Pol. J. Environ. Stud. Vol. 27, No. 2 (2018), 655-663
DOI: *10.15244/pjoes/75811* ONLINE PUBLICATION DATE: 2018-01-15

Original Research

Optimizing Toluene Degradation by Bacterial Strain Isolated from Oil-Polluted Soils

Fatemeh Heydarnezhad[1], Mehran Hoodaji[1], Mahdi Shahriarinour[2]*, Arezoo Tahmourespour[3], Shahab Shariati[4]

[1]Soil Science Department, Isfahan (Khorasgan) Branch, Islamic Azad University, Isfahan, Iran
[2]Department of Microbiology, Rasht Branch, Islamic Azad University, Rasht, Iran
[3]Basic Medical Sciences Department, Isfahan (Khorasgan) Branch, Islamic Azad University, Isfahan, Iran
[4]Department of Chemistry, Rasht Branch, Islamic Azad University, Rasht, Iran

Received: 2 May 2017
Accepted: 11 July 2017

Abstract

The presence of toxic compounds like toluene has caused extensive contamination in oil-contaminated environments. Using bacteria to degrade monoaromatic compounds could be a good approach to finding a suitable bioaugmentation agent. In this study on toluene, degrading bacterial species were isolated from oil-contaminated environments (located in Bandar-Anzali, Guilan, Iran). The strain has been molecularly identified as *Bacillus cereus* ATHH39 (Accession number: KX344721) by partial sequencing of the 16S rDNA gene. Response surface methodology (RSM) was used for biodegradation of toluene by ATHH39 by implementing the central composite design (CCD). The central composite design (CCD) was applied to optimize and investigate pH, temperature, and toluene concentrations and their interactions for enhancing cell growth and toluene degradation by ATHH39 under *in vitro* conditions. The variables (pH, temperature, and toluene concentrations) with the highest significant impacts on growth and toluene degradation were selected. According to the prediction and optimization function of the design expert software, the optimum conditions of cell growth and toluene degradation were found. When pH, temperature, and toluene concentration were adjusted to 6.72, 33.16ºC and 824.15 mg/l, respectively, cell growth and toluene degradation reached OD_{600} = 0.69 and 64.11%, respectively, which is very close to the predicted cell growth and toluene degradation of OD_{600} = 0.71 and 65.85%, indicating that the response surface methodology optimization of process parameters for cell growth and toluene degradation is reliable. Based on the results, the ATHH39 strain was introduced as a useful microorganism with the potential for bioremediation of wastewater containing toluene.

Keywords: *Bacillus cereus*, bioaugmentation, contamination, response surface methodology, toluene

*e-mail: mahdi.shahriari@iaurasht.ac.ir

Introduction

Monoaromatic hydrocarbons such as benzene, toluene, and xylene (BTX) represent an important class of environmental contaminants because of their toxicity as carcinogenic and teratogenic agents. They are widely used as chemical substances in various industrial processes, and significant amounts are found in fossil fuels [1-2]. The appearance of toluene in natural environments is usually associated with the spill or discharge of petroleum products and synthetic chemicals in the form of herbicides, pesticides, and industrial effluents [3-4]. These compounds can reduce bacteria viability and, consequently, xenobiotic biodegradation [5-6]. Compared to physiochemical methods, bioremediation offers a very feasible alternative for an oil spill response. This technique is considered an effective technology for the treatment of oil pollution. One reason is that the majority of the molecules in the crude oil and refined products are biodegradable [7-8].

Many microorganisms, e.g., *Pseudomonas aeruginosa*, *Pseudomonas putida, Bacillus subtilis, Bacillus cereus, Bacillus licheniformis, Bacillus laterospor, Klebsiella* spp., and *Acinetobacter* spp., excrete emulsifiers that increase the surface area of the substrate. These microorganisms also modify their cell surface in order to increase the affinity for hydrophobic substrates and thus facilitate their absorption [9]. Toluene degradation is sensitive to many factors such as temperature [10], pH, incubation periods, carbon and nitrogen sources, and aeration rate [11]. Response surface methodology (RSM) is a collection of statistical and mathematical techniques for designing experiments, building models, evaluating the relative significance of several independent variables, and determining the optimum conditions for desirable responses [12-13]. The use of RSM in process development reduces the number of experimental trials and hence is less time consuming [12, 14]. In addition, the one-factor-at-a-time optimization method is expensive and often leads to misinterpretation of results when there are interactions between different components [12].

The objective of this study was to isolate, characterize, and molecularly identify toluene-degrading bacteria from oil-polluted soils, and to optimize the medium composition and culture conditions for improving cell growth and toluene degradation, which can be used for any bioaugmentation study during bioremediation.

Materials and Methods

Microorganism

Different contaminated soil samples were collected from the Caspian Sea (Bandar-Anzali, Guilan, Iran). Samples were stored at 4ºC prior to use. Toluene (purity of 99.5%) was filtration-sterilized and used as the sole carbon and energy source to enrich culture media for the isolation of degrading bacteria. 5 g soil sample was added to 50 ml of mineral salt medium (MSM) supplemented with 1% (V/V) toluene as the sole carbon and energy sources. The liquid mineral salt medium (MSM) consisted of (g/l): $NaNO_3$ 4, KH_2PO_4 1.5, Na_2HPO_4 0.5, $MgSO_4.7H_2O$ 0.2, $FeSO_4.H_2O$ 0.0011, and $CaCl_2$ 0.01, and pH was adjusted to 7 before autoclaving [6]. Samples were incubated at 30ºC and shaken at 150 rpm for seven days. After an enrichment period, 1 ml of the culture was transferred into the fresh MSM medium and incubated at 30ºC and shaken at 150 rpm [6]. After three subcultures, 0.1 ml of the culture was spread on to MSM and nutrient agar plates and incubated at 37ºC for 24-48 h [15]. Toluene was provided by slow spreading from a 0.45µm sterile syringe filter from a capped 100 µl Eppendorf tube in Petri dishes after cooling down to room temperature. After isolation, the culture was characterized using a standard biochemical procedure according to the microbial identification standards, and was identified based on Bergey's Manual of Systematic Bacteriology [16].

Extraction of DNA and Amplification of 16S Ribosomal DNA Gene Fragments

The bacterial chromosomal DNA was extracted using the method of CTAB, and detected by 1% agarose electrophoresis [17]. The forward primer was 27R-ACGGCTACCTTGTTACGACT and the reverse primer was 1502F-AGAGTTTGATCCTGGCTCAG [18]. For the PCR reaction system, conditions were as follows: DNA templates (70 ng/µl) 2.5 µl; dNTP mixture (10 mM) 0.5 µL; 27 F (10 ρmol/L) 0.4 µL; 1,495 F (10 ρmol/L) 0.4 µL; 10x PCR Buffer (2.5) with $MgCl_2$ (50 mM) 1 µL; Taq DNA polymerase (5 U/µl) 0.3 µL; bringing up ddH_2O 17.4 µL. The PCR amplification conditions were as follows: force-degeneration at 95ºC for five minutes, degeneration at 95ºC for one minute, annealing at 60ºC for 30 seconds and at 72ºC for 35 seconds, 30 cycles, with another extension at 72ºC to for five minutes [19]. After purification, the PCR products were sent for sequencing.

Analysis of 16S rDNA Sequences and Grawing of Phylogenetic Tree

Resemblance analysis of the 16S rDNA sequence was done through the GeneBank database using the BLAST method. Multiple alignments were carried out among the sequences with high resemblance. Finally, a multiple alignment array was established, with gaps, and a phylogenetic tree was constructed using the MEGA (version 5.2) [20].

Growth Rate and Toluene Removal Assay

The isolated bacteria were grown at 30ºC, 150 rpm, in MSM medium containing 1% (v/v) of toluene. Cells were harvested by centrifuge at 10,000×g for 10 min and washed twice in sterile MSM and resuspended with one-tenth volume of medium. This cell suspension was used

Table 1. Experimental range and level of variables.

Variables	Level code				
	-1.68	-1	0	1	+1.68
X_1	5.32	6	7	8	8.68
X_2	21.59	25	30	35	38.41
X_3	195.46	400	700	1,000	1,204.54

X_1: pH; X_2: Temperature (ºC);
X_3: Tolueme concentration (mg/l)

as inoculum for subsequent experiments. The growth rate of the isolate was indirectly assessed by a turbidity measurement as optical density (OD) at 600 nm in a UV-visible spectrophotometer (UV-vis-3600, Mapada) [21]. The toluene removal assay was performed by dissolving residual toluene of the medium in 3 ml n-hexane and reading the optical density of the toluene against a blank at 200-400 nm wavelengths [22].

Optimizing by Response Surface Methodology (RSM)

RSM is an empirical statistical technique employed for multiple regression analysis by using quantitative data obtained from properly designed experiments to solve multivariate equations [12, 23]. It can be used to define the relationship between the response and the independent variables [12, 24]. In this work, RSM was used to assess the relationship between the responses, cell growth (Y_1), and toluene degradation (Y_2), and the independent variables including pH (X_1), temperature (X_2), and toluene concentration (X_3) to optimize the relevant variables in order to predict the best value for the responses. The central composite design (CCD) is the most widely used RSM approach, and has been employed to determine cell growth and toluene degradation.

Statistical Analysis and Modeling

Table 1 shows the independent variables, experimental range, and the levels of the design model. In order to describe the nature of the response surface in the optimum region, a central composite design with five coded levels (-1.68, -1, 0, +1, and +1.68) was performed. Twenty combinations (=2^k+2k+6), where k is the number of factors (independent variables) of experiments, were generated with six replications at the central point and the data were analyzed using Design Expert Software (version 7.1). The response value (Y_1 and Y_2) in each trial was the average of the replicate. ANOVA was used to study the relationship between the process variables and the responses. The fitness of the model was determined by R^2 coefficient and F-test was used to check the statistical significance of the model. In optimization, the chosen variables can be related to the responses of the quadratic model as shown in Eq.1:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_2 + b_{22}X_2 + b_{33}X_2 + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3 \quad (1)$$

…where Y is the predicted response factor; X_1 is pH; X_2 is temperature (ºC); X_3 is toluene concentration (mg/l); and b_0, b_1, b_2, b_3, b_{11}, b_{22}, b_{33}, b_{12}, b_{23} and b_{13} are constant regression coefficients of the model, in which b_0 is the intercept term, b_1, b_2, and b_3 are linear coefficients, and b_{11}, b_{22}, and b_{33} are squared coefficients. On the other hand, X_1, X_2, and X_3 are independent factors. Combinations of factors (such as X_1X_2) represented the interaction between the individual [25].

Results and Discussion

Identification of the Bacterial Strain

After sampling from contaminated environments and enrichment procedures in MSM toluene-containing medium, the toluene-degrading bacterial strain was isolated. Bacterial strains surviving in the presence of toluene isolated in this study was designated as *Bacillus cereus* ATHH39. *Bacillus cereus* ATHH39 cells were rod-shaped, gram-positive, motile, endospore-forming, hemolytic, aerobic, and facultative aerobic bacterium. They were catalase and reduction of nitrate positive and oxidase negative. Almost complete sequences of

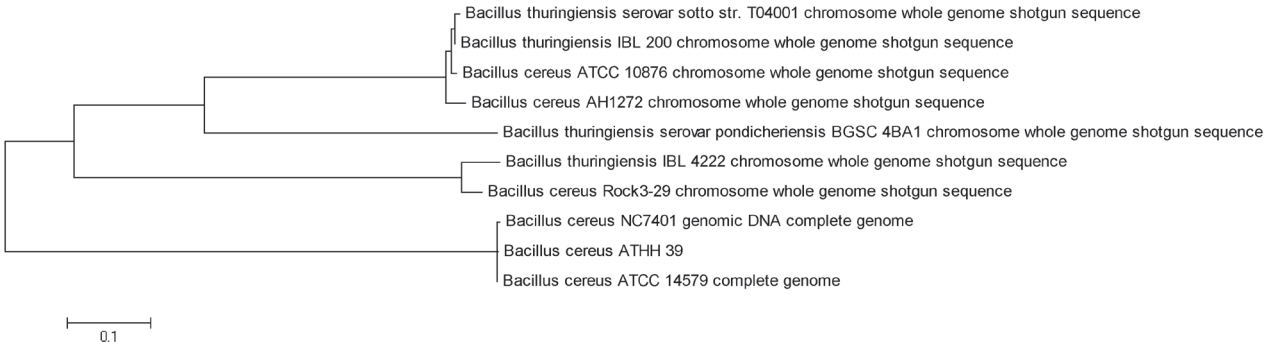

Fig. 1. Phylogenetic tree of the 16S rDNA sequence of strain ATHH39 and related strains.

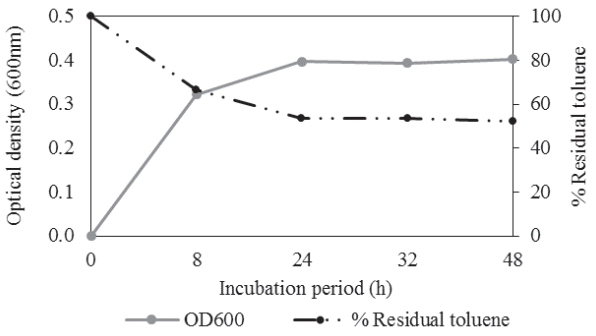

Fig. 2. Growth curve and toluene removal by bacterium *Bacillus cereus* ATHH39 in MSM broth supplemented with 100 mg/l toluene.

the 16SrDNA of the strain *Bacillus cereus* ATHH39 (1385 bases) were determined. The BLAST algorithm downloaded from the Genebank database (*blast.ncbi.nlm.nih.gov/Blast.cgi*) exhibited 99.9% identified with the closest match for *Bacillus cereus* ATCC 14579. The strain reported in this paper has been deposited in the GeneBank database under the accession number of KX344721. Fig. 1 shows phylogenetic tree of 16S rDNA sequence of the strain *Bacillus cereus* ATHH39 and the related strains using the MEGA.

Growth Rate and Toluene Biodegradation

As shown in Fig. 2, the maximum cell growth of *Bacillus cereus* ATHH39 was obtained after about 24 hours. Fig. 2 shows that the bacterium *Bacillus cereus* ATHH39 was efficiently capable of removing toluene from MSM medium. The highest toluene removal rate was also observed after 24 hours for *Bacillus cereus* ATHH39. According to these results, it can be concluded that the consumption of toluene by this strain is directly related to its growth rate.

Optimizing Significant Variables Using Response Surface Methodology

The experiments conducted in the present study were targeted toward the construction of a quadratic model consisting of 20 trials. The design matrix and

Table 2. Central composite design and its experimental and predicted values of cell growth and toluene degradation of *Bacillus cereus* ATHH39.

Trials	X_1	X_2	X_3	Y_1		Y_2	
				Experimental value	Predicted value	Experimental value	Predicted value
1	5.32	30	700	0.54	0.54	59.89	57.83
2	6	25	400	0.45	0.46	46.32	47.47
3	6	25	1,000	0.46	0.46	54.77	55.37
4	6	35	400	0.48	0.48	56.63	57.25
5	6	35	1,000	0.65	0.66	59.75	62.15
6	7	21.59	700	0.55	0.55	54.57	54.17
7	7	30	195.46	0.40	0.40	47.44	47.02
8	7	30	700	0.71	0.71	65.46	64.80
9	7	30	700	0.70	0.71	65.53	64.80
10	7	30	700	0.71	0.71	65.54	64.80
11	7	30	700	0.70	0.71	65.33	64.80
12	7	30	700	0.70	0.71	63.22	64.80
13	7	30	700	0.70	0.71	63.31	64.80
14	7	30	1,204.54	0.51	0.51	64.15	62.27
15	7	38.41	700	0.62	0.63	63.44	61.53
16	8	25	400	0.55	0.54	53.06	52.29
17	8	25	1,000	0.50	0.51	64.53	65.53
18	8	35	400	0.45	0.45	53.24	54.26
19	8	35	1,000	0.58	0.58	64.03	64.51
20	8.68	30	700	0.55	0.55	64.10	63.86

X_1: pH; X_2: Temperature (ºC); X_3: Toluene concentration (mg/l); Y_1: Cell growth; Y_2: Toluene degradation (%)

Table 3. Analysis of variance (ANOVA) for cell growth and toluene degradation of *Bacillus cereus* ATHH39 response surface-reduced quadratic model.

Source	Sum of Squares	DF	Mean Square	F Value	Prob> F
Cell growth					
Model	0.21	9	0.023	1,026.90	<0.0001***
X_1	2.390E-004	1	2.390E-004	10.77	0.0083
X_2	8.624E-003	1	8.624E-003	388.65	<0.0001
X_3	0.014	1	0.014	653.47	<0.0001
X_1^2	0.046	1	0.046	2,065.23	<0.0001
X_2^2	0.027	1	0.027	1,208.25	<0.0001
X_3^2	0.11	1	0.11	5,073.86	<0.0001
X_1X_2	7.442E-003	1	7.442E-003	335.39	<0.0001
X_1X_3	1.152E-003	1	1.152E-003	51.92	<0.0001
X_2X_3	0.014	1	0.014	643.59	<0.0001
Residual	2.219E-004	10	2.219E-005		
Lack of Fit	7.706E-005	5	1.541E-005	0.53	0.7474ns
Pure Error	1.448E-004	5	2.897E-005		
Cor Total	0.21	19			
Std. Dev.	4.71E-003		R-Squared	0.99	
Mean	0.58		Adj R-Squared	0.99	
C.V.	0.82		Pred R-Squared	0.99	
PRESS	8.13E-004		Adeq Precision	91.51	
Toluene degradation					
Model	699.84	9	77.76	26.75	<0.0001***
X_1	43.86	1	43.86	15.09	0.0030
X_2	65.43	1	65.43	22.51	0.0008
X_3	280.93	1	280.93	96.65	<0.0001
X_1^2	28.13	1	28.13	9.68	0.0110
X_2^2	86.88	1	86.88	29.89	0.0003
X_3^2	185.61	1	185.61	63.85	<0.0001
X_1X_2	30.48	1	30.48	10.49	0.0089
X_1X_3	14.27	1	14.27	4.91	0.0511
X_2X_3	4.50	1	4.50	1.55	0.2419
Residual	29.07	10	2.91		
Lack of Fit	22.57	5	4.51	3.48	0.0989ns
Pure Error	6.49	5	1.30		
Cor Total	728.90	19			
Std. Dev.	1.70		R-Squared	0.96	
Mean	59.72		Adj R-Squared	0.92	
C.V.	2.86		Pred R-Squared	0.75	
PRESS	184.41		Adeq Precision	15.36	

X_1: pH; X_2: Temperature (ºC); X_3: Toluene concentration (mg/l); Y_1: Cell growth; Y_2: Toluene degradation (%)
*Values of "Probability>F value" less than 0.05 indicates model terms are significant

the corresponding results of RSM experiments to determine the effects of three independent variables (pH, temperature, and toluene concentration) on cell growth and toluene degradation by *Bacillus cereus* ATHH39 are shown in Table 2 along with the mean predicted values. The regression analysis of the optimization study indicated that the model terms X_1, X_2, X_3, X_1^2, X_2^2, X_3^2, X_1X_2, X_1X_3, and X_2X_3 were significant ($P<0.05$) (Table 3). These results indicate that pH, temperature, and toluene concentration bear a direct relationship to cell growth and toluene degradation. The interactions between pH, temperature, and toluene concentration were significant as shown by the low P-value ($P<0.05$). The interaction between X_1X_3 and X_2X_3 was not significant for toluene degradation ($P<0.05$). In order to evaluate the model, the statistical analysis such as F-value, R^2-value, and lack of fit were determined. This is necessary for choosing an appropriate model with a significant F-value and an insignificant "lack-of-fit" at the same time. In this work, lack of fit with a probability value greater than 0.1 is desirable. If a model shows lack of fit, it should not be used to predict the response [26]. Analysis of variance (ANOVA) (Table 3) describes the P-values for the model ($P<0.01$) and for lack of fit (0.75) and for cell growth (0.99) and toluene degradation also suggested that the obtained experimental data was a good fit with the model. The regression equation coefficients were calculated and the data were fitted to a second-order polynomial equation. The simplified quadratic model with neglected insignificant terms in the general quadratic model for the responses, cell growth (Y_1) and toluene degradation (Y_2) by *Bacillus cereus* ATHH39 in coded factors can be expressed in terms of the following regression equations:

$$Y_1 = +0.71 + 4.184E\text{-}003X_1 + 0.025X_2 + 0.033X_3 - 0.056X_1^2 - 0.043X_2^2 - 0.088X_3^2 - 0.030X_1X_2 - 0.012X_1X_3 + 0.042X_2X_3 \quad (2)$$

$$Y_2 = +64.80 + 1.79X_1 + 2.19X_2 + 4.54X_3 - 1.40X_1^2 - 2.46X_2^2 - 3.59X_3^2 - 1.95X_1X_2 + 1.34X_1X_3 - 0.75X_2X_3 \quad (3)$$

…where cell growth = (Y_1), toluene degradation = (Y_2), pH = (X_1), temperature = (X_2), toluene concentration = (X_3), pH*pH = (X_1^2), temperature*temperature = (X_2^2), toluene concentration*toluene concentration = (X_3^2), pH*temperature = (X_1X_2), pH*toluene concentration = (X_1X_3), and temperature*toluene concentration = (X_2X_3).

The determination of coefficients (R^2) for cell growth and toluene degradation (0.99 and 0.96, respectively) showed the significance of the model. For good fitness of a model, the correlation coefficient should be at least 0.80 [12]. The adjusted R^2 for cell growth and toluene degradation (0.99 and 0.92, respectively) showed that more than 92% of the variability in the response were due to the changes in the factors of the model. The predicted R^2 for cell growth and toluene degradation (0.99 and 0.75, respectively) were in reasonable agreement with the adjusted R^2. Adequate precision greater than 4 is desirable [27]. Hence, the ratio of 91.51 and 15.36 for cell growth and toluene degradation indicated an adequate signal. Therefore, these models could be used to predict the response, while coefficient of variation (CV) at a lower value (0.82% and 2.86%) implied that the experiments were reliable and precise. The significance of each coefficient was observed by the F-value of the individual term (Table 3). Values of "Prob. >F" of less than 0.05 indicated that the model terms were significant [12].

Response Surface Plotting for Cell Growth and Toluene Degradation by *Bacillus cereus* ATHH39

The mathematical relationship between variables and responses to determine toluene degradation was explained using the quadratic model. In order to determine the optimal levels of each variable for maximum cell growth and toluene degradation by *Bacillus cereus* ATHH39, three-dimensional response surface plots were constructed by plotting the responses (cell growth and toluene degradation) on the Z-axis against any two independent variables (pH, temperature, and toluene concentration), while maintaining other variables at their central levels (Figs 3-4). Maximum cell growth and toluene degradation were obtained at the middle level of each pair of factors at a constant middle level of the other factor. Fig. 3a) shows a three-dimensional plot of cell growth as a function of pH and temperature, when the toluene concentration was kept constant (700 mg/l). Cell growth increased quickly as pH and temperature increased. When pH and temperature increased, cell growth rose to OD_{600} = 0.68. pH and temperature interaction had a significant positive effect on cell growth of *Bacillus cereus* ATHH39 ($p<0.001$) (Table 3). Fig. 3b) indicates that the variation in pH and toluene concentration on cell growth of *Bacillus cereus* ATHH39 significantly affected cell growth when the temperature was kept at the middle level (30ºC). Cell growth increased as pH and toluene concentration increased. When pH and toluene increased, cell growth rose to OD_{600} = 0.67. The pH and toluene concentration interaction had a significant positive effect on cell growth of *Bacillus cereus* ATHH39 ($p<0.001$) (Table 3). Fig. 3c) shows the effect of temperature and toluene concentration on the growth of *Bacillus cereus* ATHH39 pH was set at constant 7. Cell growth increased as temperature and toluene concentration increased. When the temperature and toluene concentration increased cell growth rose to OD_{600} = 0.68. It can be clearly seen that the increase in temperature and further increment in toluene concentration significantly increased cell growth of *Bacillus cereus* ATHH39 ($p<0.001$) (Table 3). A three-dimensional plot of the response of toluene degradation with respect to pH and temperature when toluene concentration was kept at the middle level (700 mg/l) is shown in Fig. 4a). Toluene degradation increased quickly as pH and temperature increased. These results indicated that the moderate pH and temperature that promoted the degradation of toluene were optimum. When pH and

temperature were increased, toluene degradation rose to 63.75%. This three-dimensional surface plot shows that the interaction between pH and temperature was significant, as indicated by the low "F values<0.05" (Table 3). Fig. 4b) shows the response of toluene degradation with respect to pH and toluene concentration when the temperature was kept at the middle level (30ºC).

It can be easily seen that toluene degradation increased very slightly with pH and toluene concentration. When pH and toluene concentration were increased, toluene degradation rose to 65.46%. The pH and toluene concentration interaction did not show a significant positive effect on toluene degradation of *Bacillus cereus* ATHH39 ($p<0.05$) (Table 3). Compared with pH, the interaction of temperature and toluene concentration was more effective on toluene degradation. Fig. 4c) shows the effect of temperature and toluene concentration on toluene degradation when pH was kept constant (7). Toluene degradation increased quickly as temperature and toluene concentration were increased. The intermediate value of temperature and toluene concentration did not show a significant positive effect on toluene degradation ($p<0.05$) (Table 3). When temperature and toluene concentration were increased, toluene degradation went up to 63.99%.

Optimal Conditions and Validation of Model

In order to determine the maximum cell growth and toluene degradation by *Bacillus cereus* ATHH39, corresponding to the optimum levels of pH, temperature and toluene concentration was conducted at optimum cultivation conditions as predicted by the RSM for results verification, a second order polynomial model was used to calculate the values of these variables. The optimum levels of variables were found to be pH 6.72, 33.16ºC, and toluene concentration of 824.15 mg/l with a prediction of OD_{600} = 0.71 for cell growth and 65.85% for toluene degradation. Fitting the experimental data to equations, determining pH (X_1 = 6.72), temperature (X_2 = 33.16°C), and toluene concentration (X_3 = 824.15 mg/l) gave maximum cell growth of (OD_{600} = 0.71) and toluene degradation (65.85%). In order to test the validity of the optimum conditions achieved by the empirical model, a confirmatory experiment was carried out using these optimal levels. The actual experimental value is OD_{600} = 0.69 for cell growth and 64.11% for toluene degradation. The experimental value for cell growth and toluene degradation in optimal conditions was comparable with those predicted in order to determine the validity of the model and keep a relative error of 3.9% and 2.6%, respectively, which indicates

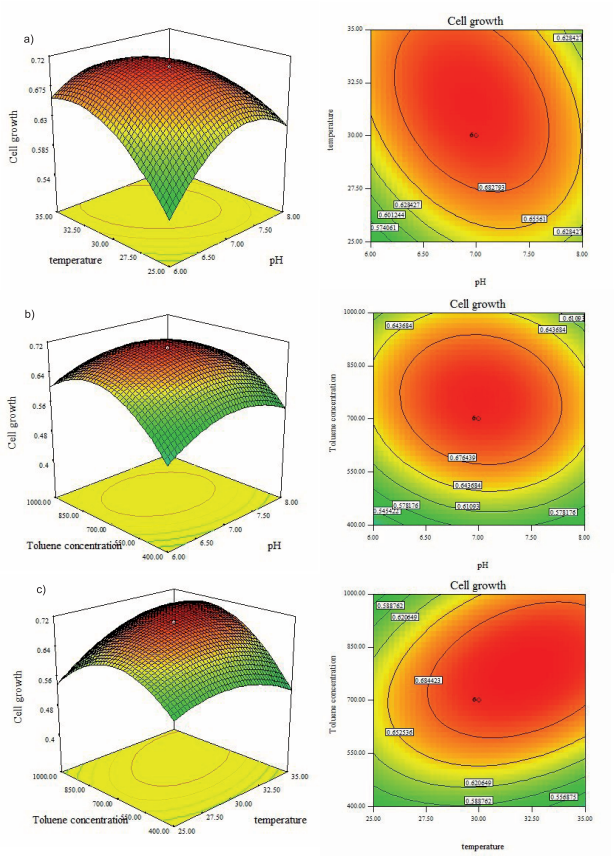

Fig. 3. Response surface plots for cell growth by *Bacillus cereus* ATHH39 versus a) pH and temperature, b) pH and toluene concentration, and c) temperature and toluene concentration.

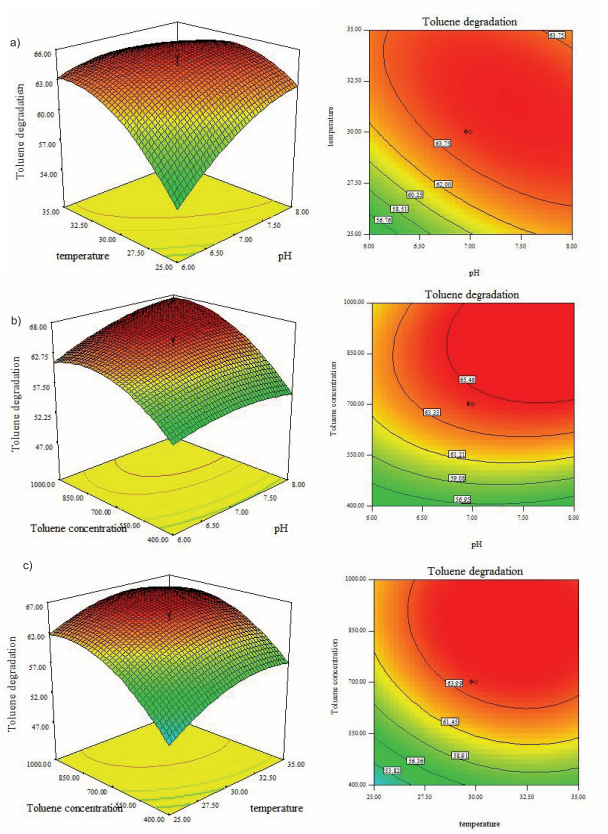

Fig. 4. Response surface plots for toluene degradation by *Bacillus cereus* ATHH39 versus a) pH and temperature, b) pH and toluene concentration, and c) temperature and toluene concentration.

that the experimental values are in agreement with the predicated one.

Conclusions

This study investigated the use of statistical experimental designs to optimize medium for cell growth and toluene degradation by *Bacillus cereus* ATHH39. Several factors, such as pollutant concentration, temperature, pH, availability of inorganic nutrients, and microbial adaptation influenced the rate and extent of biodegradation of BTEX [28].

The application of statistical experimental designs to optimize the selected factors for maximal production is an efficient method that tests the effect of factor interaction with a minimal number of experiments. The optimal medium and cultivation conditions for cell growth and toluene degradation by *Bacillus cereus* ATHH39 were found at pH 6.72, 33.16ºC, and toluene concentration of 824.15 mg/l. Oil-oxidizing microorganisms can degrade oil hydrocarbons in a relatively wide range of pH (5 to 10), but the optimal medium pH is between 7 and 8 [29]. Alagappan and Cowan [30] determined the influence of temperature on the growth rate and benzene, toluene degradation by *P. putida* over a range of 15-35ºC. The optimum temperature was found to be 33ºC for both substrates. Therefore, *P. putida* was identified to fall within the range typical for mesophilic microorganisms. Hamed et al. [31] observed that the maximum concentrations of benzene, toluene, and phenol consumed by *Pseudomonas putida* F1 (ATCC 700007) in single phase (only aqueous phase) were only 880,880 and 200 mg/l, respectively.

Acknowledgements

The authors would like to thank the Soil Science Department, Isfahan (Khorasgan) Branch, Islamic Azad University, Isfahan for financial supporting and funding this research.

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