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Isolation and Characterization of Ethylbenzene Degrading *Pseudomonas putida* E41

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***Pseudomonas putida* E41 was isolated from oil-contaminated soil and showed its ability to grow on ethylbenzene as the sole carbon and energy source. Moreover, *P. putida* E41 show the activity of biodegradation of ethylbenzene in the batch culture. E41 showed high efficiency of biodegradation of ethylbenzene with the optimum conditions (a cell concentration of 0.1 g wet cell weight/L, pH 7.0, 25°C, and ethylbenzene concentration of 50 mg/L) from the results of the batch culture. The maximum degradation rate and specific growth rate (μ_{max}) under the optimum conditions were 0.19±0.03 mg/mg-DCW (Dry Cell Weight)/h and 0.87±0.13 h^{-1}, respectively. Benzene, toluene and ethylbenzene were degraded when these compounds were provided together; however, xylene isomers persisted during degradation by *P. putida* E41. When using a bioreactor batch system with a binary culture with *P. putida* BJ10, which was isolated previously in our lab, the degradation rate for benzene and toluene was improved in BTE mixed medium (each initial concentration: 50 mg/L). Almost all of the BTE was degraded within 4 h and 70-80% of *m*-, *p*-, and *o*-xylenes within 11 h in a BTEX mixture (initial concentration: 50 mg/L each). In summary, we found a valuable new strain of *P. putida*, determined the optimal degradation conditions for this isolate and tested a mixed culture of E41 and BJ10 for its ability to degrade a common sample of mixed contaminants containing benzene, toluene, and xylene.**

***Keywords*:** ethylbenzene, *Pseudomonas putida* E41, characterization, biodegradation, aerobic condition

BTEX (benzene, toluene, ethylbenzene, and xylenes) compounds are commonly found together in crude oil and petroleum products. These non-oxygenated monoaromatic hydrocarbons are of particular concern because of their toxicity and carcinogenicity, even at low concentrations (Margesin *et al*., 2003). Their high water solubility, compared to other fuel components, contribute to their mobility, enabling them to migrate in the subsurface and eventually contaminate drinking water supplies (Blume, 1990; Leiwandowski *et al*., 1997; Chang *et al*., 2001; Margesin *et al*., 2003). In spite of governmental intervention in many countries, their emission into the environment is still increasing (Lapertot *et al*., 2007). The bioremediation of these substances has become an alternative to the traditional physical and chemical methods, which can be costly and produce hazardous products (Singleton, 1994; Abuhamed *et al*., 2004; Bordel *et al*., 2007). Among these compounds, ethylbenzene often enters the environment in the form of industrial discharges from petroleum refining, plastic, resin, and drug manufacturing or from oil spills. Ethylbenzene can also be used in a wide variety of applications as the starting material for the preparation of styrene, which is used as a solvent for coatings and in making rubber and plastic wrap (Parameswarappa *et al*., 2007).

Most of the reported works have focused on the biodegradation of BTEX mixtures (Assinder and Williams, 1990; Deeb and Cohen, 1999; Attaway and Schmidt, 2002; Parameswarappa *et al*., 2007). The presence of ethylbenzene inhibited BTX degradation rates, whereas the presence of BTX did not affect, or had little effect, on the ethylbenzene degradation rate (Deeb and Cohen, 1999). Many studies on the biodegradation of the individual components, such as benzene, xylene, and toluene, have been conducted, but there are few reports on efforts to understand the biodegradation of ethylbenzene.

We have investigated the aerobic degradation of ethylbenzene by strain E41, which was isolated from contaminated soils. Here, we report the degradation of ethylbenzene by strain E41 under various conditions and co-degradation of BTEX. Based on their degradation characteristics, a mixed culture composed of strains E41 and BJ10 was used in a bioreactor to improve the bio-degradation of BTEX.

Materials and Methods

Sampling site and enrichment cultures

The bacterial strains used in this study were isolated from contaminated soil found near a gas station in Incheon, Korea. Soil samples were stored in closed containers at 4°C prior to use. All microbial enrichment and isolation cultures were performed in a mineral salts medium (MSM) [KH_2PO_4 2.0 g, K_2HPO_4 2.0 g, KNO_3 1.0 g, $(NH_4)_2SO_4$ 2.0 g, NaCl 0.4 g, $MgSO_4 \cdot 7H_2O$ 0.4 g, $CaCl_2 \cdot 2H_2O$ 0.04 g, $FeCl_3 \cdot 7H_2O$ 0.02 g, 1.0 ml trace element solution (SL-6), and 2.0 ml of vitamin solution per L]. The final pH of the medium was 6.7±0.3. 1.0 g soil (wet weight) was applied to 100 ml MSM medium including 187.5 mg/L ethylbenzene and incubated for 7 days at 28±2°C.

Screening of bacteria degrading ethylbenzene

Isolates that grew well in the presence of ethylbenzene were assayed

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Table 1. Biodegradation of ethylbenzene during screen test with 3 strains in 6 h (Unit: mg/L)

Strain no.	Initial conc.	Residual conc.	Rem. (%)[a]
E41	80.8	ND	99.9
E51	82.4	ND	99.9
E6	86.2	6.1	92.9

[a] Removal efficiency (%)

for ethylbenzene degradation efficiency. Bacteria were grown in 500 ml bottles containing 100 ml MSM at 28±2°C in a rotary shaker (150 rpm). The removal efficiency was determined by culturing E41 (GenBank accession no.: HQ848376), BJ10 (GenBank accession no.: HQ848377) and their mixture in serum bottles containing 10 ml MSM (approx. pH 6.7) supplemented with 94.1 mg/L ethylbenzene as the sole source of carbon and energy. The serum bottle was sealed with a Teflon coated septum and an aluminum cap. The initial con-

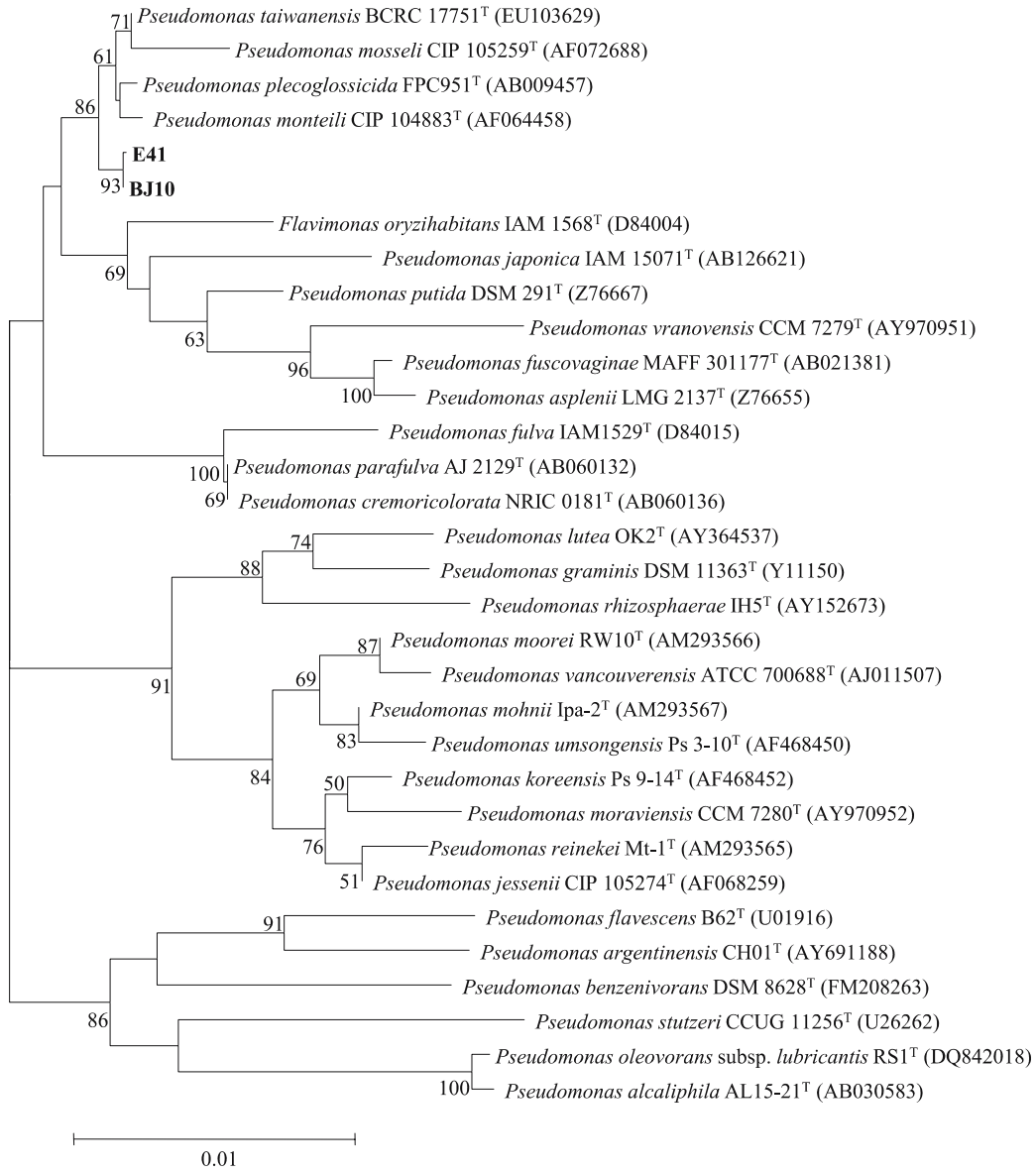

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences, showing the phylogenetic relationships between strains E41 and BJ10, and closely related species selected from the genus *Pseudomonas*. Bootstrap values >50% (percentage of 1,000 replications) are shown at branch points. The sequences used for the comparative study are included in parentheses. The neighbor-joining method was used. The bar represents 0.01 substitutions per nucleotide position.

centrations of E41, BJ10, and their mixture were 1.0 g/L (wet weight).

Identification of bacteria

Strain E41 and BJ10 were characterized by phenotypic and morphological analysis. Phenotypic analysis was carried out using an API kit 20NE (bioMérieux, France). The structural and morphological characteristics were analyzed by Gram staining and observation with an optical microscope (BX50F, Olympus, Japan), respectively. For phylogenetic characterization of this strain, we extracted DNA from strain E41 and BJ10 to amplify their 16S rRNA genes using PCR with primers 27F and 1492R in an iCycler PCR system (Bio-Rad, USA). The thermocycling conditions consisted of a denaturation step at 94°C for 5 min, 30 amplification cycles consisting of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec and a final extension for 7 min. Nucleotide sequence similarities were determined using BLAST (National Center for Biotechnology Information databases). Fatty acid methyl esters were prepared, separated and identified with the Sherlock Microbial Identification System (MIS, MIDI, Inc., USA) (Sasser, 1990).

Ethylbenzene degradation characteristics of strain E41

The influences of cell concentration, pH, temperature and substrate concentration on the growth of selected isolates were assessed using basal medium. E41 was pre-cultured in MSM containing ethylbenzene. The influence of cell concentration was examined in the range of 0.1, 0.3, 0.5, 1.0, 1.5, 2.0, and 2.5 g/L (wet weight) at pH 7.0 and 30°C with 100 mg/L ethylbenzene. To determine the influence of pH, the medium was adjusted to pH 6.0, 6.5, 7.0, 7.5, and 8.0, with cultures at 30°C, a starting cell concentration of 1.0 g/L (wet weight) cells and 100 mg/L ethylbenzene. To observe the influence of the incubation temperature, the temperatures were set as 20, 25, 30, and 37°C at pH 7.0 with 1.0 g/L (wet weight) cells and 100 mg/L ethylbenzene. To determine the effect of substrate concentration on ethylbenzene degradation by E41, tests were conducted using 10, 30, 50, 70, and 100 mg/L of ethylbenzene. The other conditions were a cell concentration of 1.0 g/L (wet weight), pH 7.0 and an incubation temperature of 30°C. Batch tests were conducted using the optimal conditions. Biodegradation rate was represented as both removal rate (mg/L/h) and degradation rate (mg/mg-DCW/h). Removal rate was determined solely as change of ethylbenzene/h, whereas degradation rate was determined as change of ethylbenzene and change of cell concentration/h, i.e., degradation rate was calculated as utilization of ethylbenzene substrate for growth of E41.

B, T, E, Xylene isomer (*m*-, *o*-, *p*-xylene) interactions during degradation by strains E41 and BJ10

The inhibitory effects of BTEX compounds were tested in 120 ml amber bottles. The E41 and BJ10 strains were first adapted to grow in MSM (500 ml bottles) with ethylbenzene and toluene as the sole carbon sources, in 500 ml bottles. The adapted cultures were then studied for their ability to degrade benzene, toluene, and ethylbenzene as the sole carbon sources and ethylbenzene mixed with benzene, toluene, and xylene isomers, respectively. BTE and xylene isomers as substrates were at concentrations of 100, 100, 100, and 10 mg/L respectively. The cell concentration was 1.0 g/L (wet weight), pH 7.0 and the incubation temperature was 30°C.

BTE biodegradation by pure cultures and a mixed culture

For comparison of the BTE degradation rate, strain BJ10, which is a highly efficient benzene and toluene removal strain (not published) was used. Strains E41 and BJ10 were first adapted to grow in MSM with toluene and ethylbenzene as the sole carbon sources, in 500 ml bottles. For kinetic studies, each serum bottle containing 10 ml MSM (pH pre-adjusted to 7.0) was aseptically injected with 50 mg/L of the BTE substrates. The cell concentrations were 1.0 g/L (wet weight) and, for a mixed culture, 0.5 g/L (wet weight) each of strains

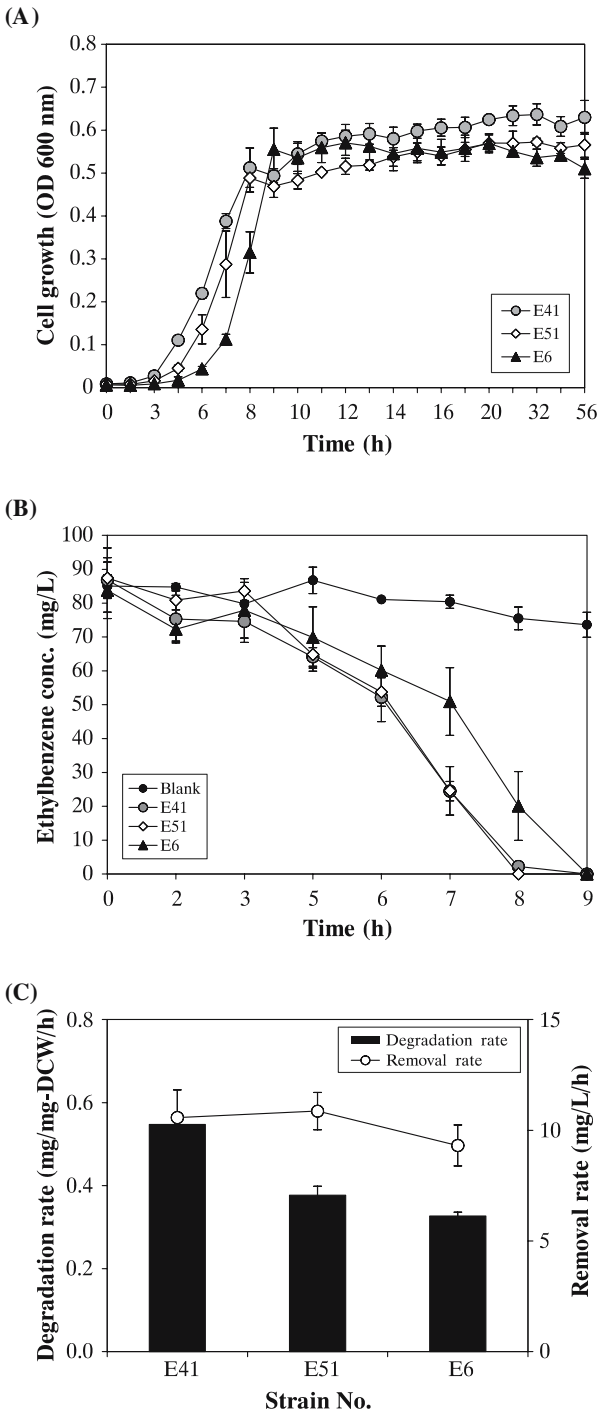

Fig. 2. Cell growth profile (A), biodegradation of ethylbenzene (B), and comparison of degradation rate and removal rate (C) of E41, E51, and E6.

BJ10 and E41 were co-inoculated. The incubation temperature was 30°C.

BTEX degradation by mixed culture (BJ10 and E41) in a bioreactor system

The lab bioreactor had two chambers, each with a maximum 5 L capacity. Each chamber was filled with 2.5 l MSM and sealed to prevent BTEX volatilization. Oxygen was supplemented to the medium until the dissolved oxygen concentration reached 7.8 mg/L. The cell concentrations of the two strains (BJ10 and E41) were 0.1 g/L (wet weight) each and the substrate concentrations were 50 mg/L for benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene, and *p*-xylene, respectively. Other conditions were D.O. 7.8 mg/L and pH 6.5. The concentration of each substrate was analyzed by gas chromatography (ACME 6000, Younglin instrument, Korea) by extracting 100 μl of gases from the headspace.

Analysis

To analyze the concentration of ethylbenzene, gas samples were periodically collected from the headspace of each bottle to monitor ethylbenzene degradation using a 250 μl gas-tight syringe (Hamilton, USA). Samples were then injected into a gas chromatograph equipped with an HP-5 capillary column (30 m×0.32 mm×1 μm) and a flame ionization detector (ACME 6000, Younglin instrument). Nitrogen (99.9% pure) was used as the carrier gas. The temperature was controlled at a constant 80°C for the oven, 150°C for the injector and 250°C for the detector. Ethylbenzene concentrations were quantified using standard curves. Cell growth was determined by measuring optical density (OD) at 600 nm using a spectrophotometer (Genequant pro, Amersham Biosciences, USA)

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain E41 is HQ848376.

Results

Isolation and screening

Fourteen bacteria were isolated from contaminated soil found near a gas station. All of them were able to grow well using ethylbenzene as the sole source of carbon. Among them, three strains (E41, E51, and E6) were chosen as the most efficient in degrading ethylbenzene (Table 1). In a comparison of bacterial growth in MSM, E41 grew more rapidly than the other two strains E51 and E6 (μ_{max}: 2.1, 1.3, and 1.1 h^{-1}, respectively) (Fig. 2A). During screening, 100 mg/L ethylbenzene was completely degraded in 9 h by these strains (Fig. 2B). The degradation rates for E41, E51, and E6 were determined to be 0.55, 0.38, and 0.33 mg/mg-DCW/h, respectively (Fig. 2C). From these results, E41 was the most efficient isolate for the removal of ethylbenzene.

Identification

E41 was a Gram-negative bacterium, with a rod shape (1.0-1.2×1.5-1.7 μm) when seen under a light microscope (Olympus BX50F, Japan). Through 16S rDNA sequencing, E41 and BJ10 were shown to be 99.0% and 98.9% identical to *P. putida* DSM 291^T, respectively. A phylogenetic tree including the BJ10 and E41 strains is shown in Fig. 1. The phenotypic characteristics that differentiate strains E41 and BJ10 from other *Pseudomonas* species are listed in Table 2. The fatty acid profile of strains E41 and BJ10 is shown in Table 3 and compared

Table 2. Phenotypic characteristics that differentiate *P. putida* E41 and BJ10 from other *Pseudomonas* species
Strains: 1, *P. putida* E41; 2, *P. putida* BJ10; 3, *P. putida* BCRC 10459^T; 4, *Pseudomonas plecoglossicida* BCRC 17517^T, *Pseudomonas taiwanensis* CMS^T. Unless indicated, all data were obtained in the course of this study.

Characteristic	1	2	3[a]	4[b]	5[c]
Enzyme activity					
Reduction of nitrates to nitrites	-	-	+	+	-
Reduction of nitrates to nitrogen	-	-	-	-	-
Arginine dihydrolase	+	+	+	+	+
Urease	-	-	+	+	-
β-Glucosidase (Esculin hydrolysis)	w	w	-	-	-
Protease (gelatin hydrolysis)	-	-	-	-	-
β-Galactosidase	-	-	-	-	-
Assimilation					
Glucose	+	+	+	+	
Arabinose	-	-	-	+	+
Mannose	+	+	-	-	
Mannitol	-	-	-	-	-
N-Acetyl-glucosamine	-	-	-	-	-
Maltose	-	-	-	-	-
Gluconate	+	+	+	-	+
Caprate	+	+	+	+	+
Adipate	-	+	-	-	-
Malate	+	+	+	+	+
Citrate	+	+	+	+	+
Phenyl-acetate	+	+	+	+	+

Results are scored as: +, positive; -, negative; w, weak reactions.
[a, b, c] Data are taken from Wang *et al*. (2010).

Table 3. Fatty acid composition of *P. putida* E41 and BJ10, and other *Pseudomonas* species
Strains: 1, *P. putida* E41; 2, *P. putida* BJ10; 3, *P. putida* BCRC 10459^T; 4, *P. plecoglossicida* BCRC 17517^T, *P. taiwanensis* CMS^T. All data were obtained in the course of this study. * Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Unless indicated, all data were obtained in the course of this study.

Fatty acids	1	2	3[a]	4[b]	5[c]
Saturated fatty acids					
10:0 3OH	11.5	9.4	3.3	8.0	10.6
12:0 3OH	5.1	4.9	3.9	4.4	4.0
12:1 3OH	2.1	1.6	0.1	1.6	1.2
12:0 2OH	5.5	5.7	3.4	4.3	3.8
12:0	-	-	3.0	2.0	1.9
14:0	0.4	0.4	0.2	0.2	0.3
16:0	19.6	21.3	25.1	23.3	23.5
18:0	0.3	0.3	0.7	0.5	0.6
Unsaturated fatty acids					
18:1 ω7c	-	-	22.4	19.9	24.9
17:0 cyclo	2.8	2.4	1.0	5.2	3.5
19:0 cyclo ω8c	-	-	ND	ND	0.2
Summed Feature 3 (16:1 ω7c/iso-15:0 2OH)	-	-	36.4	29.2	24.7

Results are scored as: ND, not detected.
[a, b, c] Data are taken from Wang *et al*. (2010).

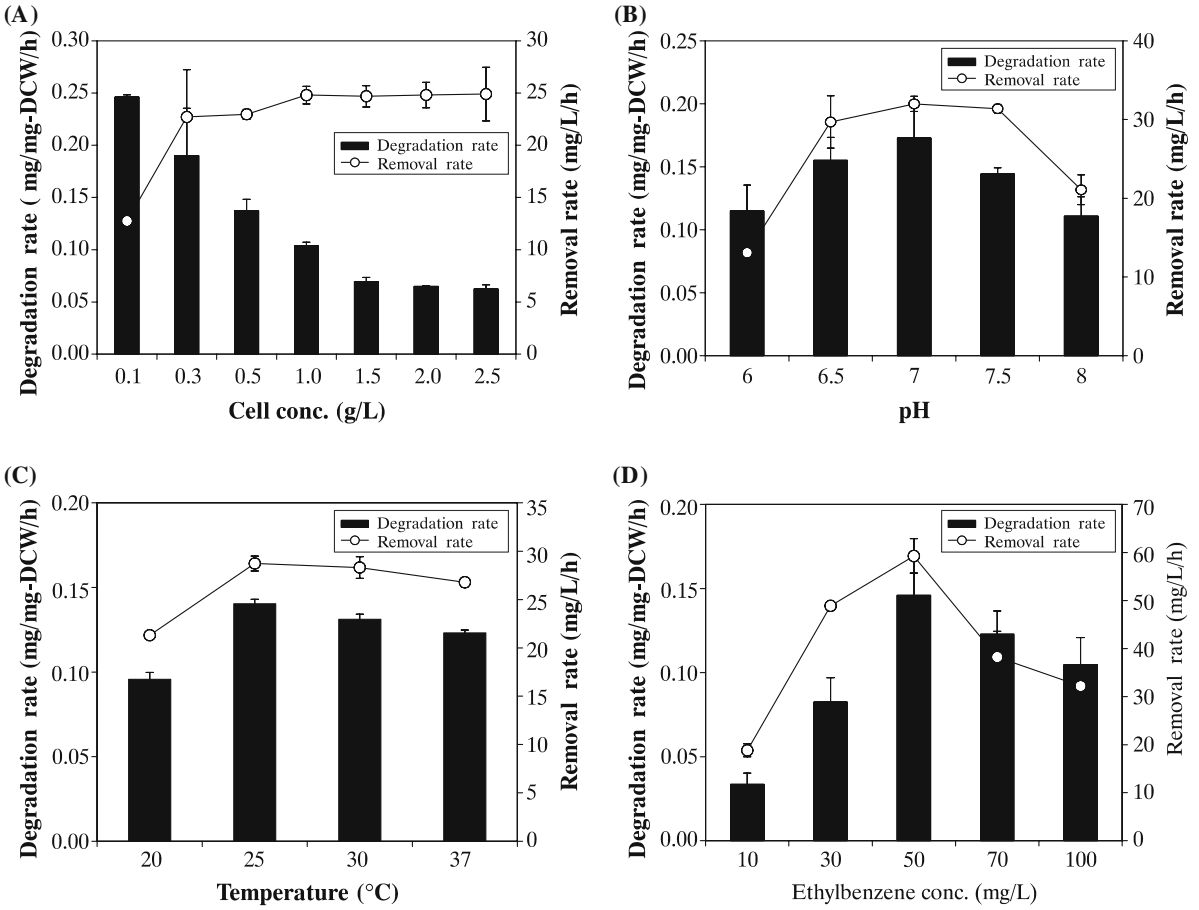

Fig. 3. Degradation rate and removal rate of ethylbenzene by E41 with the effects of different cell concentrations (A), pH levels (B), temperatures (C), and substrate concentrations (D). Others conditions were held fixed, with cell concentration of 1.0 g/L (wet weight), pH 7.0, temperature 30°C, and substrate concentration 100 mg/L.

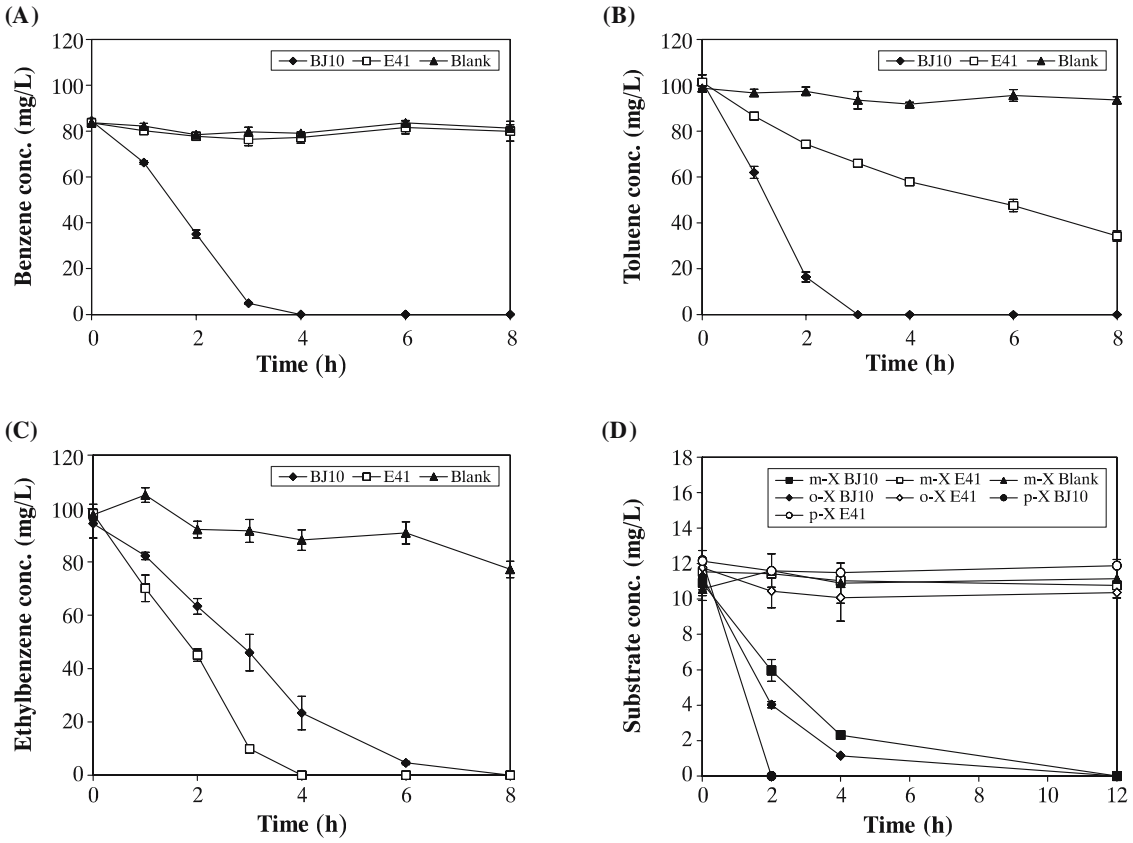

Fig. 4. Comparison of the degradations of individual BTEX compounds by *P. putida* BJ10 and *P. putida* E41. Benzene degradation (A), toluene degradation (B), ethylbenzene degradation (C), and xylene isomers degradation (D). Other conditions were held fixed, with a cell concentration of 1.0 g/L (wet weight), pH 7.0, temperature 30°C, BTE concentration of 100 mg/L, and *o*-xylene concentration of 10 mg/L.

with the profiles of other closely related *Pseudomonas* species. The five species included in the comparison contained saturated fatty acids $C_{16:0}$ as their predominant fatty acids, while unsaturated fatty acids 18:1 ω7c and summed feature 3 (16:1 ω7c/iso-15:0 2OH) were not detected, unlike other type species. We deposited the strain E41 (KEMC 2233-017) and BJ10 (KEMC 2231-020) to the Korea National Environmental Microorganisms Bank (KEMB).

Ethylbenzene degradation characteristics of *P. putida* E41

In order to determine the optimal degradation conditions for the selected isolate, E41 cell concentration (Fig. 3A), pH (Fig. 3B), temperature (Fig. 3C) and ethylbenzene concentration (Fig. 3D) were evaluated. Figure 3A illustrates the effect of the initial cell concentration inoculated into the bottles on ethylbenzene degradation. When 0.1 g/L (wet weight) were inoculated, the degradation rate was the highest (0.25 mg/mg-DCW/h; DCW: Dry Cell Weight). It took 6 h to degrade the ethylbenzene completely. Above 0.1 g/L, the degradation rates were inversely proportional and then equal from 1.5 g/L. Figure 3B shows the effect of the pH of the medium on the ethylbenzene degradation rate and removal rate. The ethylbenzene degradation rate was optimal at pH 7.0 (0.17 mg/mg-DCW/h), as was the removal rate. Figure 3C indicates the effects of temperature on the ethylbenzene degradation rate and removal rate. The optimal temperature was 25°C. At 25°C, the degradation rate was 0.14 mg/mg-DCW/h and the removal rate was 28.7 mg/L/h. The degradation rate and removal rate were optimal at an ethylbenzene concentration of 50 mg/L (Fig. 3D), at which the degradation rate and removal rate were 0.15 mg/mg-DCW/h and 59.3 mg/L/h, respectively. From these results, we determined that the optimal biodegradation conditions for ethylbenzene degradation by *P. putida* E41 were a 1.0 g/L (wet weight) cell concentration, pH 7.0, 25°C and 50 mg/L ethylbenzene concentration.

Optimum conditions were observed at cell concentrations of 1.0 g/L (the highest removal efficiency) and 0.1 g/L (the highest degradation rate), pH 7.0 and an incubation temperature of 25°C. In a batch test, four different cases were conducted to see the entire profiles of E41, at different initial cell concentration versus substrate concentration. At 0.1 g/L cell and 50 mg/L ethylbenzene concentrations, the μ_{max}, degradation rate and removal rate were 0.68 ± 0.07 h^{-1}, 0.16 ± 0.02 mg/mg-DCW/h, and 14.2 ± 1.0 mg/L/h, respectively. They were 0.34 ± 0.01 h^{-1}, 0.09 ± 0.01 mg/mg-DCW/h and 27.9 ± 2.1 mg/L/h at a 1.0 g/L cell concentration. For a 100 mg/L substrate concentration, they were 0.87 ± 0.13 h^{-1}, 0.19 ± 0.03 mg/mg-DCW/h and 22.2 ± 1.4 mg/L/h at a 0.1 g/L cell concentration and 0.37 ± 0.02 h^{-1}, 0.10 ± 0.01 mg/mg-DCW/h, 34.1 ± 1.4 mg/L/h at a 1.0 g/L cell concentration.

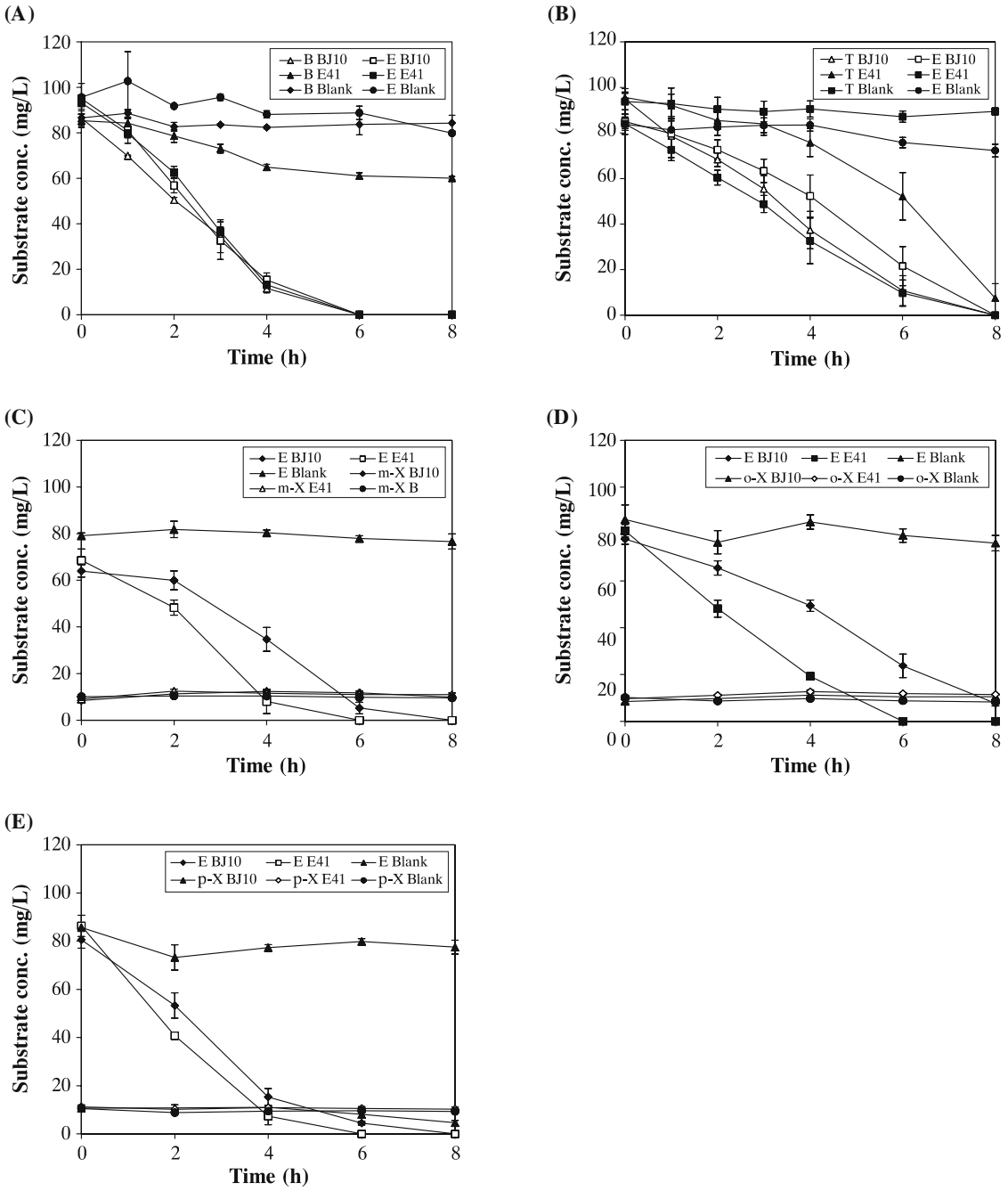

Fig. 5. Comparison of the degradations of binary mixtures by *P. putida* BJ10 and *P. putida* E41 in the presence of ethylbenzene (100 mg/L), showing benzene (A), toluene (B), *m*-xylene (C), *o*-xylene (D), and *p*-xylene (E) degradations. Others conditions were held fixed, with a cell concentration of 1.0 g/L (wet weight), pH 7.0, temperature 30°C, BTE concentrations of 100 mg/L, and xylene isomers concentration of 10 mg/L.

Binary mixed substrate utilization patterns during degradation by strains E41 and BJ10

Benzene was not degraded by *P. putida* E41 (Fig. 4A). But, when benzene was mixed with ethylbenzene, benzene was degraded by *P. putida* E41 (Fig. 5A). Ethylbenzene also had a positive effect on toluene degradation rate (Figs. 4B and 5B). The ethylbenzene was degraded by *P. putida* E41 in 4 h (Fig. 4C); however, BTX inhibited the ethylbenzene degradation rate. The xylene isomers were not degraded by *P. putida* E41 (Fig. 4D). In the case of *P. putida* BJ10, the disappearance rates for the benzene, toluene and xylene isomers were slowed by the presence of ethylbenzene (Fig. 5). In particular, *m*-, *o*-xylene were not degraded (Figs. 5C and 5D), but *p*-xylene degradation began when the ethylbenzene was almost degraded (Fig. 5E).

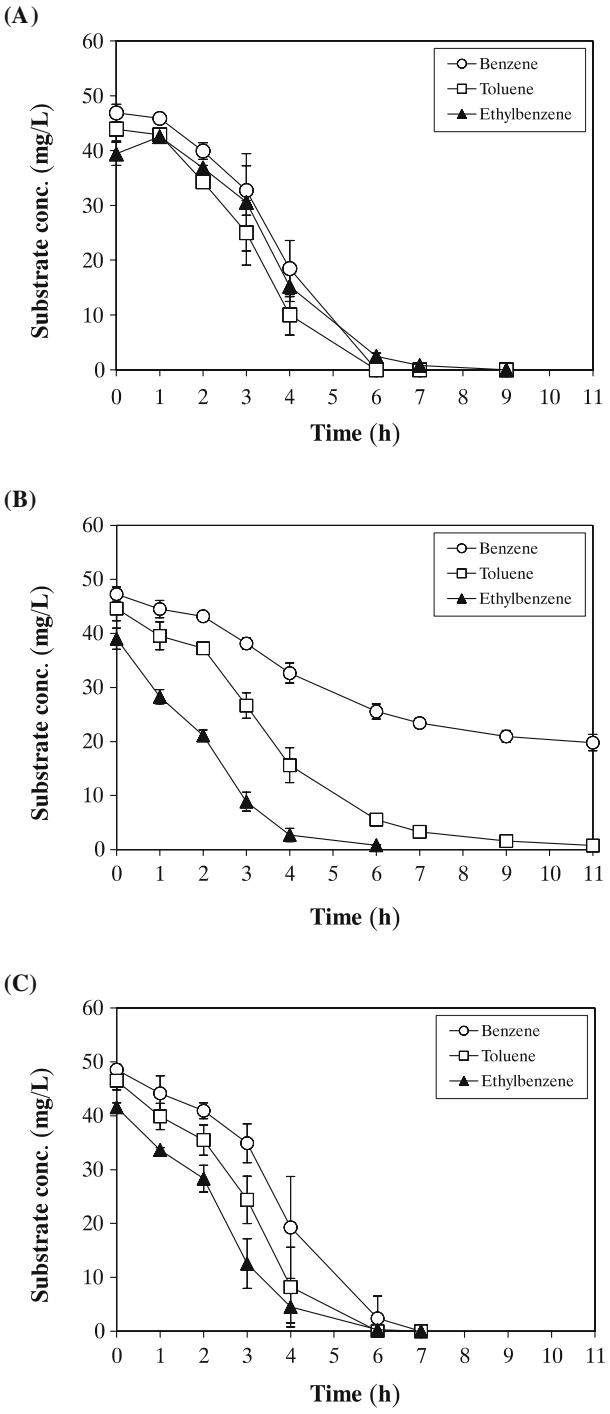

Fig. 6. Biodegradation of benzene, toluene, and ethybenzene in complex substrates by *P. p*utida BJ10 (A), *P. putida* E41 (B), and both in a mixed culture (C). The initial BTE concentrations were 50 mg/L each, with a cell concentration of 1.0 g/L (wet weight), a pH of 7.0, and an incubation temperature of 30°C.

BTE biodegradation by pure cultures and a mixed culture

The time course concentration of each BTE component in the mixture is presented in Fig. 6. Strains E41 and BJ10 were found to efficiently degrade BTE in the culture medium at 30°C and pH 7.0. But, *P. putida* strain E41 was found to degrade ethylbenzene better than BJ10. *P. putida* strain E41 removed the ethylbenzene in 6 h. The order of BTE degradation efficiencies for E41 were ethylbenzene>toluene>benzene (Fig. 6B). In contrast to E41, BJ10 removed the ethylbenzene in 7 h and it had similar removal rates for the other two substrates (Fig. 6A). In the mixed culture of strains BJ10 and E41, the order of the degradation efficiencies were ethylbenzene>toluene>benzene (Fig. 6C).

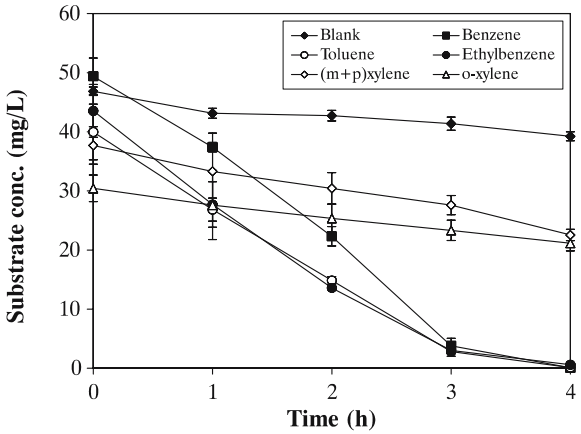

Fig. 7. BTEX biodegradation in lab bioreactor by a binary culture (*P. putida* BJ10 and *P. putida* E41). The initial BTEX concentrations were 50 mg/L each, with a cell concentration of 0.1 g/L (wet weight), pH 6.5, and D.O. 7.8 mg/L.

BTEX degradation by mixed culture (BJ10 and E41) in a bioreactor system

In the bioreactor system, almost all of the added BTE components were degraded within 4 h, with removal rates of 12.33, 9.96, and 10.74 mg/L/h, respectively, which were higher than in the bottle system (7.70, 7.75, and 6.88 mg/L/h) (Fig. 7). On the other hand, a combined peak of *m*-xylene and *p*-xylene revealed a reduction of around 78% from the initial concentration within 11 h and a separate peak of *o*-xylene showed a reduction of around 68% within 11 h (Fig. 7). Their removal rates were 2.72 and 1.88 mg/L/h in (*m*+*p*)-xylene and *o*-xylene, respectively. Because the removal rate of *o*-xylene was 0.56 mg/L/h in the bottle system (data not shown), the reactor system proved to be more effective in the same manner as it was in the BTE trials. During BTEX degradation, the pH was maintained at 6.4 (±0.1) and the dissolved oxygen concentration was decreased from 2.0 to 0.4 after 3 h. Cell growth increased for 3 h (from OD 0.06 to 0.31) and then remained steady for 11 h (OD 0.32). We determined that positive cell growth occurred only when benzene, toluene and ethylbenzene were present in the medium.

Discussion

In many studies, different bacterial strains have been capable of degrading BTEX in pure cultures (Attaway and Schmidt, 2002; Lee *et al*., 2002; Li *et al*., 2006; Plaza *et al*., 2007) and

in mixed cultures (Bielefeldt and Stensel, 1999; Deeb and Cohen, 1999). Only a few studies have reported that some bacterial strains were capable of catalyzing the conversion of ethylbenzene as the source of carbon and energy as a pure culture (Cox and Goldsmith, 1979; Utkin *et al*., 1990; Burback and Perry, 1993; Lee and Gibson, 1996; Parameswarappa *et al*., 2007). Among the BTEX compounds, ethylbenzene has been reported to be the biggest potential inhibitor of BTX degradation by a consortium (Deeb and Cohen, 1999; Chang *et al*., 2001).

By comparison of the degradation rates with ethylbenzene as the sole carbon source, we found that the degradation rate of ethylbenzene was much higher by *P. putida* E41 (15.4 mg/L/h) than by other reported bacterial species. For example, *Mycobacterium vaccae* showed a degradation rate of 1.3 mg/L/h (ethylbenzene conc.: 50 mg/L, cell conc.: 1.0 g/L) (Burback and Perry, 1993).

Mycobacterium vaccae was not able to degrade a concentration of 86.7 mg/L ethylbenzene (Burback and Perry, 1993). But, in the current work, *P. putida* E41 could degrade a wide range of ethylbenzene concentrations from 10 to 100 mg/L.

We also observed potential enhancement or inhibition effects on the degradation of binary mixed substrates. In *P. putida* BJ10 culture, ethylbenzene had negative effects on benzene and toluene degradation, similar to results from previous studies (Deeb and Cohen, 1999; Chang *et al*., 2001; Lee *et al*., 2002). Previous research also verified that the toluene degradation rate was decreased due to the fact that ethylbenzene underwent degradation first (Lee *et al*., 2002). Ethylbenzene was also known as the most potent inhibitor in BTEX degradation (Deeb and Cohen, 1999; Chang *et al*., 2001). Amazingly, ethylbenzene had a positive effect on benzene and toluene degradation in binary mixed substrates when using *P. putida* E41. The current study has yielded a unique result. Previous research showed only the positive degradation of toluene but not of benzene (Lee *et al*., 2002). For a tertiary mixture (BTE), *P. putida* E41 degraded ethylbenzene first, followed by toluene and then benzene. In contrast, *P. putida* BJ10 degraded toluene first, then benzene, with ethylbenzene last. These results showed that there can be strain specificity within a species with regard to the profile of enzymatic degradation.

In the bioreactor study, the mixed culture of *P. putida* E41 and *P. putida* BJ10 had an ethylbenzene degradation rate of 10.9 mg/L/h. These results showed that *P. putida* E41 is more effective for ethylbenzene degradation than other bacteria reported in other studies (Bielefeldt and Stensel, 1999; Deeb and Cohen, 1999; Attaway and Schmidt, 2002; Lee *et al*., 2002; Li *et al*., 2006; Lapertot *et al*., 2007; Plaza *et al*., 2007).

In conclusion, this study found an effective ethylbenzene degrader (*P. putida* E41) and achieved relatively good benzene and toluene degradation in mixed contaminants (BTE) through co-culture of *P. putida* E41 and *P. putida* BJ10. We have also observed substrate interactions in binary substrate mixtures. *P. putida* BJ10 inhibited benzene and toluene degradation in the presence of ethylbenzene, but *P. putida* E41 had a positive effect on toluene and benzene degradation.

Our observations on these different interactions can contribute to a better approach for modeling BTEX biodegradation. Further study is expected regarding their mechanism of action and *in situ* application.

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References

Abuhamed, T., E. Bayraktar, T. Mehmetoglu, and U. Mehmetoglu. 2004. Kinetics model for growth of *Pseudomonas puitda* F1 during benzene, toluene and phenol biodegradation. *Proc. Biochem.* 39, 983-988.

Assinder, S.J. and P.A. Williams. 1990. The TOL plasmids: determinants of the catabolism of toluene and xylenes. *Adv. Microb. Physiol* 31, 1-69.

Attaway, H.H. and M.G. Schmidt. 2002. Tandem biodegradation of BTEX components by two *Pseudomonas* sp. *Curr. Microbiol.* 45, 30-36.

Bielefeldt, A.R. and H.D. Stensel. 1999. Modeling competitive inhibition effects during biodegradation of BTEX mixtures. *Wat. Res*. 33, 707-714.

Blume, H.P. 1990. Handbuch des Bodenschutzes. Landsberg: Ecomed.

Bordel, S., R. Munoz, L.F. Diaz, and S. Villaverde. 2007. New insights on toluene biodegradation by *Pseudomonas putida* F1: influence of pollutant concentration and excreted metabolites. *Appl. Microbiol. Biotechnol.* 74, 857-866.

Burback, B.L. and J.J. Perry. 1993. Biodegradation and biotransformation of groundwater pollutant mixtures by *Mycobcteriumvaccae*. *Appl. Environ. Microbiol.* 59, 1025-1029.

Chang, S.W., H.J. La, and S.J. Lee. 2001. Microbial degradation of benzene, toluene, ethylbenzene and xylene isomers (BTEX) contaminated groundwater in Korea. *Water Sci. Technol.* 44, 165-171.

Cox, D.P. and C.D. Goldsmith. 1979. Microbial conversion of ethylbenzene to 1-phenethanol and acetophenone by *Nocardiatartaricans* ATCC 31190. *Appl. Environ. Microbiol.* 38, 514-520.

Deeb, R.A. and L.A. Cohen. 1999. Temperature effects and substrate interactions during the aerobic biotransformation of BTEX mixtures by toluene-enriched consortia and *Rhodococcusrhodochrous*. *Biotechnol. Bioeng.* 62, 526-536.

Lapertot, M., C. Seignez, S. Ebrahimi, S. Delorme, and P. Peringer. 2007. Mass production of bacterial communities adapted to the degradation of volatile organic compounds (TEX). *Biodegradation* 18, 343-350.

Lee, K. and D.T. Gibson. 1996. Toluene and ethylbenzene oxidation by purified naphthalene dioxygenase from *Pseudomonas* sp. Strain NCIB 9816-4. *Appl. Environ. Microbiol.* 62, 3101-3106.

Lee, E.Y., Y.S. Jun, S.C. Kyung, and H.R. Ryu. 2002. Degradation characteristics of toluene, benzene, ethylbenzene and xylene by *stenotrophomonasmaltophilia* T3-c. *J. Air Waste Manage*. *Assoc.* 52, 400-406.

Leiwandowski, J., S. Leitschun, and V. Kob. 1997. SchadstoffeimBonden. Springer, Berlin, Germany.

Li, H., Y.H. Liu, N. Luo, X.Y. Zhang, T.G. Luan, J.M. Hu, Z.Y. Wang, P.C. Wu, M.J. Chen, and J.Q. Lu. 2006. Biodegradation of benzene and its derivatives by a psychrotolerant and moderately haloalkaliphilic *Planococcus* sp. strain ZD22. *Res. Microbiol.* 157, 629-636.

Margesin, R., G. Walder, and F. Schinner. 2003. Bioremediation assessment of a BTEX-contaminated soil. *Acta Biotechnol.* 1, 29-36.

Parameswarappa, S., C. Karigar, and M. Nagenahalli. 2007. Degradation of ethylbenzene by free and immobilized *Pseudomonas fluorescens*-CS2. *Biodegradation* 10.1007/s10532-007-9121-y.

Plaza, G.A., J. Wypych, C. Berry, and R.L. Brigmon. 2007. Utilization of monocyclic aromatic hydrocarbons individually and in mixture by bacteria isolated from petroleum contaminated soil. *World J. Mocrobiol. Biotechnol.* 23, 533-542.

Sasser, M. 1990. Identification of bacteria through fatty acid analysis, pp. 199-204. *In* Z. Klement, K. Rudolph, and D.C. Sands (eds.), Methods in phytobacteriology, AkademiaiKiado, Budapest, Hungary.

Singleton, I. 1994. Microbial metabolism of xenobiotics: fundamental and applied research. *J. Chem. Technol*. *Biotechnol.* 59, 9-23.

Utkin, I.B., M.M. Yakimov, L.N. Matveeva, E.I. Kozlyak, I.S. Rogozhin, Z.G. Solomon, and A.M. Bezborodov. 1990. Degradation of styrene and ethylbenzene by *Pseudomonas* species Y2. *FEMS Microbiol. Lett.* 77, 237-242.

Wang, L.T., C.J. Tai, Y.C. Wu, Y.B. Chen, F.L. Lee, and S.L. Wang. 2010. *Pesudomonas taiwanensis* sp. nov., isolated from soil. *Int. J. Syst. Evol. Microbiol.* 60, 2094-2098.