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An analysis of synergistic and antagonistic behavior during BTEX removal in batch system using response surface methodology

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Abstract

The removal of benzene, toluene, ethylbenzene and xylene (BTEX) as quaternary mixtures were studied in batch systems using a well-defined mixed microbial culture. The synergistic and antagonistic effects of total BTEX removal ($BTEX_{T\text{-}RE}$) due to the presence of mixed substrate was evaluated through experiments designed by response surface methodology (RSM). The low and high concentrations of individual BTEX were 15 and 75 mg l^{-1}, respectively. The results showed that, increasing the concentration of xylene increased the cumulative BTEX removal ($BTEX_{T\text{-}RE}$), however the reverse occurred when benzene concentrations were increased from low to high levels. A mixed response of increasing and decreasing trend in the $BTEX_{T\text{-}RE}$ value was observed when either of toluene or ethylbenzene concentration was increased. When the concentrations of individual BTEX compounds were 30 mg l^{-1}, the $BTEX_{T\text{-}RE}$ was about 58%. Complete $BTEX_{T\text{-}RE}$ was achieved at optimal BTEX concentrations of 48.1, 45.6, 49.3 and 56.6 mg l^{-1}. The RSM approach was found efficient in explaining the main, squared and interaction effects among individual BTEX concentrations on the $BTEX_{T\text{-}RE}$ in a more statistically meaningful way.

Keywords: BTEX removal; Mixed culture; Response surface methodology; Interaction effects

1. Introduction

Benzene, toluene, ethylbenzene, and xylene collectively known as BTEX, are widely used as industrial solvents in fine chemical and petrochemical industries. They form the major aromatic component in many petroleum products and are often found in groundwater as a result of leaks in underground storage tanks and pipelines, improper waste disposal practices, inadvertent spills and leaching from landfills. The BTEXs make up the alkyl benzenes and constitute up to about 18% (w/w) in a standard gasoline blend. The USEPA classified BTEX as priority pollutants that tend to pose significant threat to human health and environment due to their toxic and carcinogenic properties. Even at low concentrations, BTEX can cause damage to the liver and kidney and paralyze the central nervous system [1,2]. The contamination of soils and aquifers with petroleum hydrocarbon is a major environmental problem. Generally these compounds are often encountered as mixtures rather than as a single compound and the fates of these compounds are strongly controlled by microbial activity [3]. Spill hydrocarbons are transported by gravity through the unsaturated zone and are usually floating on groundwater, from where they disperse horizontally along the groundwater gradient and vertically within the capillary fringe. Their poor solubility in water makes them travel hundreds of meters down stream with ground water from the contaminated site [4].

Among all remediation technologies for treating BTEX contaminated water, biodegradation appears to be an economical, energy efficient and environmentally sound approach. The biodegradation kinetics and reaction rates are kinetically controlled and influenced by parameters such as pH, temperature, initial substrate concentration, type of inoculum added, nutrient concentration, substrate inhibition and the presence of other

Abbreviations: Adj SS, adjusted sum of squares; Adj MS, adjusted mean squares; ANOVA, analysis of variance; BTEX, benzene, toluene, ethylbenzene and xylene; $BTEX_{T\text{-}RE}$, total BTEX removal; CCD, central composite design; DF, degrees of freedom; $F_{statistics}$, Fischer's variance ratio; MSM, mineral salt media; P, probability value; RSM, response surface methodology; R^2, coefficient of determination; Seq SS, sequential sum of squares; TPH, total petroleum hydrocarbon; USEPA, United States Environmental Protection Agency; *o*,*m*,*p*-X, isomers of xylene (ortho, meta and para)

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complex carbon sources [5]. These compounds are degraded effectively by naturally occurring soil microorganisms, and other mixed aerobic bacterial populations [6–8]. Most of the studies on the biodegradation of BTEX compounds have primarily been carried out with mixed cultures acclimatized to specific compounds in batch and continuous systems [6,9–12]. A few studies on the biodegradation of mixtures of BTEX compounds have also been reported. An investigation on the degradation of B, T and *p*-X individually and in mixtures by a mixed consortium and a *Pseudomonas* species showed only benzene and toluene degradation but not *p*-xylene as individual substrate, while in mixtures competitive inhibition and co-metabolic degradation were observed [8]. The kinetics of BTX biodegradation in batch systems was studied using a mixed consortium isolated from a contaminated soil and it was observed that substrate disappearance occurred in the order: toluene > benzene > xylene [13]. A microbial consortium enriched on toluene was able to degrade BTEX compounds individually and in mixtures in a chemostat [14]. Co-cultures of *Pseudomonas putida* and *Pseudomonas fluorescens* acclimatized with toluene have also shown to be capable of degrading high concentrations of BTEX [9]. However, substrate inhibition at certain threshold concentration for all the substrates was noticed. The biodegradation of BTE*o*,*m*,*p*-X, both as individual and as mixtures in batch systems using head space analysis showed high specific growth rates ($0.325\,h^{-1}$) with individual benzene, while in mixtures, benzene and toluene were rapidly utilized (<12 h) by a *Ralstonia* sp. strain PHS1 [15]. Anew, the biodegradation of benzene, toluene and phenol using *P. putida* in batch systems showed specific growth rate to be a decreasing function of concentration [16]. It has been well observed that BTEX compounds beyond certain concentrations can inhibit the activity of microbes growing on them due to complex micro- and macro-level interactions. Moreover, most of the previously reported studies have reported a complex interaction patterns during BTEX biodegradation using mixed/pure cultures, despite similarities in chemical and structural properties [7,8,15,17]. It is therefore useful to understand the interaction effects in mixtures systematically at different concentration ranges of individual BTEX compounds. For this reason, the objectives of this work were constituted as follows: (i) to conduct batch system experiments and study the removal pattern of individual BTEX compounds in mixtures and (ii) delineate the synergistic and antagonistic effects of mixed substrates through a statistically authentic approach.

2. Materials and method

2.1. Microorganism and media composition

The mixed microbial consortium previously acclimatized to total petroleum hydrocarbon (TPH) was obtained from Bio-House®, Korea, which contained a mixture of identified *Pseudomonas* sp., *Yarroia* sp., *Acinetobacter* sp., *Corynebacterium* sp., and *Sphingomonas* sp. This mixed culture was stored at 4 °C and used directly in all experiments. The mineral salt medium (MSM) had the following composition: $(NH_4)_2SO_4$: $5\,g\,l^{-1}$; K_2HPO_4: $2\,g\,l^{-1}$; $MgSO_4{\cdot}7H_2O$: $0.2\,g\,l^{-1}$; KH_2PO_4: $1\,g\,l^{-1}$; $CaCl_2{\cdot}2H_2O$: $13.2\,mg\,l^{-1}$; $FeSO_4{\cdot}7H_2O$: $10\,mg\,l^{-1}$. Two millilitres of trace mineral (MoO_3: $1\,mg\,l^{-1}$; $ZnCl_2$: $3.3\,mg\,l^{-1}$; $CuCl_2{\cdot}2H_2O$: $0.3\,mg\,l^{-1}$; H_3BO_3: $1\,mg\,l^{-1}$; $CoCl_2{\cdot}6H_2O$: $6\,mg\,l^{-1}$; $NiSO_4{\cdot}6H_2O$: $1\,mg\,l^{-1}$) was also added to the MSM. The pH of the media was adjusted to 7.0 ± 0.1. All chemicals used in this study were of analytical grade.

Table 1
Experimental range and levels of independent variables for $BTEX_{T\text{-}RE}$

Independent variable	Symbol	Level				
		$-\alpha$	−1	0	+1	$+\alpha$
Benzene ($mg\,l^{-1}$)	X_1	15	30	45	60	75
Toluene ($mg\,l^{-1}$)	X_2	15	30	45	60	75
Ethylbenzene ($mg\,l^{-1}$)	X_3	15	30	45	60	75
Xylene ($mg\,l^{-1}$)	X_4	15	30	45	60	75

2.2. BTEX removal studies

Batch removal experiments were conducted by varying individual BTEX concentrations between 15 and $75\,mg\,l^{-1}$ ($-\alpha$ to $+\alpha$, as shown in Table 1) according to the statistically significant response surface methodology (RSM), in 500 ml glass bottles (working volume, 300 ml) fitted with appropriate toggle valve based screw caps. These valves were made of Teflon that housed needles to collect headspace BTEX and biomass in suspension. BTEX was directly injected to this working volume corresponding to different initial concentrations and after water–air equilibration, 3% of the microbial suspension was added. The resulting solution was incubated in a rotary shaker at 150 rpm and maintained at ambient temperatures (28–30 °C). Samples collected at regular intervals were analyzed for biomass and residual BTEX concentration.

2.3. Analytical

2.3.1. Biomass and residual BTEX measurement

Free cell concentration was determined by measuring the optical density (OD at 660 nm) using a UV–vis spectrophotometer (Shimadzu UV-Min 1240, Japan) and reading from a standard calibration plot between OD_{660} and cell dry weight. The concentrations of BTEX in liquid phase were determined from the water–air partition coefficient values [18], and by the procedure described in Prenafeta-Boldú et al. [19]. Head space BTEX measurements were done by injecting 100 μl headspace samples collected in gas-tight syringe (Hamilton Co., Reno, NV, USA) into a DS-6200 Gas Chromatograph fitted with a HP-FFAP capillary column and flame ionization detector. Nitrogen was used as the carrier gas at a flow rate of $20\,ml\,min^{-1}$. The temperatures of the injection port, oven and detection port were 250, 80 and 250 °C.

2.4. Response surface methodology

Response surface methodology (RSM) is the combination of mathematical and statistical techniques used in empirical study of relationships and optimization, where several inde-

pendent variables influence a dependent variable or response, the goal being to secure the optimal response. By definition, RSM is an empirical technique, which employs multiple regression analysis of the quantitative data obtained from properly designed experiments to simultaneously solve multivariate equations [20,21]. The graphical representations of these equations are called a response surface contour that describes the cumulative interactions of the test variables on the response [22,23].

The full factorial central composite design (CCD) used in this study consisted of (i) a complete 2^4 factorial design, (ii) n_0, center point ($n_0 \geq 1$) and (iii) two axial points on the axis of each design variable at a distance of $\propto$ (=$2^{k/4}$ for $k = 4$) from the design centre. Hence a total number of design points of $N = 2^k + 2k + n_0$ was used. The center point was replicated seven times to give six degrees of freedom for calculation of errors in the experiments. The optimal values of the experimental conditions were obtained by solving the regression equation and by analyzing the response surface contour plots. The variables were coded according to the equation:

$$X_i = \frac{x_i - x_0}{\Delta x} \quad (1)$$

where X_i is the coded value of variable i, x_i the dimensionless uncoded (actual) value of X_i, x_0 the value of X_i at the center point, and Δx is the step change between levels -1 and 0. The behavior of the system was explained by the following second order polynomial equation:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (2)$$

where Y is the predicted response, β_0 the offset term, β_i the coefficient of linear effect, β_{ii} the coefficient of squared effect, X_j is the coded value of variable j and β_{ij} the coefficient of interaction effect.

In this study, benzene (X_1), toluene (X_2), ethylbenzene (X_3) and xylene (X_4) concentrations were chosen as the factors, while total BTEX removal values were used as the response variable. Analysis of variance (ANOVA) was applied to estimate the effects of main variables and their potential interaction effects on the BTEX removal efficiency. The ANOVA table provides information on the following terms: DF (degrees of freedom), Seq SS (sequential sum of squares), Adj SS (adjusted sum of squares), Adj MS (adjusted mean squares), $F_{\text{statistics}}$ (Fischer's variance ratio), and P (probability value). The goodness-of-fit of the regression model and the significance of parameters estimates were also determined through appropriate statistical methods. Statistical calculations and analysis were done using the software MINITAB (Version 12.2, PA, USA).

3. Results and discussion

3.1. BTEX removal in mixtures

To understand the potential of the previously acclimatized culture to degrade BTEX compounds and to estimate the interaction effects, batch experiments were conducted according to the statistically significant RSM. A total of 31 experiments were conducted at different initial concentrations of BTEX. Seven experiments (runs 25–31) were repeated at the center point ('0' level) to verify any change in the estimation procedure as a measure of the precision property. The initial concentrations of individual BTEX compounds were in the range of 15–75 mg l^{-1} (Table 1, run no. 1–31). Thus the 31 experiments, conducted for 50 h-duration would correspond to a total BTEX concentration varying between 120 and 240 mg l^{-1}. The biomass growth pattern observed at all experimental conditions (low and center point levels shown in Figs. 1 and 2) were typical of a conventional biodegradation process with lag, logarithmic growth and stationary phases. It was observed that at low and high levels of substrate concentrations, the biomass growth was significantly less. At the center point level, there was a threefold increase in the biomass growth ($\sim$1 g l^{-1}). The total BTEX removal, $\text{BTEX}_{\text{T-RE}}$

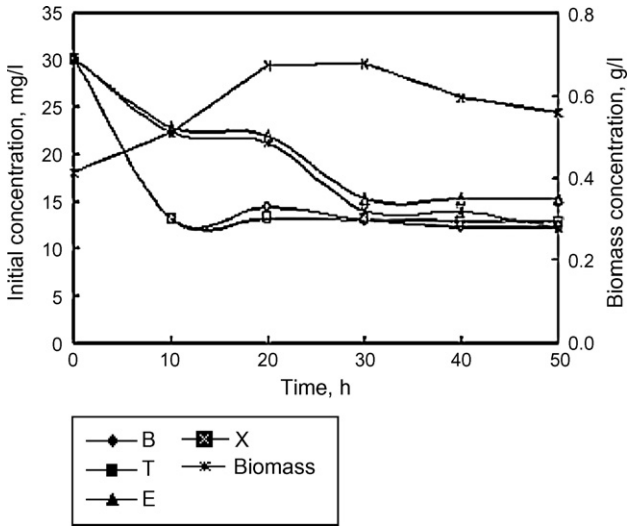

Fig. 1. Substrate utilization and biomass growth profile at low concentrations of BTEX compounds in mixture (each of B, T, E, X—30 mg l^{-1}).

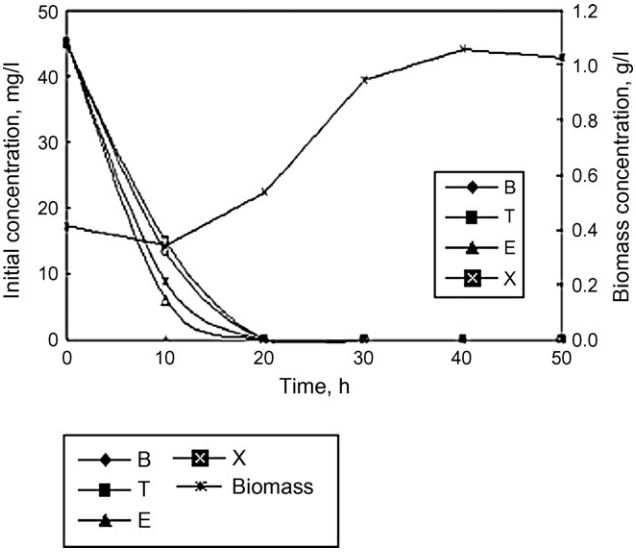

Fig. 2. Substrate utilization and biomass growth profile at the center point concentrations of BTEX compounds in mixture (each of B, T, E, X—45 mg l^{-1}).

Table 2
Full factorial central composite design for $BTEX_{T\text{-}RE}$

Run order	Concentration in coded units				$BTEX_{T\text{-}RE}$ (%)	
	X_1	X_2	X_3	X_4	Observed	Predicted
1	−1	−1	−1	−1	57.33	64.50
2	+1	−1	−1	−1	61.07	62.21
3	−1	+1	−1	−1	54.93	66.53
4	+1	+1	−1	−1	70.89	72.99
5	−1	−1	+1	−1	65.33	74.03
6	+1	−1	+1	−1	66.44	70.90
7	−1	+1	+1	−1	80.74	79.27
8	+1	+1	+1	−1	83.99	84.90
9	−1	−1	−1	+1	78.46	83.70
10	+1	−1	−1	+1	78.12	82.06
11	−1	+1	−1	+1	78.17	76.19
12	+1	+1	−1	+1	85.86	83.30
13	−1	−1	+1	+1	87.80	88.18
14	+1	−1	+1	+1	91.16	85.71
15	−1	+1	+1	+1	78.87	83.88
16	+1	+1	+1	+1	94.85	90.15
17	$-\alpha$	0	0	0	95.66	82.65
18	$+\alpha$	0	0	0	82.24	86.63
19	0	$-\alpha$	0	0	77.73	69.25
20	0	$+\alpha$	0	0	75.86	75.72
21	0	0	$-\alpha$	0	74.73	65.72
22	0	0	$+\alpha$	0	81.71	82.10
23	0	0	0	$-\alpha$	81.87	68.87
24	0	0	0	$+\alpha$	88.95	93.33
25	0	0	0	0	94.67	95.83
26	0	0	0	0	100.00	95.83
27	0	0	0	0	99.17	95.83
28	0	0	0	0	99.17	95.83
29	0	0	0	0	99.72	95.83
30	0	0	0	0	99.17	95.83
31	0	0	0	0	98.89	95.83

was calculated as follows:

$$BTEX_{T\text{-}RE}\ (\text{or})\ \text{total-RE} = \frac{(\text{Total-BTEX})_{\text{initial}} - (\text{Total-BTEX})_{\text{final}}}{\text{Total-BTEX}_{\text{initial}}} \times 100 \quad (3)$$

The total BTEX removals ($BTEX_{T\text{-}RE}$) at the three levels were 57, 99 and 94%, respectively. However, their individual removal in mixtures varied widely depending on the initial settings of other parameters ($-\alpha$, −1, 0, +1, $+\alpha$). Run no. 1 (Table 2) was chosen as the basis for this study to understand the dynamics of main (linear) and interaction effects. In run no. 2, TEX concentrations were held constant at 30 mg l^{-1}, while the concentration of benzene was increased twofold. A 24% increase in the removal of benzene was observed, while EX was inhibited by 5%. Similarly when the concentration of toluene was increased twofold, the removal of BEX was inhibited by nearly 10%. Seventy-two percent toluene was removed while the $BTEX_{T\text{-}RE}$ was 54%. The $BTEX_{T\text{-}RE}$ was 65% in run 5, which corresponds to an increase in the concentration of ethylbenzene. In run no. 9, when the concentration of xylene was increased, the removals of BT and E increased rapidly to 61, 100 and 69%, respectively. This shows severe synergistic effect due to increase in the concentration of xylene in mixtures. The removal of toluene increased by 43%, while BE were increased by 10 and 14%, respectively. The main effects plot for $BTEX_{T\text{-}RE}$ showed a very complex behavior depending on the initial concentration of individual BTEX compounds (Fig. 3). This figure could be interpreted as follows: (i) increasing the concentration of benzene from 15 to 75 mg l^{-1} decreased $BTEX_{T\text{-}RE}$; (ii) increasing the concentration of toluene from 15 to 75 mg l^{-1} increased the $BTEX_{T\text{-}RE}$ until the center point level (45 mg l^{-1}), however after that the profile of $BTEX_{T\text{-}RE}$ decreased significantly. This behavior suggests the definite existence of an optimum toluene concentration to achieve maximum $BTEX_{T\text{-}RE}$; (iii) a mixed response of increasing and decreasing $BTEX_{T\text{-}RE}$ profile was observed when ethylbenzene concentrations were varied from 15 to 75 mg l^{-1}, which also suggested the existence of an optimum concentration of xylene in BTEX mixture; (iv) the $BTEX_{T\text{-}RE}$ values increased with increase in xylene concentrations from 15 to 75 mg l^{-1} in mixtures. These profiles suggest that at both low (15 mg l^{-1}) and high (75 mg l^{-1}) levels, the biodegrading capability of the microorganisms were limited or inhibited. At high concentration levels, though there was high removal, the biomass growth profile apparently reduced (0.7 g l^{-1}). This can be attributed to changes occurring at the cellular and molecular level. On the other hand, modification in membrane permeability, lipid solubilization, adsorption and

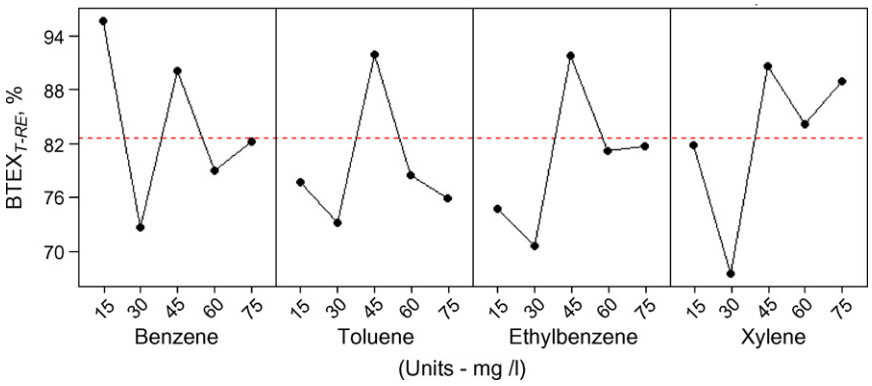

Fig. 3. Main effects plot of parameters for total BTEX removal.

inactivation of proteins also can contribute to the loss of activity leading to limiting/inhibition conditions [24]. The BEX removals were inhibited by 56, 24 and 26% when the concentration of toluene was raised to 75 mg l^{-1} in mixture. This shows a severe antagonistic behavior on the removal of other compounds, when toluene concentration was raised. Nevertheless toluene removal was also inhibited by 35%, when BEX compounds were held constant at 45 mg l^{-1}. When the concentration of xylene was low in mixture (15 mg l^{-1}), 100% removal of ethylbenzene was plausible, however at high concentrations of xylene (75 mg l^{-1}), the removal of ethylbenzene was 82%, while 100% benzene removal was achieved. The results collectively display antagonistic and synergistic effects which primarily depended on the concentrations of individual compounds. However, these results produced main, squared and interaction effects, which would be statistically interpreted in the later part of this paper. In certain cases, preferential utilization of the substrates by mixed culture could be possible, depending on the structural stability and toxicity of the compound [25]. This can be attributed to the initial oxidation mechanisms of BTEX compounds and their biodegradation pathways.

3.2. Literature comparison and degradation mechanism

Though several literatures have reported the biodegradation pattern and kinetics of mixtures of BTEX compounds, most of them focused in understanding the interaction effects at certain combinations of substrate levels without statistical interpretation. Some of the closely related works and their significant findings that can be compared with this study are discussed here. A decline in the degradation rate of BTEX as mixtures in comparison to individual degradation rates and negative substrate interaction during the biodegradation of growth substrates (BTE*o*-X) has been reported [15]. However, in binary mixtures, they observed that the presence of ethylbenzene inhibited BT*o*-X degradations. During the degradation of mixtures of benzene and toluene using *P. putida* F1 in liquid cultures, it was reported that the biomass utilized toluene better than benzene, and toluene was depleted first [26]. The presence of toluene inhibited the degradation of benzene, while the presence of benzene had little effect on toluene consumption. In batch studies using *Pseudomonas fragi*, the rate of degradation of either benzene or toluene in the presence of other substrate was slower than the degradation rate of either substrate alone. The same authors used an isolated strain of *P. fluorescens* and found that the presence of benzene did not affect the degradation of toluene [7]. The interactions observed between benzene, toluene and *p*-xylene in batch systems show that the presence of *p*-xylene slowed the utilization of toluene [8]. However, during benzene degradation in the presence of toluene, *o*-xylene and a number of poly nuclear aromatic hydrocarbons, the presence of either toluene or *o*-xylene, benzene

Table 3
Analysis of variance for total BTEX removal efficiency

Source	DF	Seq SS	Adj MS	$F_{statistics}$	*P*
Model	14	4082.9	291.6	4.81	0.002
Linear	4	1386.5	346.6	5.71	0.005
Square	4	2491.8	622.9	10.27	0.0001
Interaction	6	204.6	34.1	0.56	0.754
Residual error	16	970.5	60.6		
Lack-of-fit	10	951.2	95.1	29.41	0.0001
Pure error	6	19.4	3.2		
Total	30	5053.5			

Note: For calculations of ANOVA terms, viz., DF, Seq SS, Adj MS, $F_{statistics}$ and *P*, refer Montgomery [20].

Table 4
Estimated regression coefficients, *t* and *P*-values for total BTEX removal efficiency

Term	Coefficients	*t*	*P*
Constant	95.827	33.491	0.0001
X_1	0.996	0.627	0.540
X_2	1.619	1.018	0.324
X_3	4.096	2.577	0.020
X_4	6.114	3.846	0.001
X_1^2	−2.797	−2.395	0.029
X_2^2	−5.836	−4.481	0.001
X_3^2	−5.480	−4.236	0.001
X_4^2	−3.682	−3.002	0.008
X_1X_2	2.188	1.124	0.278
X_1X_3	−0.209	−0.108	0.916
X_1X_4	0.164	0.084	0.934
X_2X_3	0.803	0.412	0.685
X_2X_4	−2.386	−1.225	0.238
X_3X_4	−1.263	−0.649	0.526

X_1: benzene; X_2: toluene; X_3: ethylbenzene; X_4: xylene.

degradation was stimulated [17]. The prime reason for severe antagonistic effects during BTEX degradation in mixtures can be attributed to competitive inhibition [7,8,27], toxicity [28] and the formation of toxic intermediates by non-specific enzymes [29,30]. However, it has been proved that isomers of xylene can be better removed by co-metabolism due to the following reasons: (i) the ring fission products of different catechols are catabolized by different enzymatic systems [31] and (ii) possible accumulation and polymerization of intermediates such as toluic acid, tolualdehyde and methyl salicylic acid during xylene degradation [7,32]. The most commonly occurring sequence of BTEX biodegradation is as follows: the first step of benzene oxidation is a hydroxylation reaction catalyzed by dioxygenase. The product, a diol is then converted to catechol by a dehydrogenase. These initial reactions, hydroxylation and dehydroxylation are also common to degradation pathways of other aromatic hydrocarbons. The introduction of a substituent methyl group onto the benzene ring (toluene) renders alternative mechanisms possible to attack side chains or to oxidize the aromatic ring. The growth of *Pseudomonas aeruginosa* and *P. putida* F1, on toluene is an example of a catabolic pathway initiated by side chain oxidation [33]. In a monooxygenase catalyzed reaction, toluene is converted to benzyl alcohol which is further oxidized to benzoic acid by dehydrogenation. Under aerobic conditions ethylbenzene degradation involves oxygenase reactions. The aerobic degradation can proceed in either of two primary pathways. Generally this degradation is initiated by a dioxygenation of the aromatic ring, leading to an extra diol ring cleavage. Lee and Gibson [34] found that naphthalene dioxygenase exhibits highly relaxed substrate specificity and is capable of aerobically degrading ethylbenzene directly to styrene which is the process most often used in industry. Xylene isomers have been shown to be oxidized by the *tod* or *tol* pathway [32,35]. Although oxidized slowly, these substrates support the growth of bacteria that initiate degradation by ring hydrogenation and are subsequently converted to dimethyl catechols.

3.3. Statistical analysis

The results from this study helped to frame a second order polynomial equation (in coded units) that could relate $BTEX_{T\text{-}RE}$ to the initial concentrations of BTEX. This is given by

$$\begin{aligned} BTEX_{T\text{-}RE} = {} & 95.827 + 0.996X_1 + 1.619X_2 + 4.096X_3 \\ & + 6.114X_4 - 2.797X_1^2 - 5.836X_2^2 - 5.480X_3^2 \\ & - 3.682X_4^2 + 2.188X_1X_2 - 0.209X_1X_3 \\ & + 0.164X_1X_4 + 0.803X_2X_3 - 2.386X_2X_4 \\ & - 1.263X_3X_4 \end{aligned} \quad (4)$$

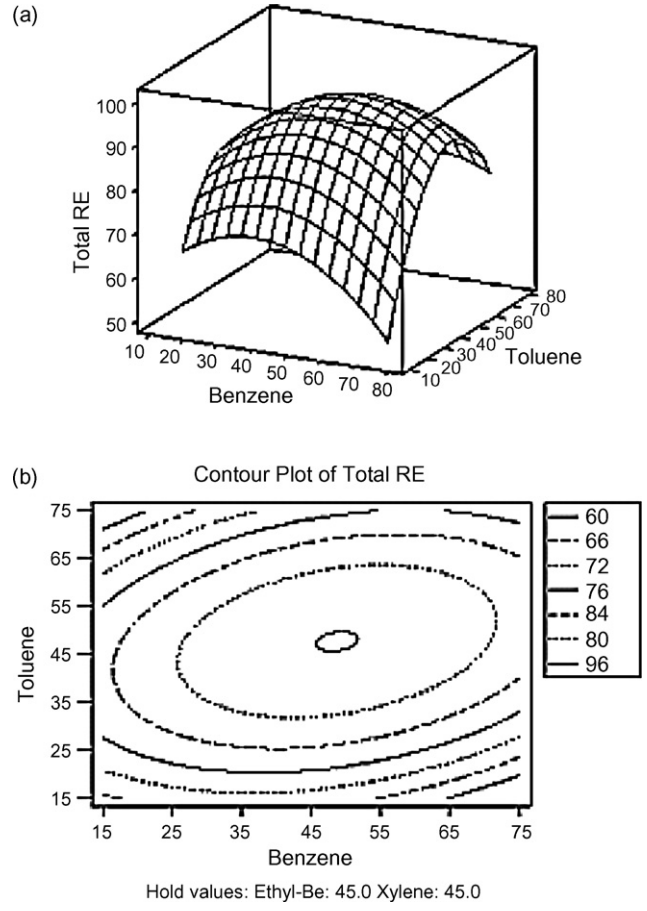

Fig. 4. Interaction effect of benzene and toluene concentration on total BTEX removal: (a) surface plot and (b) contour plot.

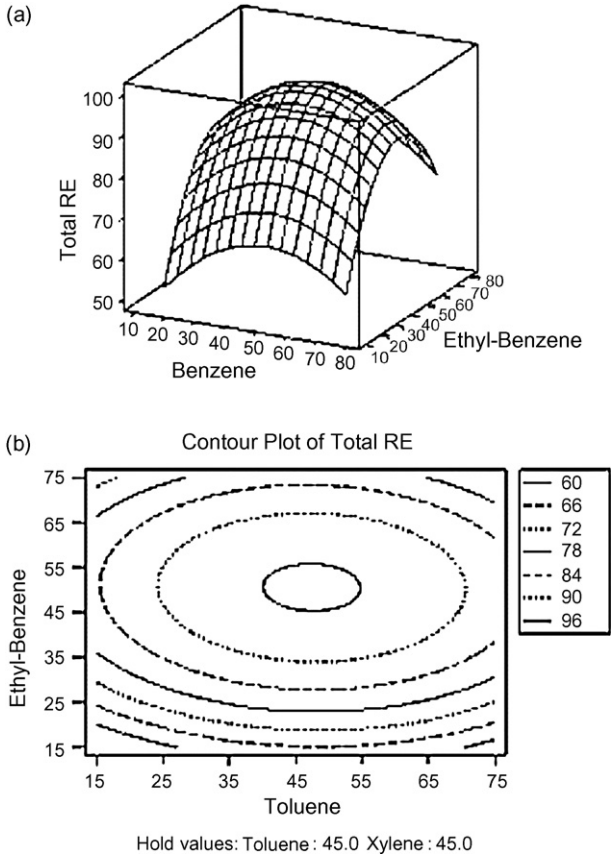

Fig. 5. Interaction effect of benzene and ethylbenzene concentration on total BTEX removal: (a) surface plot and (b) contour plot.

The predicted $BTEX_{T\text{-}RE}$ values from this equation are given in Table 2. Apart from the linear effects of the initial BTEX concentrations on $BTEX_{T\text{-}RE}$, an analysis using RSM also gives an insight into the quadratic and interaction effects of the parameters. The result of this in the form of analysis of variance (ANOVA) is given in Table 3. In general, the Fischer's '$F_{statistics}$' value with a low probability 'P' value indicates high significance of the regression model [21]. The Students 't' test can be used as a tool to check the significance of the regression coefficient of the parameter, while 'P' values signify the pattern of interaction among the factors. The larger the value of t and smaller the value of P, the more significant is the corresponding coefficient term [20]. The value of determination coefficient, ($R^2 = 0.81$) indicates that about 19% of the total variations were not satisfactorily explained by the model. Though this value would be considered low in applied statistics, it can be accepted due to complex interaction pattern observed during microbial mediated reactions, which could not be reasoned by the model equation. The ANOVA table can be used to test the statistical significance of the ratio of mean square due to regression and mean square due to residual error. From this table it is evident that, the $F_{statistics}$ values of linear and squared regression were higher. These large values imply, that the $BTEX_{T\text{-}RE}$ can be adequately explained by the model equation. Generally P-values lower than 0.01 indicates that the model is considered to be statistically significant at the 99% confidence level [36]. Thus, the squared effects of the variables were more significant ($F_{statistics} = 10.27$) than the linear (5.71) and interaction effects (0.56). The $BTEX_{T\text{-}RE}$ prediction from the model is shown in Table 2. The unexplained part of the model (19%) is represented as residual error in the ANOVA table. From Table 4, it was observed that the linear effects of ethylbenzene and xylene concentrations were significant ($P = 0.02$, 0.001), than both benzene and toluene concentrations. The coefficient of quadratic effect of BTEX concentrations ($P < 0.05$) were highly significant. Among the interaction terms, the interactions between benzene and ethylbenzene and benzene and xylene ($P = 0.916$, 0.934) were less significant in increasing the $BTEX_{T\text{-}RE}$ by mixed cultures.

The response surface plots (Figs. 4a–9a) obtained from the software provides a three-dimensional view of the $BTEX_{T\text{-}RE}$ (total-RE) surface over different combinations of independent variables. The contour plots (Figs. 4b–9b) are represented as a function of two factors at a time holding other factors at a fixed level ('0'). These plots are obtained from the model, the

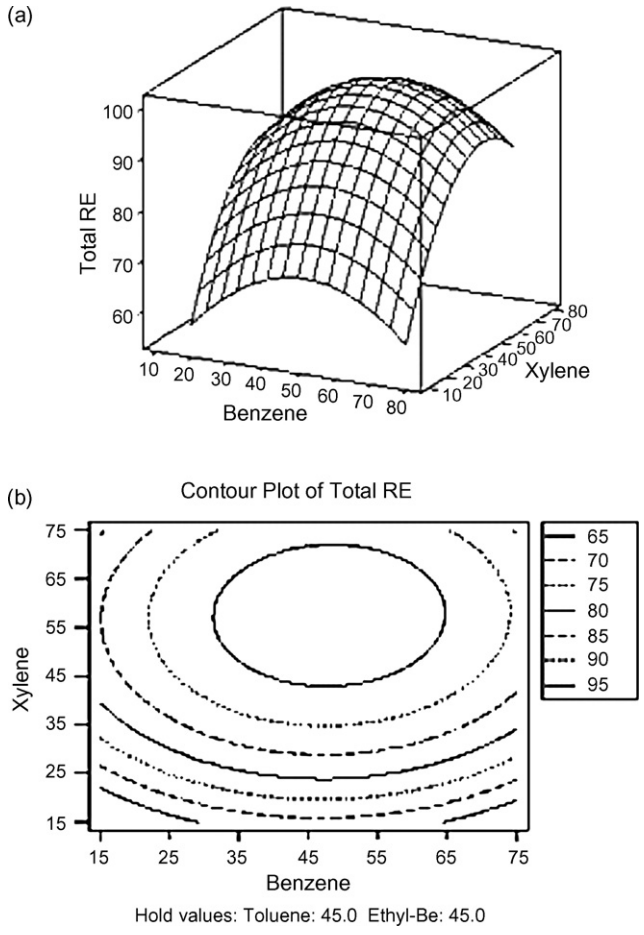

Fig. 6. Interaction effect of benzene and xylene concentration on total BTEX removal: (a) surface plot and (b) contour plot.

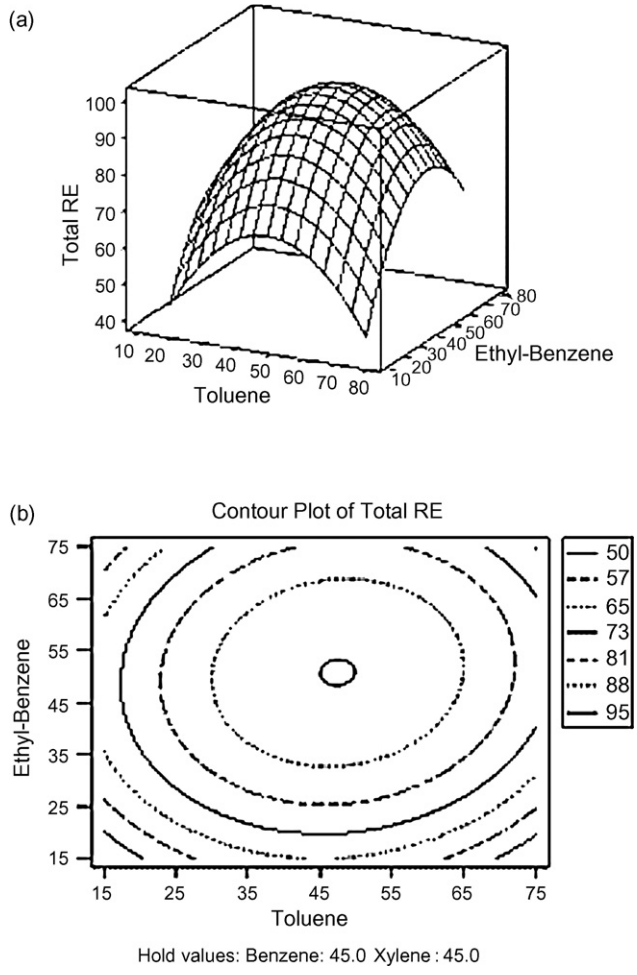

Fig. 7. Interaction effect of toluene and ethylbenzene concentration on total BTEX removal: (a) surface plot and (b) contour plot.

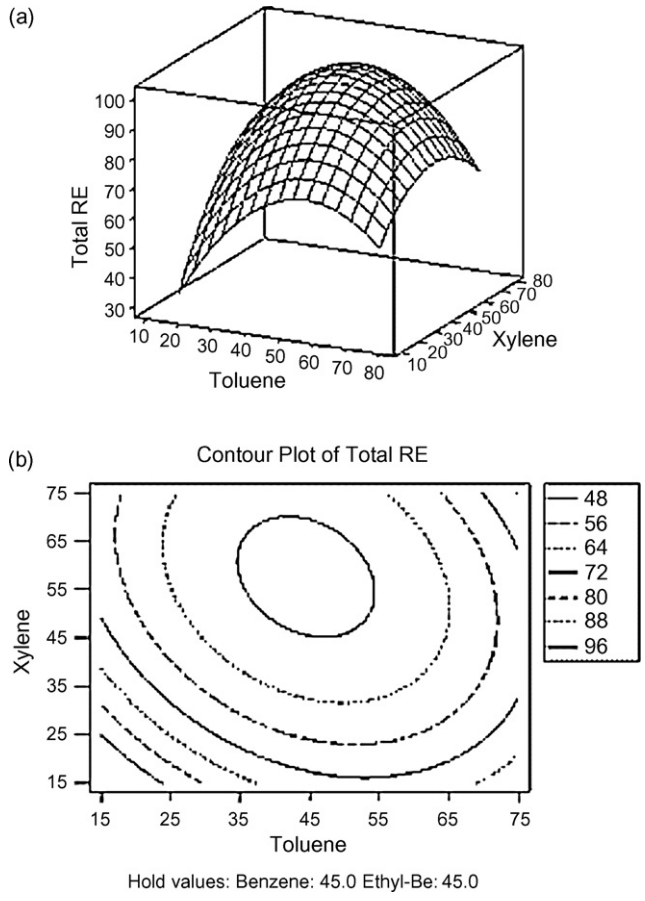

Fig. 8. Interaction effect of toluene and xylene concentration on total BTEX removal: (a) surface plot and (b) contour plot.

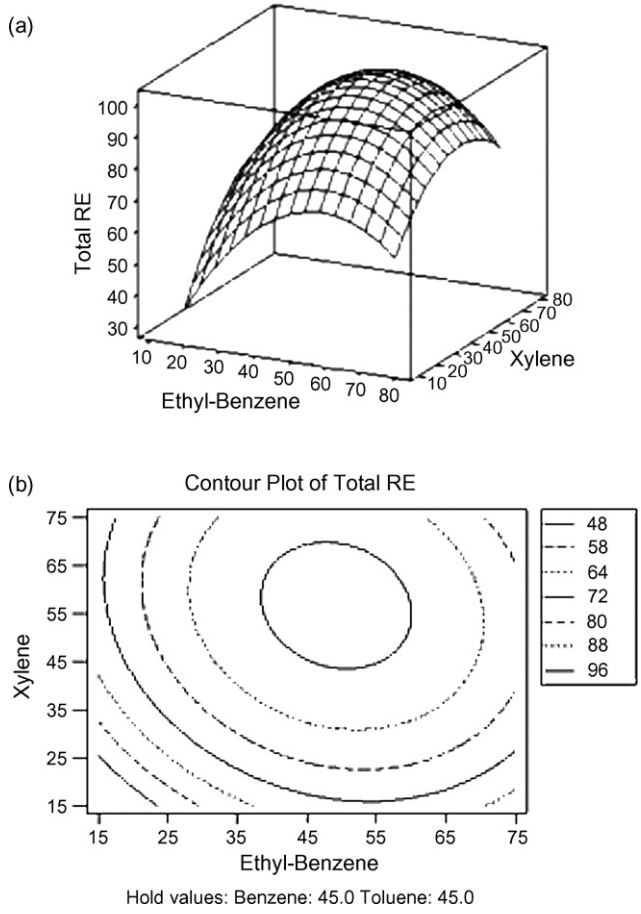

Fig. 9. Interaction effect of ethylbenzene and xylene concentration on total BTEX removal: (a) surface plot and (b) contour plot.

values taken by one factor, while the second varies from 15 to 75 mg l^{-1} with constraint of a given *Y*-value. The maximum predicted $BTEX_{T\text{-}RE}$ is indicated by the smallest ellipse that confines within the contour plot. These types of plot passes through the steepest ascent of the total BTEX removed and the optimum operating conditions and in direction of maximum decline of the response with respect to increasing or decreasing values of the independent variable. From these RS plots, it is also evident that at low and high levels of the variables the total BTEX removal was minimal, however there existed a region where neither an increasing nor a decreasing trend in the total BTEX removal was noticed. This suggests the existence of optimum concentrations of the variables to achieve maximum $BTEX_{T\text{-}RE}$. The model equation (3) was therefore solved by the Monte Carlo optimization routine [37], for finding the exact concentrations of individual BTEX compounds that could potentially yield maximum $BTEX_{T\text{-}RE}$. These concentrations were first obtained in coded units and then transformed into regular units using Eq. (1). The optimum concentrations to achieve nearly 100% removal were: benzene 48.1 mg l^{-1}, toluene 45.6 mg l^{-1}, ethylbenzene 49.3 mg l^{-1} and xylene 56.6 mg l^{-1}. These optimized values match the values that can be visually drawn from RS plots, contour plots, experimental runs (center point replicates) and with a set of duplicate runs that was later conducted at these BTEX concentrations.

4. Conclusions

A method for statistically identifying the synergistic and antagonistic removal pattern during the removal BTEX compounds as mixtures were presented in this paper. The independent variables were individual BTEX concentrations ($k=4$) ranging between 30 and 60 mg l^{-1}, while the final response was the total BTEX removal ($BTEX_{T\text{-}RE}$). At equivalent concentrations of the individual BTEX compounds held at center point level (0), the removal pattern was almost similar (~99%), however this behavior changed at high levels (+1) of different substrates. The individual removals were 97, 93, 90 and 98%, respectively. Increasing the concentrations of xylene in mixture showed good synergistic effect on the removal of other compounds, while a severe antagonistic effect was observed when benzene concentrations were increased. The effects due to toluene and ethylbenzene showed a mixed response in the total BTEX removal pattern. At high levels of toluene in mixture (75 mg l^{-1}), BEX were inhibited by 56, 24 and 26%, respectively. ANOVA for total BTEX removal gave a determination coefficient of $R^2=0.81$, suggesting that the model is a good fit. A second order polynomial equation was obtained to theoretically explain the performance of the system in terms of the concentrations of BTEX compounds.

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