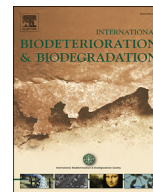




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## BTEX biodegradation in contaminated groundwater using a novel strain (*Pseudomonas* sp. BTEX-30)



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### ABSTRACT

BTEX (benzene, toluene, ethylbenzene and xylenes) compounds are the most frequently encountered subsurface contaminants among the various petroleum hydrocarbons. In this study, a new strain, able to degrade BTEX compounds, was isolated from oil contaminated groundwater. Phylogenetic analysis revealed that the isolated strain was most closely related to *Pseudomonas zhaodongensis* with 98% 16S rRNA gene sequence similarity. A series of batch experiments were carried out to investigate the ability of the new strain for removing BTEX compounds using single and mixed substrates. The optimized values of pH, temperature, and inorganic nutrients (as percent of BTEX concentration) were 7.6, 28.9 °C, and 200%, respectively which were obtained by using the response surface methodology for the biodegradation of BTEX. Results showed that the identified strain was able to completely degrade benzene, toluene, and ethylbenzene in the single substrate batch experiments while m-xylene remained non-degradable. Results of dual-substrate experiments revealed co-metabolism of m-xylene in the presence of benzene and toluene. Dioxygenase was found to be the key enzyme incorporating in the co-metabolism of m-xylene. Within the mixed substrate batch experiments all BTEX compounds can be degraded. An increase in the production of cell growth due to the degradation of benzene and toluene accelerated the degradation process of m-xylene in the mixed substrate experiments.

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### 1. Introduction

Aromatic hydrocarbons such as benzene, toluene, ethylbenzene, and xylene isomers (so-called BTEX) are found frequently in the crude oil and its derivatives are among of the most important pollutants of soil and groundwater (Nagarajan and Loh, 2015; Tabani et al., 2016). BTEX compounds are released into the environment through accidental spills of diesel fuel or gasoline during transportation, and leakages from underground storage tanks and pipelines.

BTEX compounds are listed as priority pollutants by the U.S. Environmental Protection Agency, and considered to be among the

top 100 chemicals on the priority list of hazardous substances (ATSDR, 2007). Prolonged exposure to BTEX compounds has adverse effects on both human health (e.g., damage the central nervous system) as well as ecosystem functions (e.g., inhibition effects on earthworm's survival) (Picone, 2012). Hence, developing or improving current remediation methods that minimize the environmental damages caused by BTEX compounds has drawn the attention of environmental protection agencies.

During the past several decades, a number of remediation techniques including biological (e.g., bioventing, phytoremediation), chemical (e.g., chemical oxidation, soil flushing), and physical (e.g., soil vapor extraction, thermal treatment) methods have been developed for the subsurface remediation of BTEX-contaminated soil and groundwater systems, (Firmino et al., 2015; Guo et al., 2012; Jin et al., 2013; Mahmoodlu, 2014; Mazzeo et al., 2010; Nagarajan and Loh, 2015; Stasik et al., 2015; Tabani et al., 2016; Zhang et al., 2013). Among these methods,

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biologically treatment of contaminated soil and groundwater is a well-established technique and is also known as being cost-effective and environmentally friendly (Firmino et al., 2015; Morlett-Chávez et al., 2010; Robledo-Ortiz et al., 2011; Xin et al., 2013). During the biodegradation process, microorganisms are able to directly degrade toxic hydrocarbons such as BTEX into less toxic compounds by consuming the available carbons within the structure of the hydrocarbons (Farhadian et al., 2008).

The performance of biological treatment method is affected by a range of environmental factors such as temperature, pH, and inorganic nutrients. Hence, these factors should be optimized in order to the implementation of efficient biological treatment systems in the subsurface. The One Variable at a Time (OVAT) approach has been increasingly used to optimize the multi-variable nature of the reactions involved, and consequently the bioremediation process (Li et al., 2011). However, the OVAT procedure has some disadvantages in that (1) interactions between factors are not taken into account, and many experiments are needed, (2) a large number of factors are considered, while only a small part of the experimental domain can be examined, (3) the global optimum is not easily established, and (4) the determined optimal conditions may depend upon the initial conditions (Lundstedt et al., 1998; Tarley et al., 2009).

An alternative approach would be to improve the experimental design to optimize experimental conditions as well as various factors that control the degradation reactions (Tarley et al., 2009). This approach would also include the possible use of mathematical model to evaluate the relative significance of various factors known to affect experiments (Jo et al., 2008). This method would further allow one to evaluate possible interactions between the various factors affecting degradation.

The Response Surface Methodology (RSM), is a combination of mathematical and statistical methods, is often used for the following reasons: (1) to design the experiments, (2) to approximate the complex quantitative relationships between factors and their responses, and (3) to identify the combination of factors providing optimized conditions (Fakhari et al., 2013; Kamarei et al., 2010).

*Pseudomonas* species have been extensively used for biodegradation of oil-contaminated soil and water (Heinaru et al., 2016; Sivasankar et al., 2016; Tsipa et al., 2016). However, the potential of a novel-isolated *Pseudomonas* sp. BTEX-30 strain to degrade BTEX compounds in the subsurface is still unknown. We performed for this reason a series of batch experiments with two objectives: (1) to evaluate the ability of a novel-isolated *Pseudomonas* sp. BTEX-30 strain, extracted from contaminated groundwater at the Tehran Oil Refinery Site, to degrade the BTEX compounds in the water phase, and (2) using RSM to optimize selected parameters affected the BTEX bioremediation using the newly isolated *Pseudomonas* sp. BTEX-30 strain.

## 2. Materials and methods

### 2.1. Chemicals

Chemicals used in this study included benzene, m-xylene, ethylbenzene (99% purity, Merck, Germany), and Toluene (99% purity, Scharlab, Spain). The mineral salt solution medium (MSM) used in the experiments consisted of a mixture of 500 mg NaNO<sub>3</sub>, 1000 mg NH<sub>4</sub>Cl, 21400 mg Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 1090 mg KH<sub>2</sub>PO<sub>4</sub>, 200 mg MgSO<sub>4</sub>·7H<sub>2</sub>O, and 1.0 ml of a trace salt solution per liter. A stock solution of trace salt containing 300 mg FeSO<sub>4</sub>·7H<sub>2</sub>O, 180 mg MnCl<sub>2</sub>·4H<sub>2</sub>O, 130 mg Co(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O, 40 mg ZnSO<sub>4</sub>·7H<sub>2</sub>O, 20 mg H<sub>2</sub>MoO<sub>4</sub>, 1 mg CuSO<sub>4</sub>·5H<sub>2</sub>O, 1 ml CaCl<sub>2</sub>, and 1 ml HCl (0.1 M) per liter was prepared.

### 2.2. Groundwater sampling, enrichment and bacteria isolation

BTEX-contaminated groundwater samples were collected from the monitoring wells at in the Tehran Oil Refinery (51° 25', 12" E, 35° 31', 12" N) in Iran. Ten ml of the contaminated groundwater sample was enriched in a MSM supplemented with the BTEX compounds (250 mg L<sup>-1</sup> [1:1:1:1]) serving as the sole carbon sources (Jin et al., 2013). The MSM solution was sterilized and the pH was adjusted to 7.5 with 1.0 M NaOH. Enrichment was carried out using 100 ml MSM in a 500 ml glass vial for two weeks. The glass vials were capped with a PTFE screw cap in order to prevent any leakage. All enrichment experiments were performed in a vertical rotary shaker with a speed of 100 rpm and at a temperature of 35 °C. After incubation, culture aliquots were placed on Trypticase Soy Agar plates. Bacteria with different colony morphology were selected and isolated (Di Martino et al., 2012).

The BTEX degrading bacteria were first screened by the cultivating of isolated bacteria in a sterile MSM supplemented by BTEX as the only carbon sources. The fastest degrading bacterial strain (designated as strain BTEX-30) was selected for further evaluation of its BTEX biodegrading capacity.

### 2.3. Genetic identification of BTEX degraders

Genomic DNA of the strain BTEX-30 was extracted using a genomic DNA purification kit (Fermentas, GmbH, Germany, K0512) according to the manufacturer's protocol. The universal primers PA forward (AGAGTTTGATCTGGCTCAG) and pH reverse (AAG-GAGGTGATCCAGCCGCA) were used to amplify the 16SrRNA gene. The amplification was performed in a reaction mixture of Polymerase Chain Reaction (PCR) with 10xTaq buffer, 1.25 U AmpliTaq Gold DNA Polymerase, 2 mM dNTP mixture, 25 mM MgCl<sub>2</sub>, 0.7 µg DNA, and double-distilled water, mixed in a final volume of 50 µl. The program for PCR was set as follows: one cycle of 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, with one extension cycle at 72 °C for 10 min. The amplified product was visualized using a horizontal electrophoresis of 1% agarose gel after staining with safe DNA staining. Amplified 16SrRNA genes were sequenced by Macrogen Inc. (Korea) and then analyzed using the BLAST program (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and 16S ribosomal RNA (Bacteria and Archaea) tools, which are available online. The MEGA 7.0 software was further used to construct the phylogenetic trees. The partial sequence of 16SrRNA was deposited into GenBank with accession number KU521341.

### 2.4. Analytical techniques

#### 2.4.1. Cell concentration

Cell concentrations were obtained by measuring the optical density (OD) of a sample at wavelength 450 nm (OD<sub>450</sub>) using a UV-Spectrophotometer (UV-1601, Shimadzu). The OD was correlated next to dry cell weight in order to estimate the cell concentration. Bacteria cell concentrations were estimated by filtering a known volume of the solution through 0.22 µm filter paper and then measuring the corresponding weight of the dried cells (Balasubramanian et al., 2011). A standard curve was made by plotting the OD values versus bacterial cell concentrations.

#### 2.4.2. Gas chromatography analysis

To measure BTEX concentrations in the headspace, gas samples of 1.0 ml were periodically taken from the headspace of kinetic (including the BTEX-degrading bacteria) and control (without the BTEX-degrading bacteria) batches using a 2.5 ml gastight Hamilton syringe. A gas chromatograph (GC) was used to measure the concentrations of the BTEX compounds in the headspace. The GC

(Agilent 6850) was equipped with a flame ionization detector (FID) while separation was done on an Rtx-5MS capillary column (length: 30 m, ID: 0.25 mm, film thickness: 0.25  $\mu\text{m}$ ). Nitrogen was used as the carrier gas, flowing at 15 ml  $\text{min}^{-1}$  with the injector and detector temperatures were set at 130 °C and 150 °C, respectively. An isothermal programmed run with a temperature of 55 °C was used to analyze the samples. Concentrations of the VOC compounds were determined using a headspace method as employed in previous studies (Almeida and Boas, 2004; Kubinec et al., 2005; Mahmoodlu et al., 2015; Sieg et al., 2008). The limits of quantification (LOQ) were calculated by using a signal-to-noise ratio of 10:1 (Kubinec et al., 2005; Mahmoodlu et al., 2015).

### 2.5. Optimization of effective parameters in the biodegradation experiments

Uncontaminated sterile groundwater was used as a basic solution for the optimization experiments. A stock solution of inorganic nutrients containing 5.0 g  $\text{NaNO}_3$ , 10.0 g  $\text{NH}_4\text{Cl}$ , 21.4 g  $\text{Na}_2\text{H-PO}_4 \cdot 2\text{H}_2\text{O}$  and 11.0 g  $\text{KH}_2\text{PO}_4$  in 100 ml distilled water was prepared. The amounts of inorganic nutrients added to the basic solution were calculated based on the percentage of initial BTEX concentration.

The Box-Behnken Design (BBD) was used to optimize several parameters (temperature, pH, and inorganic nutrients) and their interactions in the biodegradation process. The BBD does not contain any points at the vertices of the cubic region created by the upper and lower limits for each variable. This resulted in a reduction in the number of required runs. The approach is especially advantageous when the points on the corners of the cube represent factor-level combinations which are prohibitively expensive or impossible to test due to the constraints of physical process (Fakhari et al., 2013; Kamarei et al., 2010). A quadratic polynomial model was fitted to the experimental data, which also provided regression coefficients. The non-linear computer-generated quadratic model for the response surface had the form

$$Y(x) = \beta_0 + \sum_{i=1}^3 \beta_i X_i + \sum_{i=1}^3 \beta_i X_i^2 + \sum_{i=1}^3 \sum_{j=1}^3 \beta_{ij} X_i X_j \quad (1)$$

where  $Y(x)$  is the response,  $\beta_0$  is an intercept,  $\beta_i$  and  $\beta_{ij}$  are constant regression coefficients of the model, and  $X_i, X_j$  ( $i = 1, 3; j = 1, 3$  and  $i \neq j$ ) represent the coded level of an independent variable.

The tested levels of effective parameters in the biodegradation process are listed in Table 1. The number of experiments (N) is given by

$$N = 2k(k - 1) + C \quad (2)$$

where  $k$  is the number of variables and  $C$  is the number of centre points. In this study  $k$  and  $C$  were both equal to 3. Hence a total of 15 experiments were carried out in order to optimize the effective parameters in the biodegradation process. Normalized BTEX removal percentages (%) provided the experimental response for each run. Normalized removal (%) was the average removal (%) of

each component of BTEX.

### 2.6. BTEX batch biodegradation experiments

Biodegradation of BTEX compounds by a new strain (*Pseudomonas* sp. BTEX-30) was investigated using 500 ml glass vial reactors at a temperature of 29 °C. The glass vials were capped with a PTFE screw cap in order to prevent any leakage. A sampling port was installed near the bottom of each glass vial (bottle). Uncontaminated groundwater obtained upstream from the oil contaminated site (Tehran Oil Refinery) was sterilized and further used as a base liquid in the batch experiments. Inorganic nutrients as described earlier were added to the liquid based on the RSM results. Each batch reactor consisted of 150 ml of sterile nutritious groundwater and a headspace of 350 ml. Then, 1.0 ml of overnight culture (in its late-exponential growth phase) was added to the batches. The reactive batch experiments were performed using both single (initial concentration of each BTEX compounds was 25–250  $\text{mg L}^{-1}$ ) and quad substrates (initial concentration of each BTEX compounds was 125  $\text{mg L}^{-1}$ ). For the control experiments, we used 150 ml of the MSM, but without bacteria. All experiments were carried out in triplicates using a rotary shaker incubator at 100 rpm.

Liquid samples of 1.0 ml were taken periodically via the sampling ports placed near the bottom of the vials using sterile syringes in order to measure the OD. Gas samples of 1.0 ml were further taken regularly from the headspaces of both the reactive and the control batches using gastight syringes.

### 2.7. Enzyme assays

In this study, colorimetric techniques were used for measuring enzymatic activity. 10 ml of dual substrate cultures (benzene with *m*-xylene) in their mid-exponential phases was transferred to the falcon tubes and washed twice with a phosphate buffer solution (pH 7.2) using centrifugation (5 min at 14,000 rpm at 40C). The harvested cells were subsequently transferred to a 1.0 ml phosphate buffer and disrupted by sonication. After sonication, supernatant liquid was separated from the cell debris by centrifugation at 14,000 rpm for 2 min. Catechol 1,2-dioxygenase and catechol 2,3-dioxygenase activities were determined by measuring the production of muconic acid at 260 nm and 2-hydroxymuconic semi-aldehyde at 375 nm, respectively (Li et al., 2014). A solution of 2.0 ml phosphate buffer, 0.6 ml, 1 mM catechol, 0.375 ml deionized water, and 0.025 ml cellular lysates was prepared for the reaction mixture (Li et al., 2014). Following a study by Bradford (1976), protein contents were determined using bovine serum albumin as the standard.

## 3. Results and discussions

### 3.1. Isolation and characterization of bacteria

Phylogenetic analysis based on 16SrRNA gene sequencing showed that the strain BTEX-30 formed a phylogenetic lineage with members of the genus *Pseudomonas*. We found that the most closely species were *Pseudomonas zhaodongensis* (strain NEAU-ST5-21), *Pseudomonas knackmussii* (strain B13), *Pseudomonas chloritidismutans* (strain AW-1), and *Pseudomonas stutzeri* with 98%, 98%, 98%, and 97% 16SrRNA gene sequence similarity, respectively. The phylogenetic tree revealed the position of strain BTEX-30 to be among some members of the genus *Pseudomonas* (Fig. 1). The position of strain BTEX-30 in the constructed phylogenetic tree and also comparisons (via 16SrRNA sequence similarities) of strain BTEX-30 with closely related species suggested that strain BTEX-30

**Table 1**  
Experimental parameters and levels of the Box–Behnken design (BBD).

Variable	key	Level		
		Lower	Central	Upper
pH	A	6	7	8
Temperature	B	25	35	45
Inorganic Nutrients%	C	10	105	200

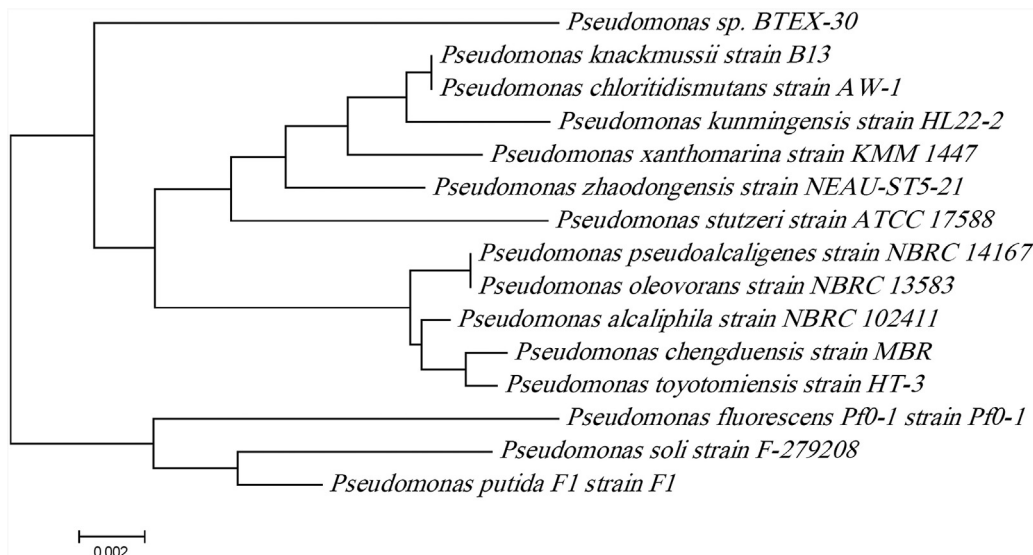


Fig. 1. Neighbor-joining phylogenetic tree shows the position of strain BTEX-30 among some members of the genus *Pseudomonas* based on the separate 16SrRNA gene sequences.

could represent a novel species of genus *Pseudomonas*. However, supplementary identification tests are still required in order to confirm this.

### 3.2. Optimization of the effective parameters for BTEX biodegradation

The following normalized quadratic polynomial model was fitted to the experimental data as follows:

$$\begin{aligned} \text{Normalized removal \%} = & -1233.26 + 279.346A + 15.0566B \\ & + 3.38947C - 17.8747A^2 \\ & - 0.70164AB + 4.15458AC \\ & - 0.139485B^2 - 0.559801BC \\ & + 3.41611C^2 \end{aligned} \quad (3)$$

where A is pH, B is temperature, and C is the inorganic nutrient content as a percent of BTEX concentration.

The goodness of fit was evaluated using the coefficient of determination,  $R^2$ . Values of the fitted  $R^2$  and adjusted  $R^2$  using the backward mode of the multiple linear regression model (MLRM) were 0.992 and 0.982, respectively, which shows that the obtained second order polynomial correlated very well with the experimental results (Loi et al., 2010). Moreover, a lack of fit test was employed to see whether the selected model was able to simulate the observed data or if a more complicated model should be used. This was done by comparing the variability of the current model residuals to the variability between observations at replicate settings of the factors. Since the P-value for the lack of fit was higher than 0.05 (see Table 2), we concluded that the model was suitable to simulate the observed data with a confidence level of around 95.0% (Ferreira et al., 2007; Ruby Figueroa et al., 2011).

To verify the existence of systematic errors, the residual was plotted versus predicted values (Fig. 2). The plot shows a random distribution of data points around the zero line. This confirms that no systematic errors affected the extraction procedure. Also, the equality of variance was not violated (Ruby Figueroa et al., 2011).

Fig. 3 depicts the main factors affecting the degradation of BTEX

Table 2  
Results of ANOVA for the fitted model.

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A = pH	965.0	1	965.0	23.4	0.04
B = T	189.4	1	189.4	4.6	0.17
C = N%	4685.1	1	4685.1	113.7	0.01
AA	1179.7	1	1179.7	28.6	0.03
AB	196.9	1	196.9	4.8	0.16
AC	145.2	1	145.2	3.5	0.20
BB	718.4	1	718.4	17.4	0.05
BC	263.6	1	263.6	6.4	0.13
CC	190.5	1	190.5	4.6	0.16
Lack-of-fit	83.8	3	27.9	0.7	0.64
Total error	82.4	2	41.2		
Total (corr.)	8711.9	14			

A is pH; B is the temperature and C is the inorganic nutrient percent.

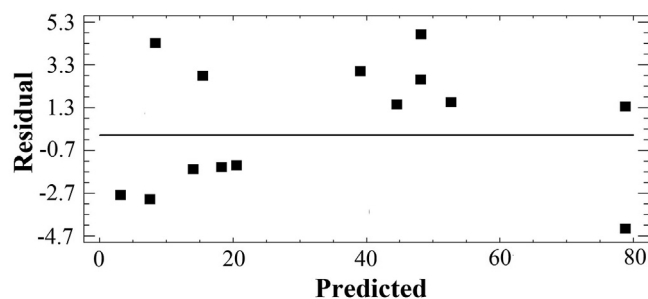


Fig. 2. The residual versus predicted values by the model. Horizontal line denotes the residual equals zero.

and their interactions using the results of the analysis of variance (ANOVA). The bar lengths in the figure are proportional to the absolute value of the estimated effects on the BTEX degradation, with the vertical line corresponding to the 95% confidence interval. A positive value for particular effect indicates that an increase in the response to the degradation of BTEX if the value of the variable increases up to its high level. However, a negative value of the estimated effect shows a better response to the BTEX degradation at especially low levels of the variable. Results of the ANOVA analysis showed that inorganic nutrient (%) and pH of the sample



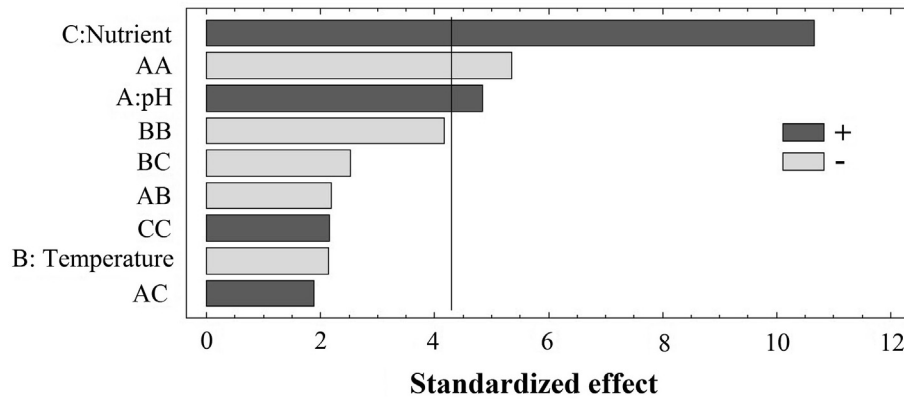


Fig. 3. Pareto chart of the effect main factors on the degradation of BTEX. A, B, and C are pH, temperature and nutrient, respectively.

have significant and positive effects on BTEX degradation. By comparison, temperature has a non-significant effect on the BTEX degradation. We furthermore used the F-ratio and P-value to evaluate the effect of the main factors and their interactions on the degradation of BTEX. The values of these two parameters are given in Table 2. If the P-value of a factor or interaction between two factors is higher than 0.05, the factor or interaction is known to not have a significant effect on the biodegradation process (e.g., for temperature (B in our study)), while the factor would have a significant effect if the P-value is less than 0.05 (such as for pH (A) and inorganic nutrient (B) in our study).

The RSM was further used to evaluate the effects of independent variables on the degradation of BTEX. Fig. 4a, c, e shows the relationship between the explanatory and response variables in three dimensional (3D) plots representing the response surfaces. To evaluate the interactive effects of independent variables on the responses, one variable was assumed to be constant while the other two variables were changed. The shapes of the response surfaces and contour plots reflect the nature and extent of the interaction between different factors (Prakash et al., 2008). Fig. 4b, d, f shows in a two dimensional (2D) contour plots of the model equations. They indicate the interaction between independent variables and help to

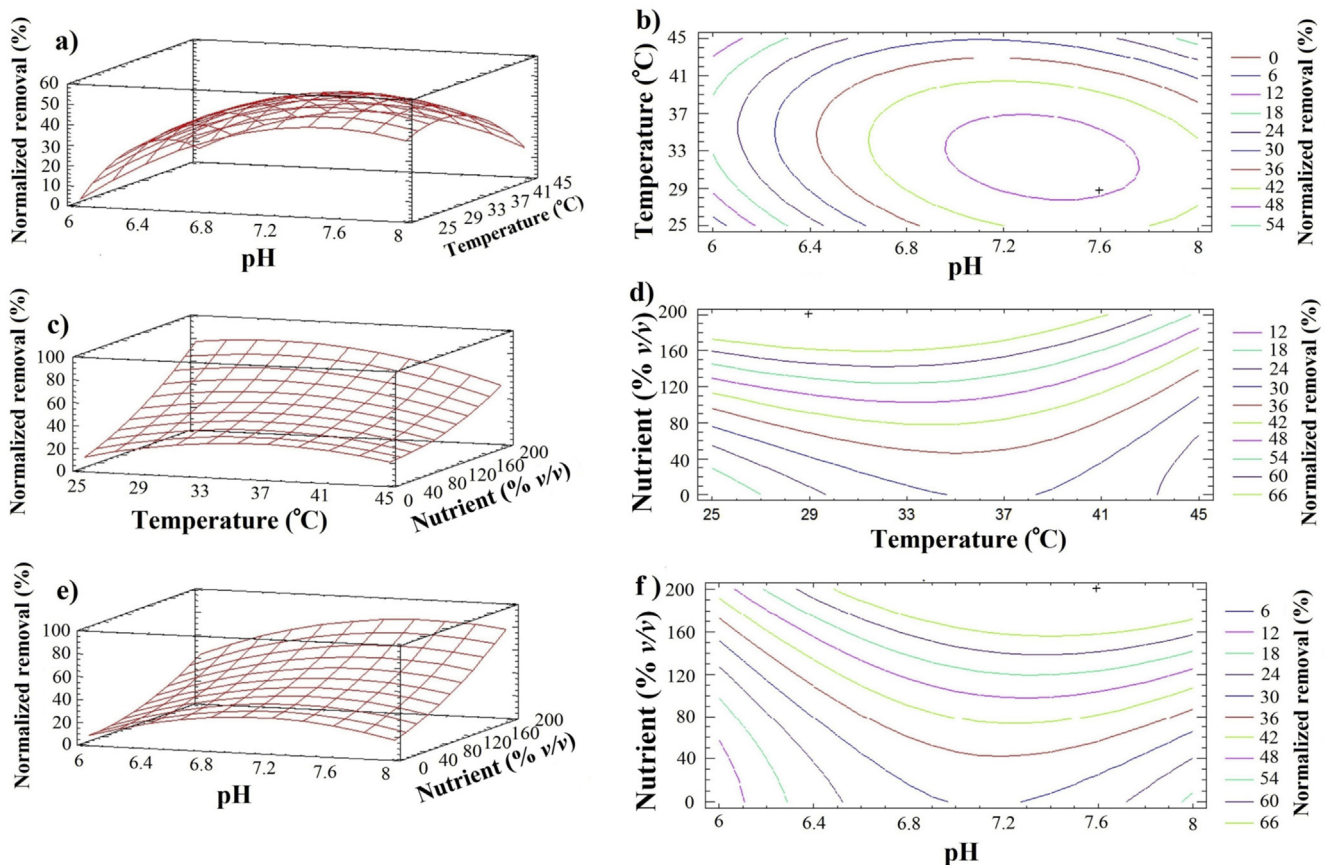


Fig. 4. Response surfaces and contour plots obtained by plotting of pH vs temperature (a, b) nutrient% vs temperature (c, d) and nutrient % vs pH (e, f) using the Box-Behnken design.

determine optimum operating condition for the desirable responses.

Results of the RSM reveal that the temperature did not have a significant effect on the degradation of BTEX. Still, the removal efficiency could be increased somewhat by reducing the temperature down to 28.9 °C. At this temperature, bacteria had the highest activity and best conditions for proliferation and hence BTEX removal. In contrast to temperature, the nutrient content had the highest influence on the removal efficiency of BTEX. Increasing the nutrient content from ambient to about two times the initial BTEX concentration increased bacterial proliferation as shown in Fig. 4. Results further indicate that BTEX degradation by *Pseudomonas* sp. BTEX-30 increased with an increase in the pH value equal to 7.6. This confirms that the physiological optimal pH is about 7.6, at which value bacteria would growth best. Our findings hence suggest the following optimal experimental conditions: pH = 7.6, temperature = 28.9 °C, and inorganic nutrients content equal two times the initial BTEX concentration.

### 3.3. BTEX degradation ability of *Pseudomonas* sp. BTEX-30

#### 3.3.1. Single substrate degradation

Fig. 5 shows the degradation versus time of the BTEX compounds and corresponding biomass growth using a single substrate. The initial concentration of all compounds was 250 mg L<sup>-1</sup>. Results indicate that toluene degraded faster than the other BTEX compounds. Biodegradation of benzene and ethylbenzene started after a 20 h lag in time, whereas the lag time for toluene biodegradation was less than 20 h. As shown in Fig. 5, the degradation of m-xylene using *Pseudomonas* sp. BTEX-30 was not significant. Benzene and toluene were degraded 95% and 96%, respectively, after 39 h, and completely degraded after 72 h, while no ethylbenzene degradation occurred 40 h. About 10% of the initial mass of

ethylbenzene remained undegradable after 72 h.

Values of the specific degradation rates (SDR) and average degradation rate (ADR) were calculated to quantitatively analyze the substrate interactions of the BTEX mixtures. Results are shown in Table 3. Following the literature (Lee and Cho, 2009), the SDR was estimated as the slope of plot of the compound concentrations versus time following the lag period, whereas the ADR included the lag period. The end time for the SDR and the ADR calculations was approximately 39 h. The value of SDR for toluene (14.2 mg L<sup>-1</sup> h<sup>-1</sup>) was slightly higher than for benzene (12.9 mg L<sup>-1</sup> h<sup>-1</sup>) and ethylbenzene (11.2 mg L<sup>-1</sup> h<sup>-1</sup>). The ADR value for toluene was the same as for benzene (3.4 mg L<sup>-1</sup> h<sup>-1</sup>), while ethylbenzene had an ADR value of 3.1 mg L<sup>-1</sup> h<sup>-1</sup>. Hence, the degradation rates in the single substrate experiments were highest for toluene, followed by benzene and ethylbenzene. The cell mass during benzene degradation and subsequently biomass productions was higher than for other compounds.

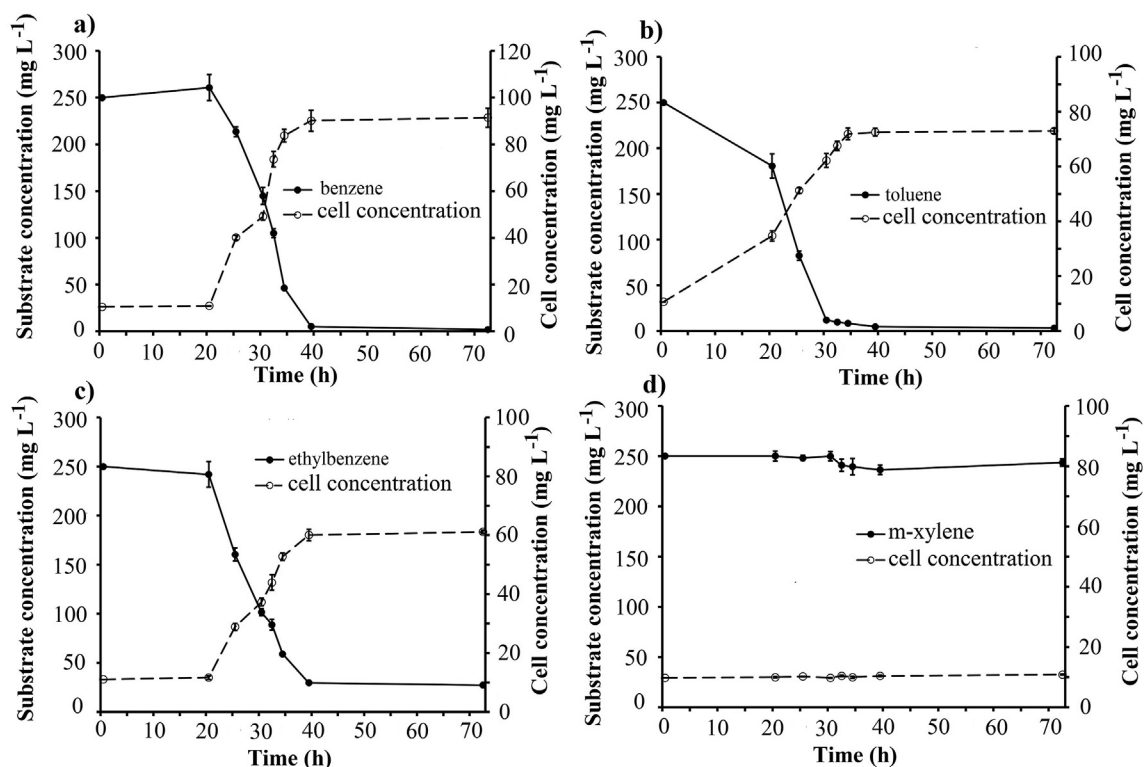
#### 3.3.2. Degradation of m-xylene by co-metabolism

Two binary-substrate (batch) experiments containing benzene/m-xylene and toluene/m-xylene were performed to evaluate the co-metabolism of m-xylene degradation and key enzymes

**Table 3**

Specific degradation rate (SDR) and average degradation rate (ADR) for BTEX compounds in single and mixed substrate experiments.

compound	Single substrate		Mixed substrate	
	SDR	ADR	SDR	ADR
Benzene	12.9	3.4	5.3	2.8
Toluene	14.2	3.4	6.8	2.7
Ethylbenzene	11.2	3.1	7.7	1.5
m-Xylene	0	0	7.6	1.5



**Fig. 5.** Degradation of benzene (A), toluene (B), ethylbenzene (C) and m-xylene (D) by *Pseudomonas* sp. BTEX-30 in the single-substrate experiments. Here, initial concentration of each BTEX compound was 250 mg L<sup>-1</sup>.

participating in the co-metabolism. Fig. 6 shows that the biodegradation of m-xylene in the presence of benzene and toluene by *Pseudomonas* sp. BTEX-30. Results are consistent with literature findings. For example, it has long been recognized that normal biodegradation or co-metabolism of BTEX compounds generally initiated by mono or dioxygenase enzymes (El-Naas et al., 2014; Karigar and Rao, 2011; Li et al., 2014). Methyl or ethyl substituents of the benzene ring will be broken down by mono-oxygenase, and are subsequently transformed by several oxidations to corresponding substituted pyrocatechols or phenyl glyoxals, respectively (Tsao et al., 1998). The dioxygenases oxidize the aromatic ring of BTEX compounds to produce 2-hydroxy-substituted compounds (Zhang et al., 2013). Intradiol or extradiol oxygenases further cleave one ring using oxygen between the hydroxyl groups (meta-cleavage) or proximal to one of the two hydroxyl groups (ortho-cleavage). The resulting products are then subjected to additional transformations before entering the Krebs cycle (Zhang et al., 2013). The first step of benzene biodegradation is hydroxylation catalysed by dioxygenase, with the main intermediate product being catechol (El-Naas et al., 2014).

In our study, m-xylene was co-metabolized by *Pseudomonas* sp. BTEX-30 in the presence of benzene. The key enzyme in the co-metabolism of m-xylene hence was dioxygenase. Fig. 7 shows that the oxidation of catechol by cellular lysates obtained from *Pseudomonas* sp. BTEX-30. The plots indicate that catechol 2,3-dioxygenase had a relatively high activity ( $9.43 \text{ IU mg}^{-1}$ ), whereas catechol 1,2-dioxygenase showed essentially no activity. This confirms that the cleavage of the benzene rings occurred mainly at the meta-position by catechol 2,3-dioxygenase.

### 3.3.3. Quaternary substrate degradation

Fig. 8 depicts the ability of *Pseudomonas* sp. BTEX-30 to degrade

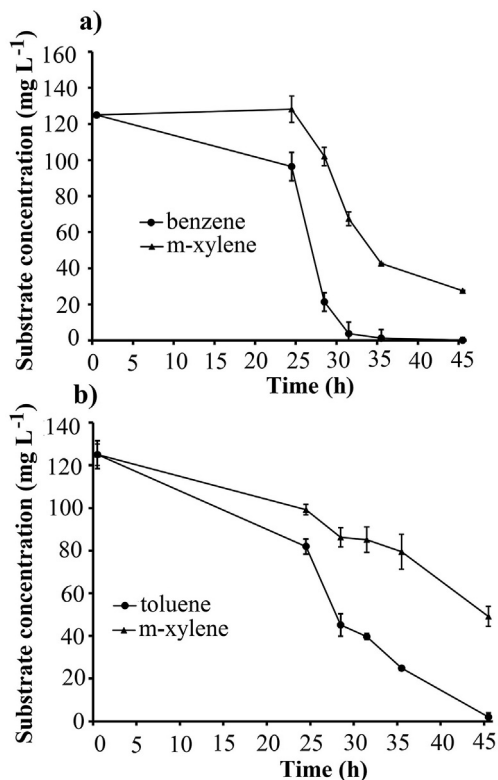


Fig. 6. Biodegradation of m-xylene by co-metabolism in the presence of benzene (A) and toluene (B). Initial concentration of each compound was  $125 \text{ mg L}^{-1}$ .

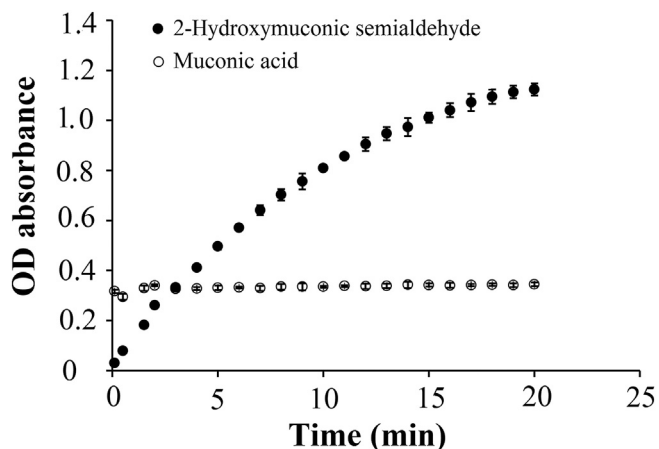


Fig. 7. Oxidation of catechol to muconic acid (by Catechol 1,2-dioxygenase) and 2-hydroxymuconic semialdehyde (by catechol 2,3-dioxygenase). Here, muconic acid and 2-hydroxymuconic semialdehyde were measured at 260 nm and 375 nm, respectively.

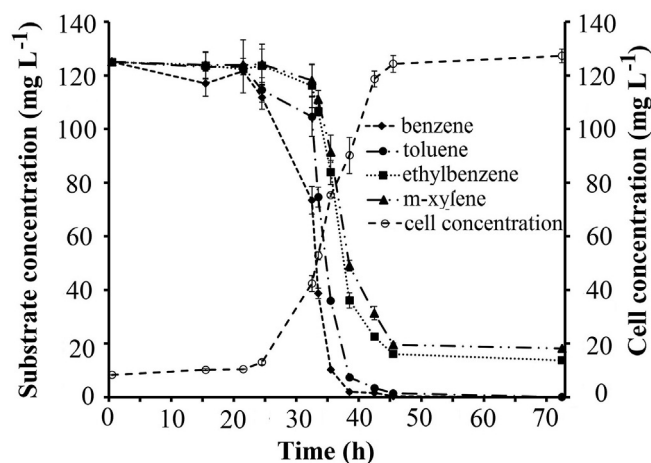


Fig. 8. Degradation of BTEX compounds by *Pseudomonas* sp. BTEX-30 in the quaternary substrate experiments.

the quaternary substrate. In contrast to the single substrate experiment, all BTEX compounds were degraded by *Pseudomonas* sp. BTEX-30. Interestingly, benzene was degraded fastest, followed by toluene and ethylbenzene and m-xylene. The lag time for benzene and toluene was about 21 h. Degradation of ethylbenzene and m-xylene was initiated when the degradation of toluene and especially benzene had progressed significantly. In contrast to the single substrate degradation experiments, m-xylene was degraded in the quaternary degradation experiments. This may be because of the catabolic enzymes, which are essential for m-xylene degradation, were being produced during the degradation of benzene and/or toluene. This hence resulted in the degradation of a non-growth substrate. An increase in cell growth due to the degradation of substrate accelerates the biodegradation of m-xylene. This type of co-metabolism of non-growth single substrates in the presence of a growth substrate was reported previously in several studies (Baboshin et al., 2003; Zhong et al., 2010; Zhou et al., 2011).

Our results (Table 4) showed that the SDR values for ethylbenzene ( $7.7 \text{ mg L}^{-1} \text{ h}^{-1}$ ) and m-xylene ( $7.6 \text{ mg L}^{-1} \text{ h}^{-1}$ ) in mixed substrate were higher than the SDR values for benzene ( $5.3 \text{ mg L}^{-1} \text{ h}^{-1}$ ) and toluene ( $6.8 \text{ mg L}^{-1} \text{ h}^{-1}$ ). Since the degradation of ethylbenzene and m-xylene started almost at the mid exponential phase

**Table 4**  
BTEX biodegradation and the corresponding experimental conditions by different *Pseudomonas* strains.

Substrate	Microorganism	Concentration (mgL <sup>-1</sup> )	pH	Temperature (°C)	Removal percent	Degradation time (hour)	References
BTEX	<i>Pseudomonas</i> sp. BTEX-30	500 BTEX (1:1:1:1)	7.4	35	99 B and T, 86 E and 82 (m)X	45	This study
BTEX	<i>Pseudomonas putida</i> F1	140–220	–	25	100 B,T and E, 75 X	14	(Nagarajan and Loh, 2015)
BTEX	<i>Pseudomonas stutzeri</i> OX1	140–220	–	25	100 B,T and E, 90 X	24	(Nagarajan and Loh, 2015)
BTEX	Mixed <i>Pseudomonas</i> sp., <i>Yarroia</i> sp., <i>Acinetobacter</i> sp., <i>Corynebacterium</i> sp., <i>Sphingomonas</i> sp.	75	7.0 ± 0.1	28–30	97 B, 93 T, 90 E, 98 X	50	(Jo et al., 2008)
BTEX	<i>Pseudomonas putida</i> F1 ATCC 700007	50 BTE, (m,p)X, 20 (o)X	7.2 ± 0.5	36 ± 2	80 B, 81 T, 80 E, 25 X	60	(Morlett-Chávez et al., 2010)
BTEX	Immobilized <i>Mycobacterium</i> sp. CHXY119 <i>Pseudomonas</i> sp. YATO411	24.68 B, 23.67, 21.97 E	–	25	97.8 B, 94.2 T, 84.7 E, 87.4 (p)X	24	(Xin et al., 2013)
BTX	<i>Pseudomonas Putida</i> F1	15, 30, 60, 90 B, T, (o)X	7	30	100 BT, 60–80 (o)X	6–14	(Robledo-Ortiz et al., 2011)

of benzene and toluene degradation, the increased cell concentrations must have caused the higher SDR values for ethylbenzene and m-xylene. However, the ADR value for benzene and toluene (2.8 and 2.7 mg L<sup>-1</sup> h<sup>-1</sup>, respectively) were larger than the ADRs for ethylbenzene and m-xylene (1.5 mg L<sup>-1</sup> h<sup>-1</sup>). The SDR and ADR values for BTEX for the mixed substrates were lower than for the single substrate experiments.

Concentrations of the BTEX compounds in the single-substrate experiments (250 mg L<sup>-1</sup>) were two times higher than for the mixed-substrate experiments (125 mg L<sup>-1</sup> for each compound). The SDR values for a single-substrate experiments were almost two times higher than those for the mixed-substrate experiments. In an experiment, the concentration of compounds in the single-substrate experiments was kept constant around 250 mg L<sup>-1</sup>, but the concentration of compounds in the mixed-substrate experiments was increased up to 500 mg L<sup>-1</sup>. Results showed that the sum of the SDR values for the mixed-substrate experiments were two times larger than that for the single substrate experiments. A mostly linear relationship between SDR and the substrate concentration was hence found.

We further compared the biodegradation ability of *Pseudomonas* sp. BTEX-30 of our study to previously published biodegradation percentages and times using other *Pseudomonas* strains. Results are summarized in Table 4. They indicate that for the mixed-substrate experiments, benzene and toluene were degraded 99% within 45 h. However, ethylbenzene and m-xylene were degraded for only 86% and 82% at the same experimental times, respectively.

#### 4. Conclusions

In this study, we evaluated the BTEX degradation performance of a novel-isolated strain (named *Pseudomonas* sp. BTEX-30) in the water phase. Results of the optimization process showed the optimum pH, temperature, and inorganic nutrient contents (as a percentage of the BTEX concentrations) for BTEX degradation using *Pseudomonas* sp. BTEX-30 to be 7.6, 28.9 °C and 200%, respectively. Toluene was found to degrade faster than other BTEX compounds in the single-substrate degradation experiments. However, the amount of biomass produced during degradation of benzene was higher than that for toluene. In contrast to toluene, the degradation of m-xylene using *Pseudomonas* sp. BTEX-30 was negligible in the single-substrate degradation experiments. Results of the dual-substrate experiments indicated co-metabolism of m-xylene in the presence of benzene and toluene. Dioxygenase was found to be the key enzyme incorporated in the co-metabolism of m-xylene. Our results further showed that all BTEX compounds were degraded in the mixed substrate experiments, with benzene

degrading fastest, followed by toluene, ethylbenzene and m-xylene. Degradation of ethylbenzene and m-xylene in the mixed substrate experiments was initiated when the degradation of benzene and toluene were almost in the mid exponential phase.

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