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Effect of substrate interaction on the degradation of methyl *tert*-butyl ether, benzene, toluene, ethylbenzene, and xylene by *Rhodococcus* sp

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ABSTRACT

It was examined the substrate interactions of benzene (B), tolulene (T), ethylbenzene (E), xylene (X), and methyl *tert*-butyl ether (M) in binary, ternary, quaternary, and quinary mixtures by *Rhodococcus* sp. EH831 that could aerobically degrade all of five single components. The specific degradation rates (SDRs) of B, T, E, X, and M were 234, 913, 131, 184 and 139 μmol g-dry cell weight (DCW)$^{-1}$ h^{-1}, respectively. In binary, ternary, quaternary, and quinary mixtures of them, ethylbenzene was the strongest inhibitor for the other substrates, and methyl *tert*-butyl ether was the weakest inhibitor. Interestingly, no degradation of benzene and methyl *tert*-butyl ether was found in the coexistence of ethylbenzene. The degradation of benzene followed only after toluene became exhausted when both was present. Ethylbenzene was least inhibited by methyl *tert*-butyl ether and most inhibited by toluene.

1. Introduction

Between the late 1970s and the 1990s, methyl *tert*-butyl ether (M) was added to gasoline to increase the efficiency of combustion and reduce atmospheric pollution [1]. Like other gasoline compounds, M was released into the environment during production, transportation, and storage. M is often found in groundwater because of its low absorption and high solubility [2]. The major aromatic component of gasoline is BTEX, a mixture of benzene (B), toluene (T), ethylbenzene (E), and xylene (X). These compounds are more soluble in water than aliphatic compounds. Recent studies have shown that M and BTEX are found together in many gasoline-contaminated sites in California [3].

Many microorganisms are known to degrade BTEX and M [1,4], but most of the initial studies have examined the degradation of single components by isolated microorganisms [5]. Recent studies have focused on developing technologies for bioremediation of groundwater and soils contaminated with M [6,7], but studies on the biodegradation of mixtures of hydrocarbons has become important because the individual compounds are rarely found alone [8–13].

The biodegradation of the different components in a mixture can be affected by the others components [14,15]. This interaction of the different compounds in a mixture has been found not only with easily degradable compounds like sugars but also with toxic compounds [16]. Because M and BTEX coexist in groundwater, it is important to investigate whether M and BTEX affect each other's degradation [1], but there have been few studies on the interaction of substrates in mixtures of BTEX and M (BTEXM) [1,17–19]. Furthermore, these previous studies have employed microorganisms that can degrade only some of the individual components in the BTEXM mixture [20–22], and the degrading organisms have been a consortium rather than a single pure microorganism [19,23]. In the current study, we investigated how BTEX and M interact in contaminated sites by quantitatively and qualitatively analyzing the degradation of different mixtures of B, T, E, X, and M using the bacterium *Rhodococcus* sp. EH831, which can degrade all five compounds [24].

2. Materials and methods

2.1. Bacterial growth conditions

Rhodococcus sp. EH831 (GenBank accession no. AY878706, KCCM 10657P) was isolated from oil-contaminated soil [24]. This bacterium was cultured in a 1.2 L serum bottle containing 50 mL of Bushnell-Hass (BH) medium (0.409 g/L $MgSO_4{\cdot}7H_2O$, 0.0265 g/L $CaCl_2{\cdot}2H_2O$, 1 g/L KH_2PO_4, 1 g/L NH_4NO_3, 6 g/L $Na_2HPO_4{\cdot}12H_2O$, and 0.0833 g/L $FeCl_3{\cdot}6H_2O$) and 50 μL trace elements (17 g/L $FeCl_3$, 0.6 g/L $CaCl_2$, 0.2 g/L $ZnSO_4$, 0.2 g/L $CuSO_4{\cdot}7H_2O$, 0.2 g/L $MnSO_4$, 0.8 g/L $CoCl_2$, 0.1 g/L H_3BO_3, and 0.3 g/L $Na_2MoO_4{\cdot}2H_2O$). After the serum bottle was sealed using a butyl rubber and aluminum cap, 0.5 mL *n*-hexane (99.5%; Duksan Pure Chemical Co., Ansan, Korea) was injected using a 1 mL syringe, and the culture was incubated at 30 °C with shaking at 180 rpm.

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2.2. Assays of BTEX and M degradation

A 10 mL preculture broth of *Rhodococcus* sp. EH831 at exponential growth phase was centrifuged at 8900 × *g* for 10 min (Supra21K; Hanil, Incheon, Korea), and the harvested cells were washed twice with distilled water and resuspended in 10 mL of fresh BH medium. The washed cells were transferred to 600 mL serum bottles and sealed using a butyl rubber and aluminum cap. BTEXM (Reagent grade, 2 μL of each component) was injected into each bottle, and the bottles were cultivated in a shaking incubator (180 rpm, 30 °C). Samples of the air in the headspace were withdrawn from the bottles every 1.5 or 2 h using a 1 mL gas-tight syringe, and the cell concentrations were determined by measuring the absorbance at 600 nm in a 300 μL sample using a Bioscreen C spectrophotometer (Labsystems, Vantaa, Finland). The serum bottles containing 10 mL of sterile BH medium without the inoculation of EH831 were used as sterile controls. The rate of BTEXM degradation was calculated from the slope of a plot of the BTEXM concentration in the headspace vs. time [25].

2.3. Analytical procedures

The concentrations of BTEXM components were analyzed using an M600D gas chromatograph (Younglin, Anyang, Korea) equipped with a SUPELCOWAX 10™ column (30 m × 0.32 mm; 0.25 μm film) and a FID (flame ionization detector). Temperatures for the oven, injector, and detector were 100, 230, and 230 °C, respectively. The analytical procedure was described in the previous study in detail [19].

3. Results

3.1. Biodegradation properties of B, T, E, X, and M

To investigate how interactions between the components of BTEXM affect each other's degradation by *Rhodococcus* sp. EH831, it was examined the degradation of pure compounds and of binary, ternary, quaternary, and quinary mixtures. Representatively, the results of B and M degradation in BTEXM mixtures are shown in Figs. 1 and 2. Although EH831 was incubated in hexane-BH medium, it could degrade pure B, T, E, X, and M without any lag period (data not shown). In binary mixtures, B was inhibited by all other components, and its degradation was initiated after the degradation of T was finished when both were present. Also, the degradation rate for B at low concentrations was reduced in the presence of M (Fig. 1). X and E also inhibited B degradation; the time for complete degradation of B was approximately 17 h in a BT mixture, and B was not completely degraded in a BE mixture. Based on the time for complete degradation, the inhibition of B in binary mixtures increased in the order of T < M < X ≪ E. These findings were similar to the results of ternary mixtures; the degradation time increased in the order of BTM ≈ BXM < BTX ≪ BEM = BEX = BTE. In particular, B was not completely degraded in the BEM, BEX, and BTE mixtures, which indicated that E inhibited the degradation of B. Inhibition of B degradation by E also occurred in quaternary and quinary mixtures (Fig. 1c and d); complete degradation of B did not occur in BTEX, BTEM, BEXM, and BTEXM mixtures, all of which contained E.

M was inhibited by all other components except for T, and T promoted the degradation of M; M was completely degraded

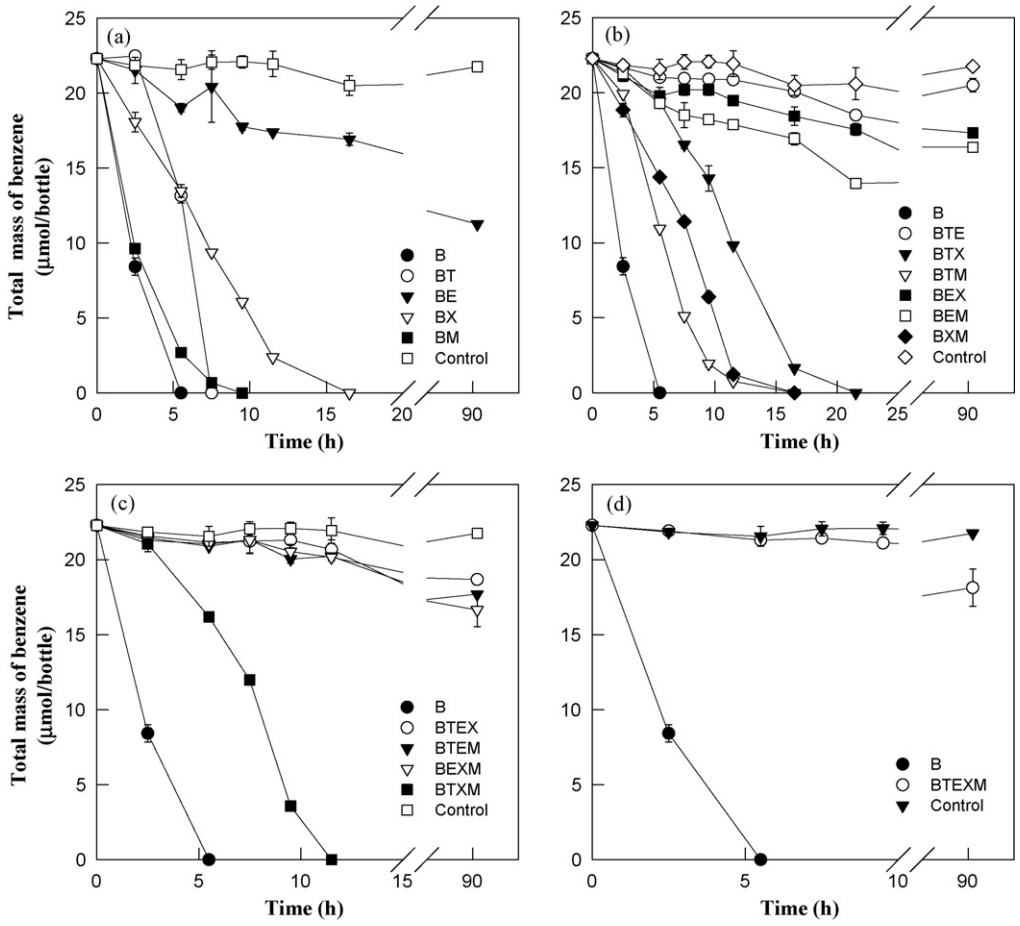

Fig. 1. Effect of substrate interactions in (a) binary, (b) ternary, (c) quaternary, and (d) quinary BTEXM mixtures on B degradation by EH831.

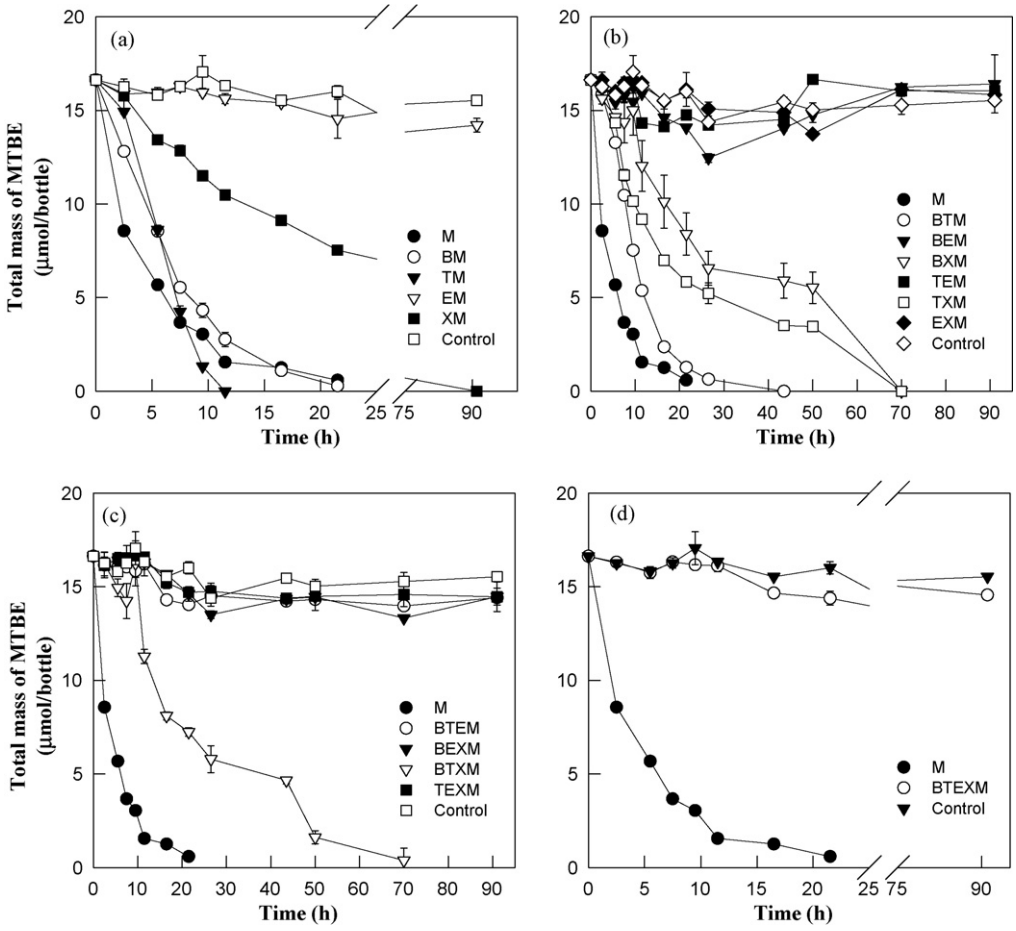

Fig. 2. Effect of substrate interactions in (a) binary, (b) ternary, (c) quaternary, and (d) quinary BTEXM mixtures on M degradation by EH831.

within 11.5 h in the presence of T, which was faster than for pure M (Fig. 2a). The degradation of M was inhibited in binary tests in the order of BM < XM ≪ EM and in ternary tests in the order of BTM < TXM < BXM ≪ BEM = TEM = EXM. Also, M could not be degraded in quaternary and quinary tests containing E (Fig. 2c and d).

3.2. Comparison of degradation rates

To quantitatively analyze the substrate interactions of BTEXM mixtures, we calculated the specific degradation rate (SDR) and the average degradation rate (ADR) (Table 1). The SDR was obtained as the slope of the plot of the compound concentration vs. time following the lag period, and the ADR was calculated using time including the lag period [26].

The SDR and ADR for B by EH831 were 234 and 171 μmol g-dry cell weight (DCW)$^{-1}$ h^{-1}, respectively. In a binary mixture of BT, the ADR of B (127 μmol g-DCW^{-1} h^{-1}) was lower than for B alone because the initiation of degradation for B was delayed until the degradation of T was complete; however, the SDR of B (280 μmol g-DCW^{-1} h^{-1}) was slightly higher than for B alone. The degradation of B was decreased by 71 μmol g-DCW^{-1} h^{-1} for SDR and 73 μmol g-DCW^{-1} h^{-1} for ADR. In mixtures containing M, the SDR and ADR were slightly diminished to 211 and 120 μmol g-DCW^{-1} h^{-1}, respectively. The SDR of B in BTX (108 μmol g-DCW^{-1} h^{-1}) was higher than that for a binary mixture of BX (71 μmol g-DCW^{-1} h^{-1}). It seemed that this enhancement was due to a reduction of the inhibitory effect of X by T because T promoted the degradation rate of B in the BT mixture. The ADR of B in the BTX mixture, however, was lower than for binary mixtures of BT, because T caused a delay in the initiation of degradation for B. In all tests containing E (from binary to quinary), the degradation of B was not observed.

The SDR of T alone was 913 μmol g-DCW^{-1} h^{-1}, and the ADR was 310 μmol g-DCW^{-1} h^{-1}. In the binary mixtures, the rate of T degradation was reduced when B or X were present; the SDRs were 121–218 μmol g-DCW^{-1} h^{-1}, and the ADRs were 121–145 μmol g-DCW^{-1} h^{-1}. In the presence of E, the SDR and ADR for T were both decreased more than 90%. These trends were also observed in BTE, TEX, TEM, BTEX, BTEM, TEXM, and BTEXM mixtures; the SDR and ADR for T were significantly decreased to 58–135 and 34–100 μmol g-DCW^{-1} h^{-1}, respectively. M rarely affected the degradation of T in MT mixtures, but the SDR of T was slightly reduced to 711 μmol g-DCW^{-1} h^{-1}, and its ADR was slightly increased to 345 μmol g-DCW^{-1} h^{-1}. Thus, M appears to have substantial effects on the degradation of T.

EH831 showed the lowest degradation rate for B alone, with an SDR of 131 μmol g-DCW^{-1} h^{-1} and an ADR of 81 μmol g-DCW^{-1} h^{-1}. In the presence of B, the degradation rate of E was 115 μmol g-DCW^{-1} h^{-1} (SDR), indicating only a weak inhibition. The SDR and ADR for E were decreased by 54–57% and by 61–71% in the presence of T and X, respectively. However, M promoted the degradation of E, with an increase in the SDR of 32% to 173 μmol g-DCW^{-1} h^{-1}. In ternary mixtures of EBM, SDR and ADR for E were almost similar with that in E alone.

The SDR and ADR for X alone were 184 and 115 μmol g-DCW^{-1} h^{-1}, which are lower than the values for T and B. The SDR and ADR for X in the BX mixture were similar with those for X alone. Also, the degradation of X in BXM and TXM mixtures was similar or slightly more rapid than for X alone. Although T decreased the SDR of X by 115 μmol g-DCW^{-1} h^{-1} compared to pure X, the ADR did not

Table 1
Effects of BTEXM mixtures on the rate of degradation by EH831.

Cond.[a]	SDR[b]	ADR[c]	Cond.	SDR	ADR	Cond.	SDR	ADR	Cond.	SDR	ADR	Cond.	SDR	ADR
B	234	171	T	913	310	E	131	81	X	184	115	M	139	56
B_T	280	127	T_B	218	145	E_B	115	56	X_B	181	114	M_B	61	56
B_E	0	0	T_E	66	29	E_T	57	24	X_T	115	115	M_T	88	63
B_X	71	73	T_X	121	121	E_X	60	32	X_E	76	57	M_E	0	0
B_M	211	120	T_M	711	345	E_M	173	74	X_M	168	90	M_X	34	9
B_{TE}	0	0	T_{BE}	75	34	E_{BT}	53	28	X_{BT}	91	81	M_{BT}	57	37
B_{TX}	108	53	T_{BX}	129	129	E_{BX}	88	39	X_{BE}	97	70	M_{BE}	0	0
B_{TM}	129	82	T_{BM}	287	287	E_{BM}	131	91	X_{BM}	188	121	M_{BX}	65	18
B_{EX}	0	0	T_{EX}	58	38	E_{TX}	53	53	X_{TE}	90	55	M_{TE}	0	0
B_{EM}	0	0	T_{EM}	85	70	E_{TM}	62	52	X_{TM}	205	116	M_{TX}	60	13
B_{XM}	119	84	T_{XM}	200	140	E_{XM}	80	63	X_{EM}	79	56	M_{EX}	0	0
B_{TEX}	0	0	T_{BEX}	65	42	E_{BTX}	59	31	X_{BTE}	94	54	M_{BTE}	0	0
B_{TEM}	0	0	T_{BEM}	135	100	E_{BTM}	89	70	X_{BTM}	152	124	M_{BTX}	114	16
B_{TXM}	195	91	T_{BXM}	222	155	E_{BXM}	79	39	X_{BEM}	92	37	M_{BEX}	0	0
B_{EXM}	0	0	T_{EXM}	91	42	E_{TXM}	67	30	X_{TEM}	65	57	M_{TEX}	0	0
B_{TEXM}	0	0	T_{BEXM}	68	26	E_{BTEX}	64	37	X_{BTEM}	83	39	M_{BTEX}	0	0

[a] Cond., condition.
[b] SDR, specific degradation rate.
[c] ADR, average degradation rate (μmol g-DCW^{-1} h^{-1}).

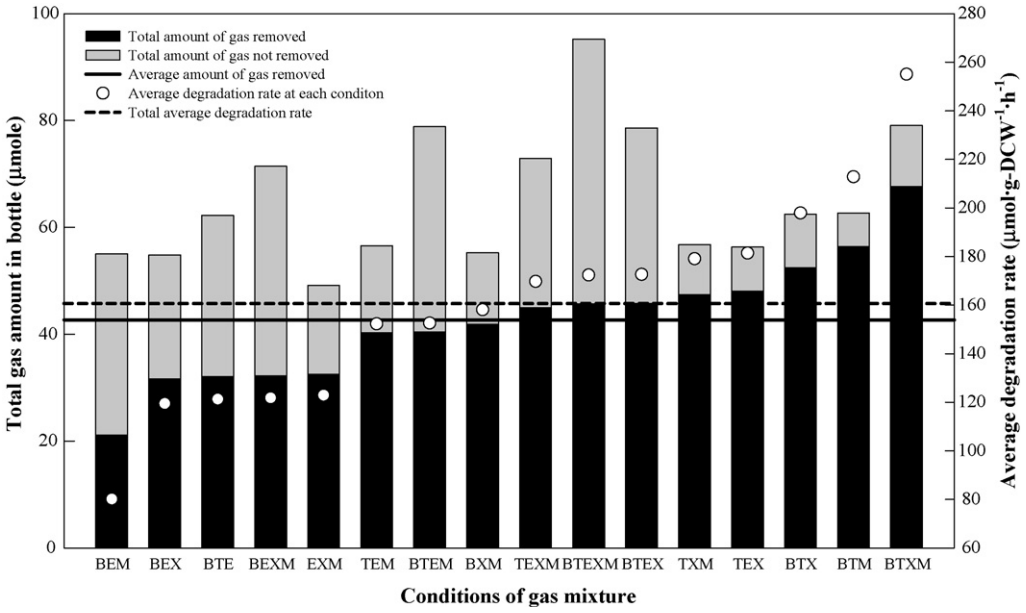

Fig. 3. Total amount of gas removed and average degradation rate in BTEXM mixtures within the culture time of 11.5 h.

change significantly. On the other hand, the strongest inhibitor of X degradation was E, with a 50% reduction in the rate of X degradation. This was also found in ternary, quaternary, and quinary mixtures.

EH831 could degrade M with an SDR of 139 μmol g-DCW^{-1} h^{-1} and an ADR of 56 μmol g-DCW^{-1} h^{-1}. In binary tests, T was the weakest inhibitor of M degradation. T or B did not reduce the ADR for M even though it reduced the SDR values for M. In the coexistence of E, no degradation of M was found.

3.3. Comparison of the total amount of gas removed

Total amount of gas removed in the tested bottle within the culture time of 11.5 h was calculated and shown in Fig. 3. The average amount of gas removed in the ternary and more complex mixtures was 42.7 μmol/bottle. The amount of gas removed in BEM, BEX, BTE, BEXM, and EXM mixtures was remarkably less than the average value. In BTX, BTM, and BTXM mixtures, however, the amount of gas removed was significantly higher than the average value. The degradation rate of total gas in each condition showed a similar tendency. These results indicated that E was the strongest inhibitor for the degradation of the other gases.

4. Discussion

To evaluate the effect of substrate interaction on the biodegradation of BTEX and M, *Rhodococcus* sp. EH831 was employed in this study because this bacterium could degrade all of B, T, E, X, and M. The specific degradation rate for each substrate by EH831 increased in the order of E < M < X < B < T (Table 1). du Plessis and Strauss had been reported that the degradation by a consortium increased in the order of *p*-X < *m*-X < *o*-X < B < E [27]. In addition, Yadav and Reddy showed that degradation by *Phanerochaete chrysosporium* increased in the order of B < X < T < E [28]. These previous studies are different from our result because they used different strains, which have different BTEX degradation pathways [19].

Although B, T, E, and X are structurally similar, they have both positive and negative effects on each other's degradation when in a mixture. The positive effects are due to co-metabolism [8,29] and

induction of degradative enzymes [8], and the negative effects are due to competitive inhibition [10,11,30], toxicity [31], enzyme inactivation by intermediates [32,33], competitive enzyme inhibition, mechanism suppression, and exhaustion of electron acceptors [8]. In the BTEX mixture, E was the strongest inhibitor for the other substrates (Table 1, Fig. 3). Deeb and Alvarez-Cohen also reported that E is the strongest inhibitor of degradation of binary mixtures of B, T, E, X by *Rhodococcus rhodochrous* [20]. On the other hand, it was found that the degradation of B began only after T was degraded. Burback and Perry also reported that T causes a delay in the degradation of B in a BT mixture using *Mycobacterium* sp. JOB-5 [34]. Also, *Pseudomonas putida* F1 degrades B and T at the same time but degrades T faster than B [16].

The degradation of M by *Rhodococcus* sp. EH831 was inhibited by the coexistence of other substrates of BTEX (Fig. 2, Table 1). There are some previous studies on the effect of BTEX on the degradation of M. In a batch test with a BTEX- and M-enriched consortium, BTEX was not found to affect the degradation of M, but it increased the biomass in a continuous flow system [35]. Moreover, BTEX did not affect the degradation of M in an aerobic fluidized bed reactor [17,18]. Pruden and Suidan also found that BTEX did not significantly affect the degradation of M degradation by *Pseudomonas* sp. PM1 [22]. Other studies, however, show that BTEX affects the degradation of M. For example, using the M-degrading bacterium strain PM1, Deeb et al. found that the degradation of M was prevented by the addition of 20 mg/L E and X and reduced by 5 mg/L of these two compounds [1]. Also, the rate of M degradation by an M-enriched consortium was slightly decreased by BTEX [35]. Thus, BTEX inhibits or has no affect on the degradation of M depending on the microorganism, the concentration of compounds, and the experimental system (e.g., batch or continuous). In particular, different results have been obtained for BTEX depending on whether each component was analyzed individually or within a mixture. For example, the degradation of M by PM1 was inhibited by B, T, E, and X [1] but not by a BTEX mixture [22].

Interestingly, the degradation of M by *Rhodococcus* sp. EH831 was completely inhibited in the presence of E (Fig. 2 and Table 1). Deeb et al. reported that The M-degrading bacterium strain PM1 was also completely inhibited by E [1]. They reported that this inhibition was not due to a metabolite of E but rather competitive enzyme inhibition.

It seems that *Rhodococcus* sp. EH831 might have multiple pathways for the degradation of B, T, E, X, and M because the degradation of only B was delayed by T, and because E completely blocked the degradation of M and B. These results suggest that, although EH831 can degrade structurally similar compounds (B, T, E, and X), the pathways mediating their degradation appear to be different. Further studies are needed to clarify the interactions between the different components in these metabolic pathways. Investigation of the degradative pathways for each substrate in EH831 will provide additional information about how the different components affect each other's degradation.

5. Conclusions

Methyl *tert*-butyl ether (M) and BTEX (mixture of benzene (B), toluene (T), ethylbenzene (E), and xylene (X)) are often found together in gasoline-contaminated groundwater. It is important to study how the different components affect each other's degradation to understand the dynamics in the environment and the similarities and differences in the biodegradation of the components in a BTEXM mixture. In this study, the substrate interaction in BTEXM mixtures by a single microorganism, *Rhodococcus* sp. EH831 was quantitatively and qualitatively analyzed. Our findings should aid in the development of bioremediation methods and in prediction of the dynamics between the different components *ex situ* and *in situ*.

Acknowledgements

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