

## Kinetic optimization of biodegradation and debromination of 2,4,6-tribromophenol using response surface methodology

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### A B S T R A C T

#### Keywords:

Biodegradation  
2,4,6-Tribromophenol  
Kinetic optimization  
Central composite design  
Response surface methodology

2,4,6-Tribromophenol (2,4,6-TBP) is one of hazardous brominated flame retardants with acute toxicity which had an enormous applications in electronic devices and wood preservation. A bacterium *Bacillus* sp. GZT isolated in our lab was used to degrade 2,4,6-TBP and the response surface methodology was applied to optimize the biodegradation and debromination kinetics of 2,4,6-TBP. The central composite design model was chose to mathematically describe those affecting parameters of biodegradation. Three factors were determined to be the function of parameters, such as 2,4,6-TBP concentration ( $X_1$ ), initial pH value ( $X_2$ ) and the inoculum volume ( $X_3$ ). The results showed that two responses, degradation efficiency (%) ( $Y_1$ ) and debromination efficiency (%) ( $Y_2$ ), were coincidentally affected by significant factors of the linear terms of  $x_2$  and  $x_3$  with synergistic effect, but quadratic terms of  $x_2^2$  and  $x_3^2$  with antagonistic effect, and linear terms of  $x_1$  also had antagonistic effect on those two responses. The optimal conditions for  $Y_1$  and  $Y_2$  were 3.0 mg/L 2,4,6-TBP with 20.7 ml inoculum volume at pH value 7.3, and the estimated maximum responses were 94.6% and 88.6%, respectively. The experimental values were agreed with the theoretical results very well, indicating that the models employed to optimize the biodegradation and debromination of 2,4,6-TBP were effective.

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### 1. Introduction

2,4,6-Tribromophenol (2,4,6-TBP) is widely used as a reactive flame retardant intermediates and wood preservatives in industry manufacture (Gutiérrez et al., 2005). It can be detected in many environmental samples as well as human plasma (Thomsen et al., 2001) because of its persistent and bioaccumulation property. Researches on the toxicity effects of this chemical revealed that 2,4,6-TBP might possess estrogen-like property (Meerts et al., 2001; Legler and Brouwer, 2003) and it could cause developmental neurotoxicity, embryotoxicity, and fetotoxicity (Lyubimov et al., 1998; Rios et al., 2003). As a chemical with acute toxicity, it was already listed as the hazardous waste by the Environmental Protection Agency, USA (EPA, 1998). However, to date, only few works have investigated the degradation of 2,4,6-TBP by various technologies, such as photocatalysis (An et al., 2008) and biodegradation. Both activated sludge (Brenner et al., 2006) and some pure strains (Arnon et al., 2004; WHO, 2005) have been reported

their degradation ability to 2,4,6-TBP. But most of works only focused on the debromination rather than the complete degradation. For instance, strain *Desulfovibrio* sp. (Boyle et al., 1999) and *Aplysina aerophoba* (Ahn et al., 2003) were isolated to debrominate TBP under anaerobic condition, while the strain *Ochrobactrum* sp. TB01 could debrominate TBP under aerobic condition. Nevertheless, the ultimate products in these biodegradation works were all found as phenol. Recently, a pure strain *Bacillus* sp. GZT (Gene bank No.HQ603747) was newly-isolated in our lab by using 2,4,6-TBP as the start carbon and energy source, and the primary study showed that it could debrominate and mineralize of 2,4,6-TBP efficiently.

As it is well known, the energy resource concentrations and the cultivation conditions were very important factors for the micro-organism to biodegrade organics (Cuozzo et al., 2012; Giles et al., 2012). Conventionally, the biodegradation conditions optimization was usually carried out by the single-variable-at-a-time (SVAT), the most common practice holding all other variables constant) method (Kaneco et al., 2009). However, SVAT approach has lots of drawbacks, such as time-consuming, the absence of interactions between different variables and the inefficiency to predict the true optimum. However, an alternative method, the central composite design (CCD) based on response surface methodology (RSM) can

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overcome all these above mentioned shortcomings to optimize the kinetics of organics (Yao et al., 2009).

Therefore, in this paper, the biodegradation and debromination kinetics of 2,4,6-TBP by this newly-isolated strain *Bacillus* sp. GZT in our lab was optimized with CCD, and RSM was applied to mathematically describe the main and interactive effects of different variables on the debromination and biodegradation of 2,4,6-TBP. Furthermore, a predict CCD model was also developed to efficiently evaluate these processes.

## 2. Materials and methods

### 2.1. Chemicals and medium

2,4,6-TBP (purity: 99%) was obtained from Acros Organics (New Jersey, USA). The growth medium (GM) of the strain *Bacillus* sp. GZT consisted of peptone 10.0 g, beef extract 3.0 g, NaCl 5.0 g per liter of distilled water at the pH value 7.0, autoclaved at 121 °C for 15 min. The mineral medium (MM) used in the isolation of TBP-degrading strains contained the following (in g L<sup>-1</sup> of distilled water): 1.73 K<sub>2</sub>HPO<sub>4</sub>, 0.68 KH<sub>2</sub>PO<sub>4</sub>, 1.00 NH<sub>4</sub>Cl, 0.10 MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.03 MnSO<sub>4</sub>, 0.03 FeSO<sub>4</sub> and 0.02 CaCl<sub>2</sub>·2H<sub>2</sub>O. The pH value of the medium was adjusted to 7.0 and TBP with the initial concentration of 5.0 mg L<sup>-1</sup> was added to the medium.

### 2.2. 2,4,6-TBP biodegradation and analytical methods

The biodegradation experiments by strain *Bacillus* sp. GZT were performed by inoculating pre-cultivated strains in growth medium for 15 h, into 100 mL mineral medium containing 2,4,6-TBP as the degradation substrate under the conditions of 35 °C, 200 rpm and 120 h. Bromide concentrations were determined using the method 4500-Br<sup>-</sup> (APHA, 1995) with slight modification. The concentration of 2,4,6-TBP was measured by high performance liquid chromatography (HPLC) (Agilent 1200) equipped with a DAD detector set at 286 nm as described in our previous work (An et al., 2008).

### 2.3. Experimental design

CCD model based on RSM was adopted to optimize the 2,4,6-TBP concentration, the initial pH value and the inoculum volume on both the debromination and degradation efficiencies. The free Design-Expert software (trial version 8, Stat-Ease, Inc., MN, USA) was employed in the analysis of the experimental data.

## 3. Results

### 3.1. CCD for 2,4,6-TBP biodegradation and debromination

In order to find the optimum conditions for 2,4,6-TBP biodegradation and debromination, the experimental design as a function of the main factors was developed. According to our preliminary experiments, three factors ( $n = 3$ ) such as 2,4,6-TBP concentration ( $X_1$ ), initial pH value ( $X_2$ ) and the inoculum volume ( $X_3$ ) were chosen as the variables of the function at low value (-1), central (0) and high (+1) levels. Table 1 summarizes the levels and variables involved in the design strategy in this paper. Two responses were selected as the degradation efficiency ( $Y_1$ ) and debromination efficiency ( $Y_2$ ). Thus, the three significant independent variables  $X_1$ ,  $X_2$ , and  $X_3$  and the mathematical relationship of the response  $Y$  on these variables can be fitted by quadratic polynomial equation as below:

**Table 1**  
Experimental ranges and levels of independent variables.

Variable	Symbol	Coded levels		
		Low (-1)	Center (0)	High (+1)
2,4,6-TBP concentration	$X_1$	5	7	9
pH value	$X_2$	6.5	7	7.5
The inoculum volume	$X_3$	10	15	20

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 \quad (1)$$

where  $a_0$  is the constant,  $a_1$ ,  $a_2$ , and  $a_3$  are the linear coefficients,  $a_{12}$ ,  $a_{13}$ , and  $a_{23}$  are the cross-product coefficients, and  $a_{11}$ ,  $a_{22}$ , and  $a_{33}$  are the quadratic coefficients. Accordingly, 20 experiments which determined by consisting 8 ( $2^n$ ) full factorial points, 6 ( $2^n$ ) axial points located at the central and both extreme levels and 6 center points designed as replications were showed in Table 2. All 20 experiment runs data including the experimental values and theoretical values were all summarized in Table 3. The experimental values against the theoretical responses by the model for degradation and debromination efficiencies of 2,4,6-TBP (%), with a good correlation ( $R^2 = 0.9864$  and  $0.9866$ , respectively) indicated that this model explains the experimental range studied very well, as shown in Fig. 1.

### 3.2. The experimental model and analysis of variance

Two response regression equations which composed of three independent variables by the coded levels were analyzed by the free Design-Expert software and demonstrated as follows:

$$Y_1 = 76.24 - 8.31x_1 + 0.97x_2 + 2.75x_3 - 0.25x_1x_2 - 0.5x_1x_3 + 0.45x_2x_3 - 0.46x_1^2 - 1.29x_2^2 - 1.09x_3^2 \quad (2)$$

$$Y_2 = 65.04 - 11.22x_1 + 2.22x_2 + 9.49x_3 - 0.41x_1x_2 + 1.09x_1x_3 + 0.99x_2x_3 - 0.62x_1^2 - 3.30x_2^2 - 4.70x_3^2 \quad (3)$$

The negative and positive signs of regression coefficients indicate the antagonistic effect and synergistic effect of each variable, respectively. To evaluate the quantitative effect of three variables,

**Table 2**  
The 2<sup>3</sup> factorial and central composite design for experiment.

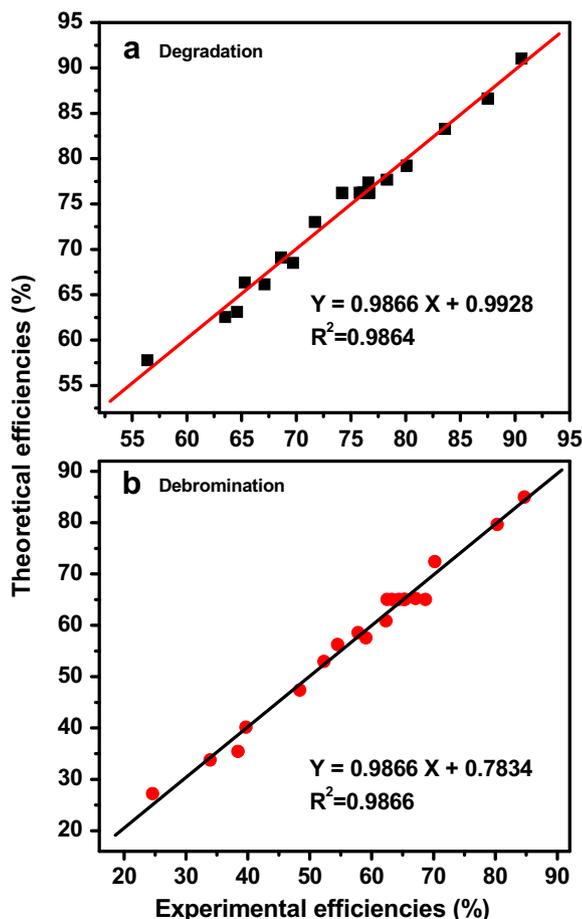
Standard no.	Variables in coded levels			Comment
	$x_1$	$x_2$	$x_3$	
1	-1	-1	-1	Full factorial
2	1	-1	-1	Full factorial
3	-1	1	-1	Full factorial
4	1	1	-1	Full factorial
5	-1	-1	1	Full factorial
6	1	-1	1	Full factorial
7	-1	1	1	Full factorial
8	1	1	1	Full factorial
9	-2	0	0	Axial
10	2	0	0	Axial
11	0	-2	0	Axial
12	0	2	0	Axial
13	0	0	-2	Axial
14	0	0	2	Axial
15	0	0	0	Center
16	0	0	0	Center
17	0	0	0	Center
18	0	0	0	Center
19	0	0	0	Center
20	0	0	0	Center

**Table 3**  
Experimental values of CCD for degradation and debromination efficiency of 2,4,6-TBP.

Pattern	Variables in uncoded levels			Responses			
	X <sub>1</sub> (2,4,6-TBP concentration)	X <sub>2</sub> (pH value)	X <sub>3</sub> (the inoculum volume)	Y <sub>1</sub>		Y <sub>2</sub>	
				Experimental value	Theoretical value	Experimental value	Theoretical value
-1-1-1	5	6.5	10	78.3	77.68	59.1	57.58
+1-1-1	9	6.5	10	63.5	62.55	33.9	33.80
-1+1-1	5	7.5	10	80.1	79.23	62.3	60.87
+1+1-1	9	7.5	10	64.6	63.10	38.4	35.43
-1-1+1	5	6.5	20	83.6	83.28	70.2	72.42
+1-1+1	9	6.5	20	67.1	66.15	52.3	52.98
-1+1+1	5	7.5	20	87.5	86.63	80.3	79.66
+1+1+1	9	7.5	20	69.7	68.50	57.8	58.57
-α00	3	7	15	90.6	91.03	84.7	85.01
+α00	11	7	15	56.4	57.78	39.7	40.14
0-α0	7	6	15	68.6	69.11	48.4	47.39
0+α0	7	8	15	71.7	73.01	54.5	56.26
00-α	7	7	5	65.3	66.36	24.6	27.24
00+α	7	7	25	76.6	77.36	67.1	65.21
000	7	7	15	76.6	76.24	68.7	65.04
000	7	7	15	74.2	76.24	62.5	65.04
000	7	7	15	75.8	76.24	65.2	65.04
000	7	7	15	76.7	76.24	65.4	65.04
000	7	7	15	76.1	76.24	63.3	65.04
000	7	7	15	76.2	76.24	64.4	65.04

the *p*-values of the *t*-test were calculated and showed in Table 4. The significance of variables is revealed by the *p*-value, if it less than 0.05 means that the term is significant. So, in this case, the *p*-values of two models were both <0.0001, which implied the models

corresponding to the degradation and debromination efficiencies are both very significant. Additionally, the two responses possessed the same significant factors of linear terms  $x_1$ ,  $x_2$  and  $x_3$  and the quadratic terms  $x_2^2$  and  $x_3^2$ . An antagonistic effect associated with terms of  $x_1$ ,  $x_2^2$  and  $x_3^2$ , and the synergistic effect with  $x_2$  and  $x_3$  can be also concluded through the regression coefficients.



**Fig. 1.** Correlation between the experimental and theoretical biodegradation (a) and debromination (b) efficiencies of 2,4,6-TBP (%) in uncoded values for  $t = 120$  h.

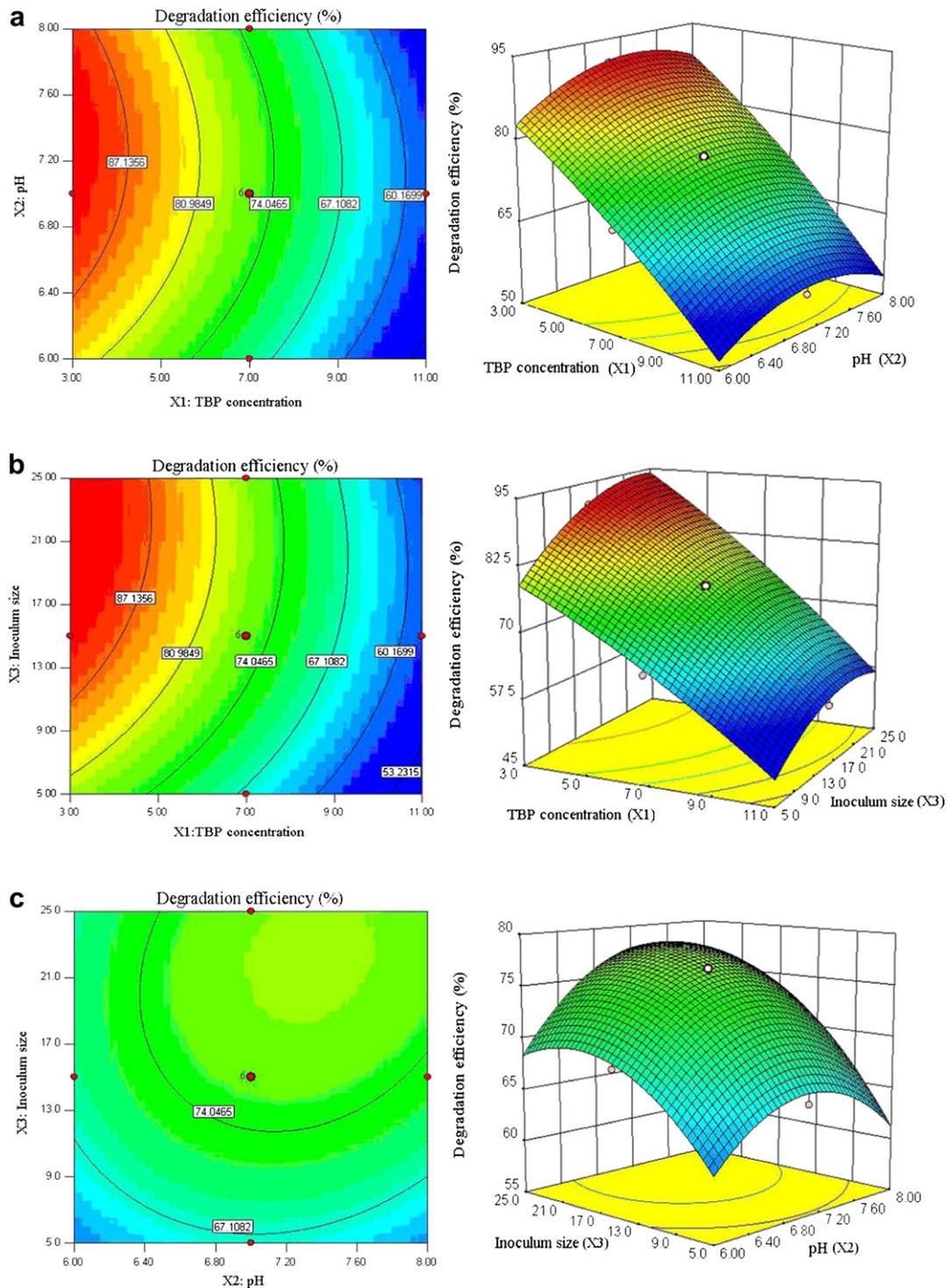
### 3.3. Response surface and optimization conditions

To better understand the relationship between the two responses ( $Y_1$ ,  $Y_2$ ) and the independent variables ( $X_1$ ,  $X_2$ ,  $X_3$ ), two-dimensional contours and response surface plots were also analyzed. Figs. 2a and 3a showed that the highest value of  $Y_1$  and  $Y_2$  could achieve at the minimum  $X_1$  (2,4,6-TBP concentration) with  $X_2$  (initial pH value) keep greater than about 6.8. The effect of  $X_1$  (2,4,6-TBP concentration) and  $X_3$  (the inoculum volume) showed that the maximum  $X_3$  and the minimum  $X_1$  would obtain the highest value of the two responses (Figs. 2b and 3b). The two response surfaces had the maximum points demonstrated in Figs. 2c and 3c, indicating that the influence of  $X_3$  (the inoculum volume) on the efficiencies of the degradation and debromination was dependent on the initial pH value.

To confirm the validity and accuracy of the model, experiments were also carried out at the optimal conditions for the highest

**Table 4**  
Analysis of variance for two responses.

Source	Y <sub>1</sub>		Y <sub>2</sub>	
	F value	<i>p</i> -value	F value	<i>p</i> -value
Model	80.81	<0.0001	81.82	<0.0001
$x_1$	615.84	<0.0001	347.63	<0.0001
$x_2$	8.47	0.0155	13.60	0.0042
$x_3$	67.40	<0.0001	248.94	<0.0001
$x_1 x_2$	0.28	0.6092	0.24	0.6383
$x_1 x_3$	1.11	0.3160	1.63	0.2301
$x_2 x_3$	0.90	0.3645	1.35	0.2728
$x_1^2$	2.92	0.1181	1.65	0.2276
$x_2^2$	23.46	0.0007	47.40	<0.0001
$x_3^2$	16.77	0.0022	96.06	<0.0001

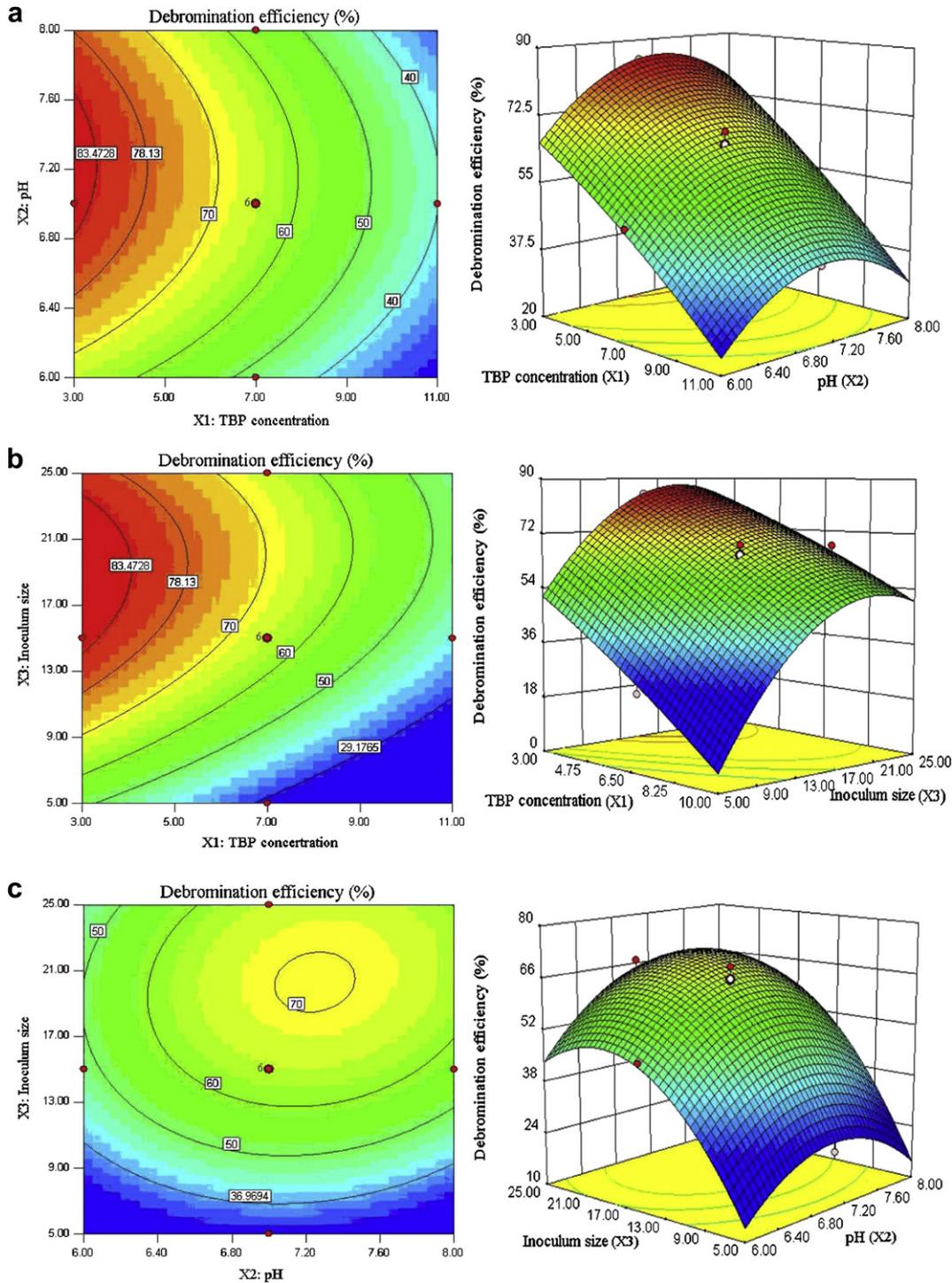


**Fig. 2.** Contour and surface plots of 2,4,6-TBP degradation efficiency. (a)  $X_1$  (TBP concentration) and  $X_2$  (pH value) in fixed  $X_3$  (the inoculum volume, 15 ml); (b)  $X_1$  (TBP concentration) and  $X_3$  (the inoculum volume) in fixed  $X_2$  (pH value, 7.0); (c)  $X_2$  (pH value) and  $X_3$  (the inoculum volume) in fixed  $X_1$  (TBP concentration, 7.0 mg/L).

biodegradation and debromination efficiencies of 2,4,6-TBP. The software optimized biodegradation and debromination efficiencies were 94.6% and 88.6%, respectively, at the optimized conditions of  $3.0 \text{ mg L}^{-1}$  2,4,6-TBP with 20.7 ml inoculum volume at pH value 7.3 (Fig. 4). These two data of the experimental biodegradation and debromination efficiencies of 94.4% and 89.7% were very close to the predicted values, respectively, indicating the adequacy of the obtained model to optimize biodegradation and debromination processes of 2,4,6-TBP.

#### 4. Discussion

The analysis of variance showed the effect of variables for each response, respectively. However, the significance of the model for each response were also different, and the  $p$ -values of  $x_2$ ,  $x_2^2$  and  $x_3^2$  for response  $Y_1$  were 0.0155, 0.0007 and 0.0022, respectively, greater than the values of 0.0042, <0.0001 and <0.0001 for response  $Y_2$ . The  $F$ -value of the two models were 80.81 and 81.82, respectively (Table 4), indicating that the model for the



**Fig. 3.** Contour and surface plots of TBP debromination efficiency. (a)  $X_1$  (TBP concentration) and  $X_2$  (pH value) in fixed  $X_3$  (the inoculum volume, 15 ml); (b)  $X_1$  (TBP concentration) and  $X_3$  (the inoculum volume) in fixed  $X_2$  (pH value, 7.0); (c)  $X_2$  (pH value) and  $X_3$  (the inoculum volume) in fixed  $X_1$  (TBP concentration, 7.0 mg/L).

debromination of 2,4,6-TBP was more significant than the model for the biodegradation of 2,4,6-TBP.

From the response surface analysis of the effect for  $X_1$  (the TBP concentration), it can be concluded that the substrate 2,4,6-TBP had an obvious inhibition on this biodegradation process. But a notable different effect on the response of  $Y_1$  (the degradation efficiency) and  $Y_2$  (the debromination efficiency) could be demonstrated by the cross correlation of  $X_1$  (the TBP concentration) and  $X_3$  (the

inoculum volume). It can be found that  $X_1$  had a predominant antagonistic effect on  $Y_1$  rather than the synergistic effect of  $X_3$ , while for  $Y_2$ ,  $X_3$  showed more impact than  $X_1$ . This result could also be verified by the signs of the two cross terms regression coefficients presented above. So, it indicated that the substrate inhibition could be reduced by the increase of the inoculum volume as well as the bacteria amount, and the release was more effective for the debromination than for the biodegradation. Furthermore, the effect

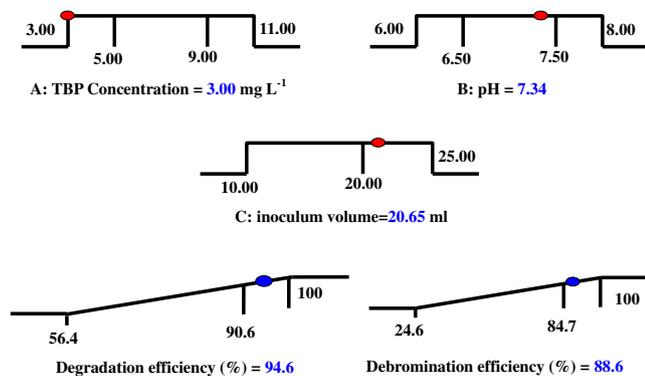


Fig. 4. Desirability ramp for numerical optimization.

of  $X_1$  and  $X_2$  (initial pH value) showed that the optimal pH value should keep above 6.8 (Figs. 2a and 3a), and this result consisted with a previously report which showed that the  $pK_a$  of 2,4,6-TBP was 6.8 (Franzen et al., 2007). When the pH value was lower than 6.8, the low solubility could decrease the utilization of the bacterial cell which would reduce the efficiencies of the debromination and biodegradation. Additionally, the effect of  $X_2$  and  $X_3$  also suggested that the bacterial cell activity was mainly affected by the pH value: lower pH value would result lower cell utilization and the higher one might not benefit for the microorganism growth. And the pH value affecting the activity of the bacterial strain can be also found in a previous report (Forte Giacobone et al., 2011).

## 5. Conclusions

The kinetics optimization of two models associated with the efficiencies of the biodegradation and debromination of 2,4,6-TBP was performed by the CCD based on RSM. The significant terms were determined as  $x_1$  (the TBP concentration),  $x_2$  (initial pH value),  $x_3$  (the inoculum volume),  $x_2^2$  and  $x_3^2$  by the  $p$ -value of each response, and the model for  $Y_2$  was considered more significant than the model for  $Y_1$ . The experimental values were very close to the predicted theoretical values indicating that the models could be validated for the kinetics optimization of the biodegradation and debromination processes of 2,4,6-TBP in water.

## Acknowledgments

This is contribution No. IS–1415 from GIGCAS. Financial support from NSFC (21077104), the Sci. & Technol. Project of Guangdong Province, China (2009A030902003 and 2005A30401001) and Guangdong Natural Science Foundation (S2011010001101).

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