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Response surface optimization of dissolved oxygen and nitrogen sources for the biodegradation of MTBE and BTEX

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Abstract Response surface methodology (RSM) using central composite design was applied to obtain the optimal dissolved oxygen (DO) and nitrogen (N) concentrations for biodegrading MTBE (Methyl *tert*-butyl ether) and BTEX (benzene, ethylbenzene, toluene, *p*-xylene). Moreover, the effects of DO, N, and their interaction on the degradation process were evaluated. It was found that N, N^2, DO and DO^2 have significant effects on the efficiency of MTBE and BTEX removal. The removal efficiency when using biostimulation with bioaugmentation (BwB) is higher than with other processes, being greater than 82% at concentrations of 12 and 48 mg l^{-1} for DO and N, respectively. However, it was also found that the interaction term of DO $\times$ N has no significant effect on the degradation processes.

Keywords Bioaugmentation · Biostimulation · Methyl *tert*-butyl ether (MTBE) · Response surface methodology (RSM)

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Introduction

Bioremediation is a biological process in which microorganisms are used to decompose or transform targeted contaminant compounds to less toxic forms (Evans and Furlong 2003). This can be a natural process known as bioattenuation, a method for monitoring the natural degradation of toxic compounds to ensure that the concentrations gradually decrease (Iwamoto and Nasu 2001). If natural degradation cannot occur or if the process is too slow, the environment must be manipulated so that biodegradation is stimulated and the reaction rates increased. This biostimulation method includes supplying the environment with nutrients such as nitrogen and phosphorus, having electron acceptors such as oxygen, and with substrates (Iwamoto and Nasu 2001; Vidali 2001). In addition, bioremediation via bioaugmentation is a way to enhance the biodegradative capacities of contaminated sites by inoculation of bacteria with the desired catalytic capabilities, which is considered to be an effective approach in cases of very recalcitrant chemicals for which bioattenuation or biostimulation does not work (Whiteley and Lee 2005; Yu et al. 2005). Both biostimulation and bioaugmentation have been commonly used to enhance biodegradation at soil and groundwater sites (Hamdi et al. 2007; Olaniran et al. 2006; Smith et al. 2005).

Contamination of groundwater with the gasoline additive methyl *tert*-butyl ether (MTBE) is often accompanied by many aromatic components such as benzene, toluene, ethylbenzene, *o*-xylene, *m*-xylene and *p*-xylene (BTEX) which are all considered as resistant substrates to most physical methods of treating fuel-contaminated water (Happel et al. 1998). Thus, there were more investigations have paid much attention in increasing bio-utilization of MTBE and BTEX under various conditions and bioprocessing designs over the last decade.

Studies using single strain microorganism reported that pure culture showed the better capability in degradation MTBE than mixed culture (Nikpey and Nikpey 2006; Okeke and Frankenberger 2003). Moreover, *tert*-butyl alcohol (TBA) was formed as a metabolic intermediate during the breakdown of MTBE (Lin et al. 2007). The BTEX mixture was observed not to affect either the rate or the degradation of MTBE but had positive effect on bio-utilization of TBA by pure culture UC1 which was known as MTBE-degrader PM1 (Wang and Deshusses 2007).

Mixed culture and microbial community were also widely used to investigate biodegradation of MTBE in order to evaluate efficiency of bio-utilization process. It was found that removal efficiencies of MTBE can be reached high percentages even in the presence of other substrates. Toluene, ethyl benzene, *p*-xylene, benzene, MTBE, ethyl ether, *tert*-amyl methyl ether, and ethyl *tert*-butyl were arranged as the sequence of the target compounds in order of removal rate by mixed culture (Wang and Deshusses 2007). In addition, single BTEX compound or BTEX mixtures slightly inhibited MTBE degradation but *tert*-butyl alcohol (TBA) is as a frequent co-contaminant of MTBE had no inhibitory effect on MTBE degradation using microbial consortium (Pruden and Suidan 2004). Culture composition and reactor configuration were reported as key factors that effect on removal efficiency of MTBE. Results of the study showed that biodegradation of MTBE was completely inhibited by BTEX in the batch reactors using microbial consortium enriched on MTBE and BTEX, and severely retarded in those inoculated culture enriched on MTBE, originally. However, it was obviously that the semi-batch reactor had positive effects on removal efficiencies of MTBE, the MTBE biodegradation rate in the presence of BTEX was almost three times as high as in the batch reactors but slower than MTBE biodegradation in the absence of BTEX in batch reactors inoculated MTBE-enriched culture (Raynal and Pruden 2008). Relating to MTBE degradation in the presence of other fuel components such as toluene, benzene, ethanol, methanol, or gasoline, it was reported that removal efficiency of MTBE was not affected by these compositions. However, many aryl *O*-methyl ethers, such as syringic acid, that are *O*-demethylated by acetogenic bacteria, were also *O*-demethylated by the MTBE-utilizing enrichment cultures and the addition of these compounds as co-substrates increasing the rate of MTBE degradation. Study of MTBE microbial utilization was continued using ethanol as co-substrate. It was found that MTBE can be effectively degraded by aerobic granules under a co-substrate condition in sequencing batch reactor (SBR) (Zhang et al. 2008).

As overviewed about, most recent studies conducted investigations under various conditions with or without presence of other contaminated compounds which are considered as co-substrates of MTBE. There were also more studies that investigated effect of nutrient compositions on removal efficiency of MTBE by variously biological processes. However, very less study applied statistic in design and process experimental data in study of treatment MTBE using biological process.

Response surface methodology (RSM) is an empirical modeling and mathematical technique useful for developing, improving and optimizing processes. The method usually consists of three stages: (1) the central composite design of experiments, (2) response surface modeling through regression, and optimization (Montgomery 2005). The central composite design (CCD) technique is widely used to construct second-order response surfaces. Several studies have applied RSM using CCD to obtain optimal experimental parameters because this method shows the advantage of a reduced number of experimental trials needed to evaluate multiple parameters and their interactions

(Liu and Chiou 2005; Myers and Montgomery 1995; Sheeja and Murugesan 2005).

In our study, the biodegradation of MTBE and BTEX (benzene, ethylbenzene, toluene, *p*-xylene) was studied using mixed cultures in a five liter liquid-agitated bioreactor and accomplished through the processes of biostimulation and bioaugmentation (B&B). A CCD of the experiments was used to construct second-order response surfaces with substrate removal as an optimization parameter and dissolved oxygen (DO) and nitrogen (N) contents as design factors. The results were modeled using RSM, which determines the dependence of the maximum percentage of MTBE and BTEX degraded as a function of the concentrations of the nitrogen source and the oxygen. This study attempted to identify the optimal regions of DO and N concentrations and evaluate their interaction in the biodegradation processes of MTBE and BTEX using a response surface model.

Materials and methods

Experimental apparatus, microorganisms and culture media

The microorganism cultures used in this study were obtained from a petrochemical wastewater treatment plant and subsequently adapted in a biotricking filter fed with the substrates MTBE and BTEX as carbon sources for approximately 5 months. Pure isolates of *Ralstonia* sp. YABE411, *Pseudomonas* sp. YATO411, *Pseudomonas* sp. YAET411, and *Pseudomonas aeruginosa* YAMT521 (Lin and Cheng 2007) were obtained after a series of screening and isolating procedures and maintained for subcultures in shaker flasks at 30°C, 125 rpm before being used in the following design experiments.

This study was implemented in a five-liter fed-batch bioreactor. Four liters of media, microorganisms and substrates (MTBE and BTEX) were added to the bioreactor, when the pH and the temperature of the solution were automatically controlled at 7.0 and 30°C, respectively. The dissolved oxygen concentration was determined by maintaining a pure oxygen input rate at 10–11 l min^{-1} using a mass-flow controller, the rotary speed of the impeller in the bioreactor being kept at 300 rpm. The substrates (MTBE and BTEX having a volumetric ratio of 0.85–1) were added daily, the fixed concentration of each being 30 mg l^{-1}. The composition of the medium was as follows: (mg l^{-1}) K_2HPO_4 330, KH_2PO_4 1270, $MgSO_4 \cdot 7H_2O$ 100, $FeSO_4 \cdot 7H_2O$ 1, $CaCl_2 \cdot 2H_2O$ 45, $CuCl_2 \cdot 2H_2O$ 0.25, $CoCl_2 \cdot 6H_2O$ 0.25, $ZnSO_4 \cdot 7H_2O$ 1, $MnCl_2 \cdot 4H_2O$ 1, $Na_2MoO_4 \cdot 2H_2O$ 0.1, $NiCl_2 \cdot 6H_2O$ 0.02, $NaNO_3$ 8–100.

The biodegradation capability was evaluated using two stages of experiments. In the first, biostimulation was implemented by varying the DO and N contents in the reactor; in the second, further bioaugmentation was conducted by adding 100 mg l^{-1} pure cultures (YABE411, YATO411, YAET411, and YAMT521) to the bioreactor while in a biostimulated condition.

Analytical methods

The physical and chemical properties of MTBE and BTEX compounds including Henry's law coefficient, vapor pressure, and water solubility, reveal that these are volatile organic compounds (VOC_S); therefore, the change in substrate concentrations in the gaseous and liquid phases in each bottle can be related using Henry's law. Since the mass transfer rates between both phases are rapid, the gas concentrations approach equilibrium with the liquid phases. Hence, use of the headspace method for biodegradation experiments can capture the substrate concentrations in the liquid. In all experiments, mixed compounds of MTBE and BTEX were sampled from the bioreactor by a 250 μl stainless steel needle fitted to a gas-tight syringe.

The sampled compound concentrations were analyzed by using a model GC-14B gas chromatograph (Shimadzu Corp., Japan) equipped with an RTX-1 capillary column (30 m × 0.53 mm) and a flame ionization detector. Nitrogen (99.98% pure) was used as the carrier gas, hydrogen and air, as a makeup gas. The oven temperature was controlled at a constant of 105°C; whereas, the injector and detector temperatures were set at 200 and 250°C, respectively. The concentrations were quantified against primary standard curves. The removal efficiency was determined by using the relationships between the influent and effluent concentrations.

RSM experimental design

RSM consists of a collection of mathematical and statistical techniques that are useful for the modeling and analysis of problem in which a response of interest is influenced by several variables, the objective being to optimize this response. In most RSM problems, the form of the relationship between the response and independent variables is unknown. Therefore, the first step in RSM is to find a suitable approximation for the true functional relationship between the response variable and the set of independent variables. After estimating the parameters in the approximating polynomials, the response surface analysis is performed using a fitted surface. If the surface is an adequate approximation of the true response function, then analysis of this surface will be approximately equivalent to an analysis of the actual system. The model parameters can be estimated most effectively if proper experimental designs are used to collect the data. The eventual goal of RSM is to determine the optimum operating conditions for the system or to identify a region of factor space in which the requirements are satisfied.

RSM using CCD was applied in several of the reviewed reports (Chiang and Chang 2006; Kumar and Satyanarayana 2007; Lin and Chou 2002; Rigas et al. 2005). CCD is widely use for fitting a second-order response surface. If the assumption that interactions between more than two factors are negligible is correct, this procedure allows unbiased estimation of all main effects and two-factor interactions. In the present study, RSM using CCD was applied to determine the optimum levels of the significant factors (DO, N) and the effect of their interaction on efficiency in removing MTBE and BTEX. A 2^2 factorial design augmented by five replicates at the center point was implemented in 13 experiments wherein the efficiency in removing each compound was taken as a response. First, the independent input variables (DO, N) and the responses with design constraints were defined. Second, the CCD for the independent variables was implemented. Third, the experiments with DO and N concentrations from the CCD results were conducted. Fourth, a statistical analysis of variance (ANOVA) for DO and N was calculated to determine which concentration level significantly affects the removal efficiency, after which the optimal design parameters with design constraints were obtained. Fifth, confirmatory experiments to confirm and verify the setting of the optimal design parameters were conducted for each biological process.

Results and discussion

Removal efficiency under biostimulation and bioaugmentation (B&B)

The results indicate that the efficiency in removing MTBE and BTEX decreased in the order of ethylbenzene, *p*-xylene, toluene, benzene, and MTBE. Additional removals of 10–30% for both MTBE and BTEX were observed via biostimulation and bioaugmentation (B&B), thereby demonstrating the significant contributions of nitrogen source, oxygen and substrate-degrading pure cultures.

RSM modeling of results

CCD was implemented for the independent variables (DO, N) having concentrations in the ranges of 3.6–20.4 and 0–104 mg l^{-1}, respectively, by using the Statistica 6 software package (STATISTICA, Inc.). Table 1 shows the experimental design. It should be noted that the design used to collect this data was a 2^2 factorial augmented by five center points, both having concentrations of DO and N at 12 and 48 mg l^{-1}, respectively. Thirty nine experiments were conducted for three cases (“biostimulation with bioaugmentation” (BwB) or “biostimulation without bioaugmentation” (BwoB), and “biostimulation + bioaugmentation” (B&B)) with DO and N concentrations resulting from the CCD. The obtained results indicate that the actual (observed) values of MTBE and BTEX removal efficiency are similar to the predicted values. In addition, Table 1 shows that there were no significant differences between the actual and predicted values of the removal efficiencies of these three biological processes.

Table 2 summaries the effect estimates and *P*-values. The column labeled “effects” measures the effect contribution of each model term to the response of the model. It should be noted that the main variable DO with its effect contribution of 12.95 is a little bit more significant than N with effect contribution of 8.05 in the BwoB process, whereas the quadratic interactions N^2 and DO^2 account for their effects of 12.20 and 13.21 (absolute values), respectively, seem to have the same significance. However, DO and N could be considered as two main terms having equal effect contributions to response of BwoB.

Table 1 Experimental design and results of central composite design

Run	Factors		Actual concentrations		Removal efficiency (%)					
	N	DO	N (mg l^{-1})	DO (mg l^{-1})	MTBE + BTEX[a]		MTBE + BTEX[b]		MTBE + BTEX[c]	
					Actual value	Predicted value	Actual value	Predicted value	Actual value	Predicted value
1	0	0	48	12	60.33	60.19	82.45	82.15	71.39	70.94
2	0	0	48	12	59.43	60.19	83.97	82.15	71.10	70.94
3	0	0	48	12	61.24	60.19	81.24	82.15	70.08	70.94
4	0	0	48	12	60.96	60.19	82.98	82.15	69.89	70.94
5	0	0	48	12	58.78	60.19	79.94	82.15	72.04	70.94
6	1	−1	88	6	44.37	45.32	60.90	64.25	52.64	54.79
7	−1	−1	8	6	36.74	36.68	52.18	54.54	44.46	45.64
8	−1	1	8	18	48.91	50.22	76.41	75.08	62.66	62.67
9	1	1	88	18	55.38	57.70	79.13	78.78	67.26	68.24
10	1.4142	0	104	12	55.78	53.87	78.54	76.78	67.16	65.32
11	−1.4142	0	0	12	47.12	46.57	71.14	70.73	59.13	58.61
12	0	1.4142	48	20.4	58.45	56.31	75.47	77.08	66.96	66.69
13	0	−1.4142	48	3.6	38.35	38.17	56.20	52.53	47.30	45.36

[a] Biostimulation (without bioaugmentation)

[b] Biostimulation (with bioaugmentation)

[c] Biostimulation + bioaugmentation

In the BwB and B&B process the ANOVA indicated that N, N^2, DO and DO^2 are still the significant effects (data not shown). Furthermore, as results shown in Table 2, DO is considered as dominative effects of these two processes, accounting for 17.53 for BwB and 15.23 for B&B, respectively, which are over two times of N effects for BwB (6.70) and B&B (7.36), respectively. Moreover, two variables N^2 and DO^2 were estimated as significant effects having the same effect contributions to response of these two processes. Effects of N^2, DO^2 are 10.27, 17.70 for BwB and 7.36, 15.22 for B&B, respectively. However, the interaction term of $N \times DO$ is not an important factor for these two processes. Its effect contribution is much smaller than other significant terms (Table 2). Furthermore, the *P*-values of lack-of-fits are 0.07 for both BwoB and BwB, and 0.05 for B&B, respectively, thereby indicating that there are no significant differences between the central points (data not shown).

Table 3 shows that the overall fit of the equation is very good, with adjusted R-squares of 0.96, 0.94 and 0.97 for BwoB, BwB and B&B, respectively. The modeling terms (N, N^2, DO and DO^2) are statistically significant as indicated by the t-statistics and *P*-values in the three processes. The results of the response surface regression analysis, the *P*-values of the modeling terms being less than 0.05, indicate that these terms have a significant effect on the MTBE and BTEX removal efficiency. In the three processes, DO has the highest estimated coefficients of 5.54, 7.66 and 6.52 for BwoB, BwB and B&B, respectively, thus revealing that this modeling term is more important than the others. Moreover, DO also have effect contributions of 12.95, 17.53 and 15.23 for BwoB, BwB and B&B, respectively, are almost over effect contributions of N in consistent processes. Thus, DO is considered as dominatively significant factor in these three processes, especially, in BwB and B&B.

Figure 1 shows the response surface of the removal efficiency in the BwoB process. It should be noted that the efficiency in removing the compounds in this process is over 60% at concentrations of DO and N at 12 and 48 mg l^{-1}, respectively (data not shown). However, these plots and output results of Statistic 6.0 indicate that the removal efficiency in this process is the highest values of 62.4% for DO, at

Table 2 Effect estimates

Variables	Effects	Standard error	*t*-Statistic	*P*-value
Biostimulation (without bioaugmentation)				
Intercept	60.19	0.46	130.78	0.0000
N*	8.05	0.76	10.49	0.0004
N^2*	−12.20	0.87	−13.92	0.0001
DO*	12.95	0.73	17.64	0.0000
DO^2*	−13.21	0.79	−16.68	0.0000
N × DO	−0.58	1.03	−0.56	0.6045
R^2	0.97			
adj R^2	0.96			
Biostimulation (with bioaugmentation)				
Intercept	82.15	0.69	117.86	0.0000
N	6.70	1.16	5.77	0.0044
N^2	−10.27	1.32	−7.73	0.0015
DO*	17.53	1.11	15.76	0.0000
DO^2*	−17.70	1.19	−14.76	0.0001
N × DO	−3.00	1.56	−1.91	0.1277
R^2	0.96			
adj R^2	0.94			
Biostimulation + bioaugmentation				
Intercept	70.94	0.40	176.11	0.0000
N*	7.36	0.67	10.96	0.0003
N^2*	−10.98	0.76	−14.32	0.0001
DO*	15.23	0.64	23.70	0.0000
DO^2*	−15.22	0.69	−21.96	0.0000
N × DO	−1.79	0.90	−1.97	0.1189
R^2	0.98			
adj R^2	0.97			

* $P < 0.05$, significance

Table 3 Results of regression analysis

Variables	Coefficients	Standard error	*t*-Statistic	*P*-value
Biostimulation (without bioaugmentation)				
Intercept	6.48	2.10	3.07	0.0371
N*	0.48	0.03	12.34	0.0002
N^2*	−0.003	0.0002	−13.92	0.0001
DO*	5.54	0.28	19.11	0.0000
DO^2*	−0.18	0.01	−16.68	0.0000
N × DO	−0.001	0.002	−0.56	0.6045
R^2	0.97			
adj R^2	0.96			
Biostimulation (with bioaugmentation)				
Intercept	14.18	3.19	4.44	0.0113
N	0.46	0.05	7.90	0.0013
N^2	−0.003	0.0004	−7.73	0.0015
DO*	7.66	0.43	17.44	0.0000
DO^2*	−0.24	0.01	−14.76	0.0001
N × DO	−0.006	0.003	−1.91	0.1277
R^2	0.96			
adj R^2	0.94			
Biostimulation + bioaugmentation				
Intercept	10.77	1.84	5.83	0.0042
N*	0.46	0.03	13.66	0.0001
N^2*	−0.003	0.0002	−14.32	0.0001
DO*	6.52	0.25	25.70	0.0000
DO^2*	−0.21	0.009	−21.96	0.0000
N × DO	−0.003	0.001	−1.97	0.1189
R^2	0.98			
adj R^2	0.97			

* $P < 0.05$, significance

14.90 mg l^{-1}; whereas, N is approximately 60.74 mg l^{-1}. Furthermore, the plot indicates that both DO and N have significant effects on the removal efficiency.

Figure 2 illustrates the response surface of the removal efficiency in the BwB process; moreover, it indicates that both DO and N have significant effects on the removal efficiency. Previous results showed that the MTBE and BTEX removal efficiency in this process is averagely greater than 80% at concentrations of DO and N at 12 and 48 mg l^{-1}, respectively. However, outcome results of Statistic 6.0 and these plots also indicate that the removal efficiency in this process reaches the highest value of 84.66% at concentrations of DO and N of approximately 14.84 and 58.29 mg l^{-1}, respectively.

Figure 3 indicates that both DO and N have significant effects on the removal efficiency in the B&B process. Previous results showed that the removal efficiency in this process is averagely greater than 70% at concentrations of DO and N at 12 and 48 mg l^{-1}, respectively. However, output results of Statistic 6.0 and these plots also indicate that the removal efficiency reaches the highest value of 73.33% at concentrations of DO and N at approximately 14.90 and 59.83 mg l^{-1}, respectively.

Confirmation experiments

To verify the adequacy of the quadratic models obtained, confirmatory experiments were conducted

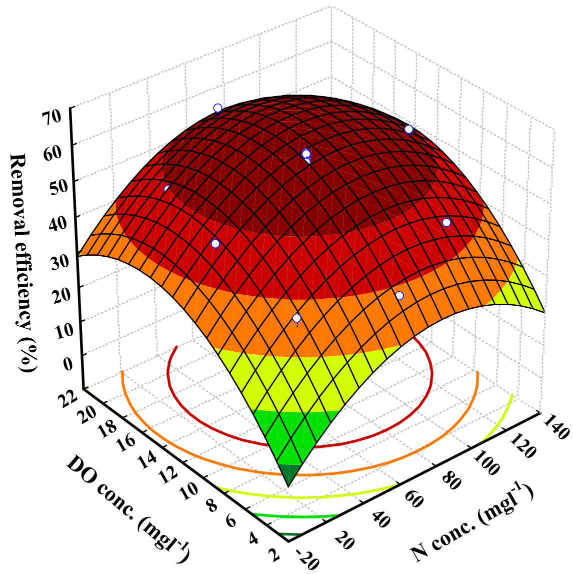

Fig. 1 Response surface for NO_3^-–N and DO concentrations depicted as removal efficiency contour in biostimulation without bioaugmentation (BwoB)

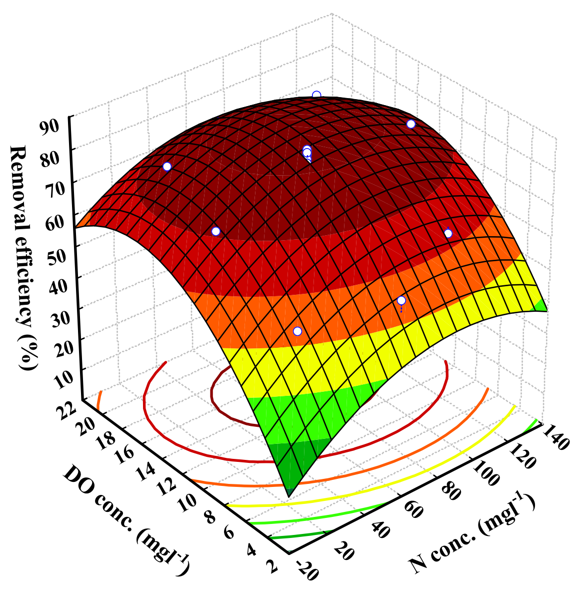

Fig. 2 Response surface for NO_3^-–N and DO concentrations depicted as removal efficiency contour in biostimulation with bioaugmentation (BwB)

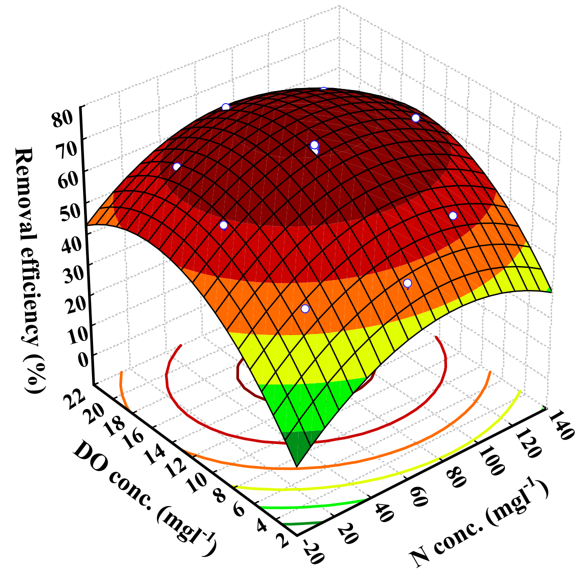

Fig. 3 Response surface for NO_3^-–N and DO concentrations depicted as removal efficiency contour in biostimulation + bioaugmentation (B&B)

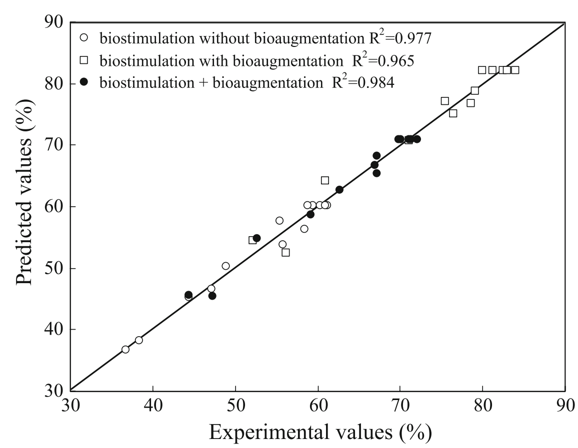

Fig. 4 Plot of predicted versus experimental values for MTBE and BTEX removal efficiency in case of biostimulation without bioaugmentation (BwoB), biostimulation with bioaugmentation (BwB), and biostimulation + bioaugmentation (B&B)

for measuring the MTBE and BTEX removal efficiency in the BwoB, BwB and B&B processes. For BwoB, duplicate experiments were carried out at optimal point of the design parameters of DO and N at 14.90 and 60.74 mg l^{-1}, respectively. With this optimal concentration, the observed results and the predicted value in the two duplicates for the removal efficiency were 61.34, 62.04 and 62.4%, respectively, as plotted in Fig. 4. The differences between the experimental and predicted values in duplicates are 1.57 and 0.57%, respectively. For BwB, confirmatory experiments were carried out at optimal point of DO and N at concentrations 14.84 and 58.29 mg l^{-1}, respectively. At this optimal concentration, the actual results and the predicted value in the two duplicates for the removal efficiency were 80.12, 82.43 and 84.66%, respectively, as plotted in Fig. 4. The differences between the experimental and predicted values in two duplicates are 5.36 and 2.63%, respectively. Similarity, in B&B, duplicate is carried out at DO and N concentrations of 14.90 and 59.83 mg l^{-1}, respectively. At this optimal point, the observed results and the predicted value in the two duplicates for the removal efficiency were 70.31, 72.22 and 73.33%, respectively, as plotted in Fig. 4. The differences between the experimental and predicted values in two duplicates are 4.11 and 1.51%, respectively. The predicted values were highly correlated with the experimental values for BwoB ($r = 0.988$, $P < 0.001$), BwB ($r = 0.982$, $P < 0.001$), and B&B ($r = 0.992$, $P < 0.001$). The high values of correlation coefficients and R^2 obtained for three processes, indicate the consistency between the measured and the model-predicted removal efficiencies.

Results from RSM indicated that interactions of N and DO have no large effect on removal efficiency of MTBE and BTEX. Practically, these interactions have countable effects which make slight difference between data from individual investigation and combinative investigation as reported previously. As results obtained in individual investigation, RSM data, and confirmatory experiments, three quadratic models for three biological processes in treatment of MTBE and BTEX obtained appear to be accurate.

Conclusions

RSM is a very powerful tool for the optimization of the MTBE and BTEX removal efficiency measured in this study. The optimal concentrations of DO and N for degradation of the compounds were obtained and their interactions evaluated. The resulting information allows the maximal biodegradation to be obtained with the minimal experiments performed.

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