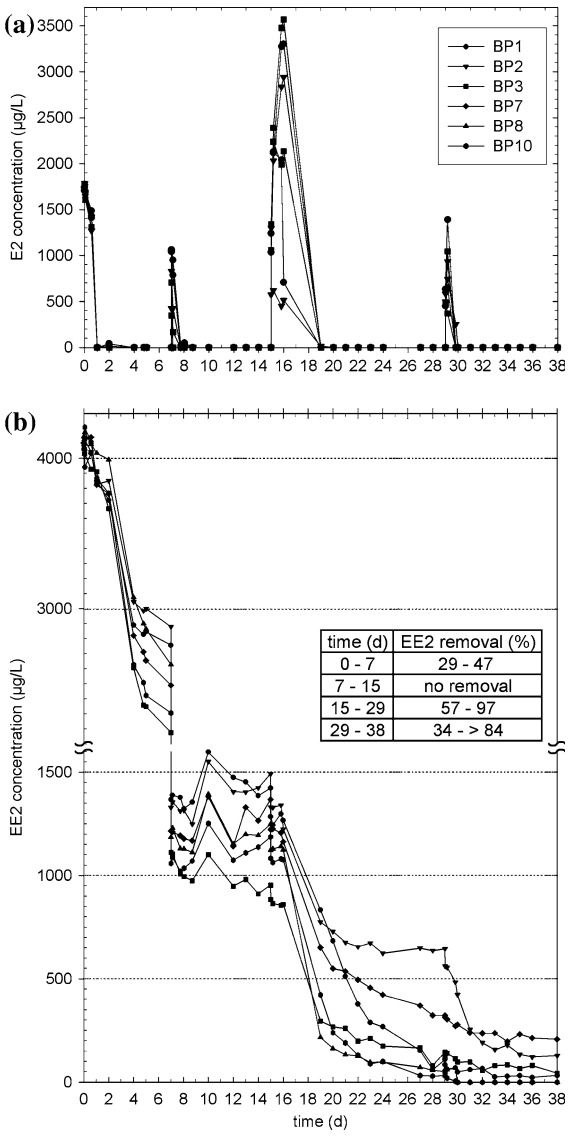

Fig. 2 E2 and EE2 degrading capacity of the selected strains BP 1, 2, 3, 7, 8 and 10; (**a**) E2 concentration in function of time, (**b**) EE2 concentration in function of time (fresh E2 medium addition on day 7, 15 and 29)

EE2 was dosed in all cultures at an initial concentration of 1 mg/l. For all ratios E2 was removed between 48 and 120 h (Fig. 3a). The half-lives derived from first order equations fitted to the E2 removal curves are 46 ± 6 h ($k = 0.015 \pm 0.002$ h^{-1}; $R^2 = 0.95$), 19 ± 5 h ($k = 0.036 \pm 0.009$ h^{-1}; $R^2 = 0.99$), 18 ± 3 h ($k = 0.039 \pm 0.006$ h^{-1}; $R^2 = 0.97$) and 17 ± 4 h ($k = 0.04 \pm 0.01$ h^{-1}; $R^2 = 0.89$) for E2/EE2 ratios 0.1, 0.5, 1 and 5, respectively. The EE2 cometabolic removal was related

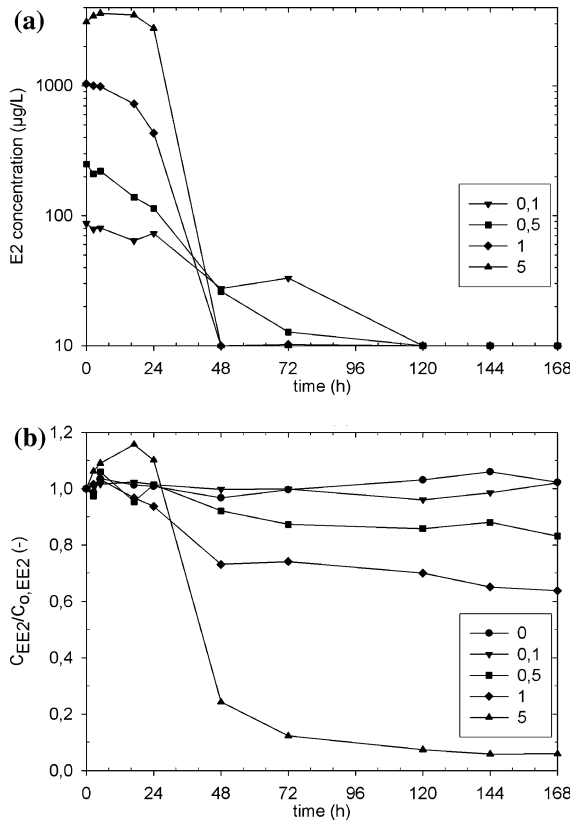

Fig. 3 (**a**) E2 concentration profile over time at different E2/EE2 ratios, (**b**) EE2 concentration profile over time at different E2/EE2 ratios

to the initial E2 concentration. The higher the E2/EE2 ratio was, the higher was the EE2 removal (Fig. 3b). Exponential degradation functions could be fitted to higher E2/EE2 ratios of 1 and 5. The resulting half-lives were 41 ± 14 h ($k = 0.017 \pm 0.006$ h^{-1}) and 14 ± 3 h ($k = 0.05 \pm 0.01$ h^{-1}), respectively ($R^2 = 0.98$ in both cases).

Growth-related degradation

A degradation assay was performed to examine if the degradation of E2 could result in an increase of ATP by the cells (Fig. 4). ATP measurements were preferred to optical density (OD) measurement, because the bacteria grew in flocs, which made OD measurement unreliable. After about 50 h a removal of E2 was visible. During E2 transformation E1 was formed, but to a lower extent (about 50%) than expected from the stoichiometric conversion. Concomitantly a clear

increase in ATP concentration in the culture medium was observed. If an ATP content of 4 mg ATP/g CDW (cell dry weight) is assumed (Verstraete et al. 1983), a cellular yield coefficient of 0.2 g CDW/g E2 converted can subsequently be calculated. The E1 slowly decreased after reaching a maximal concentration of about 700 µg E1/L.

The degradation profile of a BP10 culture growing on 5 mg E2/l and also supplemented with 1 mg EE2/l is presented in Fig. 4b. A similar E1 production and ATP concentration increase ($Y = 0.1$ g CDW/g E2 converted) was observed as in the E2 degradation assay. The maximal measured ATP concentration was of the same order as in the E2 experiment. E1 and EE2 were removed from the culture liquid at the same time (onset occurred at 112 h) and at the same rate, i.e. 55% removal in 75 h.

Figure 4c shows the degradation profile of strain BP10 when E1 and EE2 were present in the culture liquid at 5 mg/l and 1 mg/l, respectively. From the beginning of the experiment a clear increase in ATP concentration was observed. The ATP concentration decreased after 112 h, with a concomitant decrease in E1 and EE2 concentration (k-value of 0.08 ± 0.01 h^{-1}; $Y = 0.06$ g CDW/g E1).

The E1, E2, EE2 and ATP concentration profiles of the other strains (BP 2, BP 3, BP 7 and BP 8) gave similar trends (data not shown). In the experiment with E3 as the substrate for EE2 biodegradation, a slow (>100 h) and simultaneous biodegradation of both estrogens was observed (data not shown).

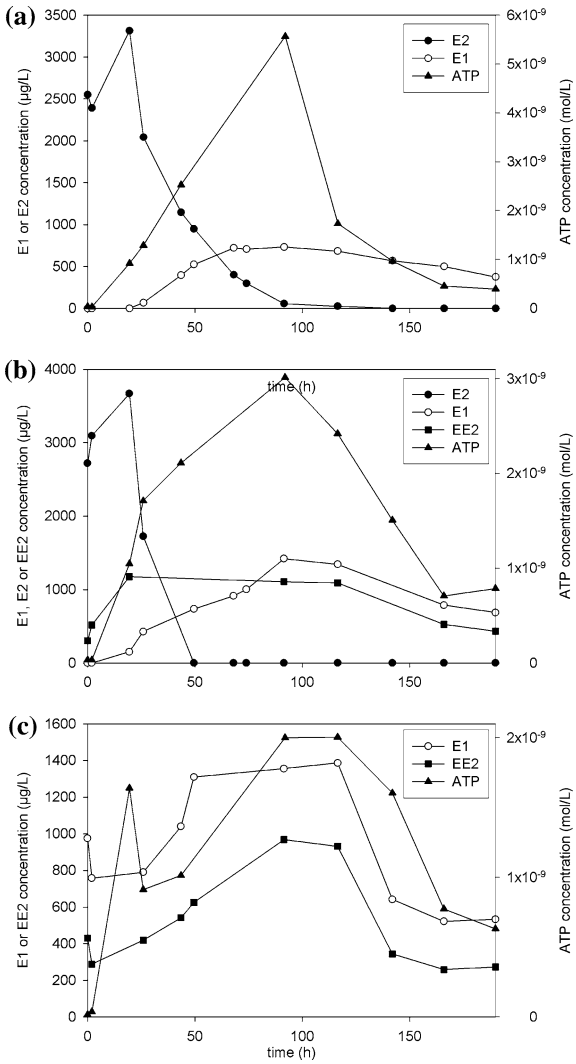

Fig. 4 Degradation profile of strain BP10 with (**a**) E2, (**b**) E2 and EE2 and (**c**) E1 and EE2 as the sole carbon sources

Degradation assay at lower concentrations

To examine the biodegradation capacity of the different strains, incubation experiments at the µg to ng/l level were performed. Doses of 0.5 µg E2/l and 0.6 µg EE2/l were degraded within 4 days (Fig. 5). E1 was formed on day 2 and subsequently degraded over time. The E2, EE2 and E1 concentrations at day 10 were 1.8 ± 0.4 ng/l, 85 ± 16 ng/l and 262 ± 51 ng/l, respectively. E3 was degraded as well within 4 days to reach a concentration of about 51 ± 16 ng/l. Abiotic controls showed no significant changes (= >5%) in estrogen concentrations.

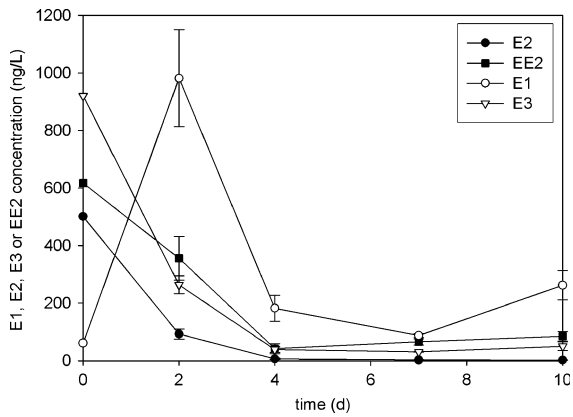

Fig. 5 E1, E2, E3 and EE2 concentration profile by a strain BP10 culture in 0.5 µg E2/l and 0.6 µg EE2/l

Discussion

In this work six bacterial strains were isolated from compost which were capable to biodegrade natural and synthetic estrogens. To the best of our knowledge this is the first report on cometabolism of EE2 induced by the structural analogues E1, E2 and, to a lower extent, E3. As evidenced by previous research E2 is converted to E1 under aerobic conditions (Ternes et al. 1999b). Subsequently E1 was removed from the culture liquid and no other biodegradation products were detected. For EE2, biodegradation products neither could be detected. This work describes biodegradation of estrogens at microgram per litre level by bacterial isolates. Ternes et al. (1999b) who worked with activated sludge could detect biodegradation of E2 and E1 but not EE2 at starting concentrations of 1 μg/l.

All the isolated strains belong to genera described to contain metabolic properties towards phenolic structures. *Ralstonia pickettii* strain has been previously described by Weber et al. (2005) as an E2, but not EE2 degrading bacterium. When E2 and EE2 were incubated together with this strain, cometabolism was not observed (Hollender, EAWAG, Switzerland, personal communication). *Phyllobacterium myrsinacearum* is known as a leaf associated nitrogen fixing bacterium. The other four isolates comprise members of the order of the *Pseudomonadales*, belonging to the genera *Pseudomonas* and *Acinetobacter*. These bacteria are well known for their capacity to degrade environmental pollutants (Feisti and Hegeman 1969; Kim and Hao 1999).

All strains were capable of degrading four subsequent doses of E2 with a concomitant removal of EE2 (Fig. 2). Ammonia oxidizing bacteria have been reported to cometabolize EE2 when oxidizing ammonia. This was associated to the aspecificity of the ammonium mono-oxygenase enzyme (Vader et al. 2000; Joss et al. 2004; Yi and Harper 2005, 2007). However, cometabolism of EE2 using E1, E2 or E3 as the inducing substrate has not been demonstrated to date. The E2 over EE2 concentration ratio is of importance for the occurrence of EE2 biodegradation, as shown in Fig. 3. Given the fact that the EE2 concentration was kept constant at 1 mg/l, a lower E2 concentration, and hence a lower E2/EE2 ratio, resulted in a lower k value for EE2 degradation and a lower overall EE2 degradation efficiency. The k-value for EE2 biodegradation of E2/EE2 ratio 5 was about 2.5 times the k-value of E2/EE2 ratio 1. An E2/EE2 ratio of 5 was needed to yield a 95% EE2 removal in 5 days, and an E2 removal below detection limit. It has to be stressed that sorption is considered to be of minimal importance in this removal process. This is based on the data for E2/EE2 ratio 0, where no significant removal of EE2 was observed within 168 h. Furthermore Fig. 4b shows no EE2 degradation during the growth and ATP generation of new active biomass on E2. EE2 degradation only starts after the depletion of the E2 substrate. The ethinylgroup of EE2 is assumed to sterically hinder the receptor-substrate binding, enzyme expression and EE2 metabolism. Kim and Hao (1999) observed similar phenomena as in this study. They studied the 3- and 4-chlorophenol cometabolism by phenol metabolizing *Acinetobacter* sp. isolates. They found that the mass ratio of the growth and the non-growth substrate, i.e. phenol and chlorophenol respectively, was determinative for the success of the chlorophenol biodegradation. At lower mass ratios, chlorophenol degradation was incomplete and a phenol over chlorophenol ratio of 4 was needed to guarantee full breakdown of chlorophenol. This form of substrate synergism explains the findings presented in Fig. 3 and the cascaded EE2 degradation pattern as observed in Fig. 2b.

The ATP concentration in a microbial culture is considered to be a measure for the amount of the biomass present and its activity (Verstraete et al. 1983). The ATP concentration was not higher in the presence of both E2 and EE2 than in the presence of only E2. Hence, EE2 appeared to be a non-growth substrate for these strains. The trend in Fig. 3, i.e. the more E2 is present, the more complete the EE2 degradation was, can be attributed to the higher extent of E1 formation as a primary biodegradation product of E2, and the higher extent of energy generation by E1 breakdown. EE2 is apparently not capable of inducing the metabolic gene expression. Interestingly, when one converts the levels of ATP attained to cell dry weight (CDW), assuming an average ATP content of 4 mg ATP per g cell dry weight, the cell yields per g E2 or E1 removed are at the order of 0.1–0.2 g CDW/g E2 or E1. The latter values correspond to data normally reported for microbial growth on biotic substances (Verstraete et al. 1983).

From the above, it seems logical that the isolated strains start the degradation with the most accessible functional group of the steroidal estrogens, the 17-hydroxylgroup of E2 or the 17-ketogroup of E1 (Fig. 1) (Pruneda-Paz et al. 2004). As a result, E1 is produced when E2 is biodegraded. Several authors have found E1 as a metabolite of E2 in batch tests with activated sludge or pure cultures (Ternes et al. 1999b; Lee and Liu 2002; Shi et al. 2004a, b; Weber et al. 2005). This in situ production of E1 explains the low overall removal efficiencies encountered in WWTPs. However, other authors (Fujii et al. 2002; Yoshimoto et al. 2004) could not detect E1 as an E2 metabolite in batch tests. This dehydrogenation of a hydroxyl group to a ketone function is not supposed to yield energy. We suggest that the ATP is generated during breakdown of E1 and its metabolites. In case of EE2, the 17-hydroxylgroup is not accessible because of the sterical hinderance by the ethinylgroup.

In our work, the EE2 biodegradation starts after the E2 biodegradation. During the experiments, a temporary build up of E1 and subsequent decrease was detected when E2 was degraded (see Fig. 4). In the cometabolism experiments (Fig. 4b, c), a clear relation between E1 and EE2 degradation can be discerned. The onset of both E1 and EE2 degradation occurred at the same time, i.e. after 112 h in both cases. Based on these findings, we suggest that E1 is the trigger during EE2 cometabolism. The conversion of E1 and/or its metabolites and EE2 presumably proceeds by a cleavage of the A-ring, probably by the same enzyme. These E1 metabolites could even comprise E3 if the further E3 breakdown would be fast in comparison to the E3 formation from E1.

The relevance of these findings in terms of environmental hygiene warrants further research. Indeed, the observed phenomena suggest that as long as a sufficient level of the natural estrogens is present, there is a chance that the xenobiotic EE2 will be cometabolized with the natural products. Since normally in urine the level of natural estrogens is a factor 2–3 higher than that of EE2, it will be of interest to monitor if this ratio is sufficient under normal non-axenic conditions to sustain adequate cometabolism of EE2. The findings as reported in Fig. 3 suggest that the E2/EE2 ratio will be too low. Hence, for such conditions, one might have to rely on the supplementation of an environmentally friendly cosubstrate capable to properly drive the cometabolism of EE2 in these natural matrices.

Conclusions

In this work six bacterial strains that can metabolize the natural estrogens estrone (E1), estradiol (E2) and estriol (E3) have been isolated from compost. Ethinylestradiol was not metabolized by these bacteria, but it was cometabolized in the presence of E1, E2 and E3. This transformation also occurs at lower concentrations (500 ng/l initial concentration). To the best of our knowledge these strains are the first to be reported to grow on natural estrogens and to cometabolize EE2, even at μg/l to ng/l concentrations.

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