Desalination and Water Treatment
www.deswater.com
doi: 10.1080/19443994.2013.769746

51 (2013) 6058–6069
September

Taylor & Francis
Taylor & Francis Group

A statistical approach for optimization of media components for phenol degradation by *Alcaligenes faecalis* using Plackett–Burman and response surface methodology

Subhasis Mandal[a], Biswanath Bhunia[b], Anuj Kumar[a], Dalia Dasgupta[b], Tamal Mandal[a,*], Siddhartha Datta[c], Pinaki Bhattacharya[c]

[a]*Department of Chemical Engineering, National Institute of Technology, Mahatma Gandhi Avenue, Durgapur-713209, India*
Tel. +91 343 2754083; Fax: +91 343 2547375; email: tamal_mandal@yahoo.com
[b]*Department of Biotechnology, National Institute of Technology, Mahatma Gandhi Avenue, Durgapur-713209, India*
[c]*Department of Chemical Engineering, Jadavpur University, Kolkata-32, India*

Received 30 March 2012; Accepted 12 January 2013

ABSTRACT

In the present study, phenol degrading bacterial species was isolated from coke oven wastewater of Durgapur steel plant, India. The biochemical, morphological, and 16s rDNA study were performed to identify the isolated microbes. The studies revealed that the high concentration phenol (2,100 mg L^{-1}) degrading isolating microbe is *Alcaligenes faecalis*. Optimization of media components and incubation time for phenol degradation was carried out by two-step statistical approach. Six key determinants such as phosphate, iron sulfate, calcium chloride, magnesium chloride, ammonium sulfate concentrations, and incubation time were screened using Plackett–Burman design and were further optimized by response surface methodology (RSM) using practical central composite design. The experimental results of the RSM were fitted via a second-order polynomial regression equation having the correlation coefficient (R^2) 0.9998 that indicates appropriate predictions of the above variables for significant phenol degradation. The results showed that there was 100% phenol degradation of 2100 mg/L which could be achieved at the optimized media composition as analyzed from RSM to appreciate the efficiency of the process using composition 0.42 g L^{-1} phosphate, 0.46 g L^{-1} iron sulfate, 0.08 g L^{-1} calcium chloride, 0.18 g L^{-1} magnesium chloride, and ammonium sulfate 0.25 g L^{-1} and 90.8 h incubation time.

Keywords: Modeling; Response surface methodology; Plackett–Burman design; Optimization; Fermentation; Biodegradation

1. Introduction

Phenol and its derivatives are very common products in several industries. The gas and coke oven industries, polymeric resin production, petroleum refineries, fiber glass units, pharmaceuticals, explosive, plastic and varnish industries, textile industries making use of organic dyes, smelting, and related

*Corresponding author.

metallurgical operations [1,2] are most common representatives. Phenol when present even at a low concentration level (5–25 mg L^{-1}) in wastewater is a potential threat to marine life [3]. The pretreatment alternatives such as activated carbon adsorption, ion exchange, and solvent extraction [4] are prohibitively constrained by high cost and the formation of hazardous by products. On the other hand, diverse micro-organisms, including bacteria [5], yeasts [6,7], algae [8], and filamentous fungi [9] have shown the metabolic capacities to degrade phenol of various concentrations. In addition, biodegradation is environmental friendly, cost effective, low power consumption, and safe process [10]. The main objective of this study are the optimization media composition and other physiochemical parameters like incubation time etc, as these parameters play a vital role in the improvement of biodegradability of phenol and have a great influence on bioreactor design and economics [11].

The traditional "one-variable at a time" technique used for optimizing a multivariable system is not only time consuming and expensive but also often easily misses the interaction effects among variables [12]. Recently, many statistical experimental design methods have been used in bioprocess optimization due to the variety of documented advantages [13–16]. The Plackett–Burman factorial designs can be applied in preliminary studies for screening the important factors from large number of process variables by identifying the effect of individual variables. This will lead to select variables that can be fixed or eradicated in further optimization processes. Thus, the design fulfills the principal preliminary objectives [17]. Additionally, response surface methodology (RSM) is an efficient strategic experimental tool to determine the optimum conditions by considering both the effect of primary factors and their mutual interactions for a multivariable system efficiently. The central composite design (CCD) is most commonly used for response surfaces determination of both linear and quadratic models, since it is evolved from a two-level factorial design augmented with center points and axial points. Recently, it has been successfully used to improve bioprocess application [18,19].

In the present investigation, an attempt has been made to optimize the important variables of phenol degradation using Plackett–Burman factorial design technique along with RSM. The system selected in this investigation is a challenging one since wastewater from different industries contains different extent of phenol. Significant variables were identified by two-level factorial technique using Plackett–Burman design methodology. Finally, optimization of these selected variables has been carried out by RSM using practical CCD on optimization of phenol degradation by *Alcaligenes faecalis*.

2. Materials and methods

2.1. Chemicals and analysis

Methanol for HPLC (E-Merck, Germany) and Phenol for HPLC (E-Merck, Germany) were used in this study. Water used for the HPLC analysis was prepared by Ultrapure Water System (Arium®, 611UF, Sartorius, Germany). All other chemicals used were of analytical grade commercially available in India. The statistical software package "Design Expert"® 7.0.0, Stat-Ease Inc., Minneapolis, USA was used to analyze the experimental design and the regression analysis of the experimental data.

2.2. Source of microorganism and isolation

All microbial enrichment and isolation procedures were performed in media with an inorganic culture media having the following composition (g L^{-1}): $MgCl_2$, 0.1; $CaCl_2$, $2H_2O$, 0.05; K_2HPO_4, 0.3; KH_2PO_4, 0.3; NaCl, 0.25; NH_4Cl, 0.15; $FeSO_4$, $7H_2O$, 0.35; $MnSO_4$, H_2O, 0.005; Na_2MoO_4, $2H_2O$, 0.005; $(NH_4)_2SO_4$, 0.10; NH_4NO_3, 0.005. The pH of inorganic culture media was adjusted to 7.4. The phenol contaminated water was collected from Durgapur (India) steel plant wastewater and synthetically phenol containing wastewater was used in the present study. The series of dilutions technique was carried out to obtain single pure colonies. After obtaining pure strains, each strain was checked for its ability to degrade phenol.

2.3. Identification of the phenol degrading bacterium

Biochemical and microscopical identification of the bacterial isolate with maximum phenol degrading potential this strain was carried out using following tests: oxidase, catalase, Simmons citrate, urea, motility, and nitrate–nitrite reduction [20,21]. Genomic identification of the strain was done using 16S rDNA technique from Bangalore Genei, India. Genomic DNA was isolated from the pure culture pellet [22]. The ~1.5 kb 16S rDNA fragment was amplified using the primers; 27f (50-AGA GTT TGA TCC TGG CTC AG-30) as forward and 1492r (50-TAC GGT TAC CTT GTT ACG ACT T-30) as reverse primer [23]. Sequence data were aligned and analyzed for finding the closest homologs for the microbe.

2.4. Culture harvesting and phenol degradation

For using the bacteria in phenolic wastewater treatment, fresh culture was grown. This was done by

adding a loop full of pure culture into a 50 mL inorganic media (pH−7.4). The 10% of fresh culture ($A_{600nm} = 0.01$, 10^7 colony forming unit per ml) at 37°C was used as inoculum for phenolic wastewater treatment. The composition of the simulated wastewater was used for experiment. Initial phenol concentrations used in all experiments are 2,100 mg L^{-1}. The treatment system was kept in a BOD incubator shaker at 120 rpm for various time periods. The culture was centrifuged (Model Centrifuge 5810 R, Eppendorf, Germany) at 12,000 × *g* for 15 min at 4°C. The cell pellet was discarded and the supernatant was used for residual phenol analysis using high-performance liquid chromatography (HPLC).

2.5. Assay of phenol

The supernatant filtered through 0.45 μm nylon membrane filters. After appropriate dilutions with water, sample was analyzed. For the analysis of sample, Waters™ 600 Pump-based HPLC system equipped with waters quaternary pump, Waters manual injector, Waters on-line degasser AF, CTO-10 AS VP column oven, and Waters 2489 UV/Visible detector were used. Water Empowered software (Version: Empower 2 software Build 2,154) was used for data acquisition and mathematical calculations. Chromatographic separation of protease was performed on a C_{18} hypersil column (4.6 mm × 250 mm; 5 μm particle size; Waters, USA). Mobile phase used was methanol–water (5% v/v acetic acid) (60:40 v/v), at a flow rate of 1 mL/min. Temperature of the column oven was maintained at 30°C. The sample (20 μL) was injected and analyzed at 270 nm using UV–Visible detector.

2.6. Optimization of phenol degradation

2.6.1. Screening of significant physico-chemical determinants by Plackett–Burman factorial design

The effect of eleven factors known to influence phenol degradation, viz. phosphate (A), sodium chloride (B), iron sulfate (C), sodium molibodate (D), calcium chloride (E), magnesium chloride (F), manganus sulfate (G), ammonium chloride (H), ammonium nitrate (J), ammonium sulfate (K), and incubation time (L), was studied [24–26] using statistical approach. All the variables were investigated at two widely spaced levels and selected by one way variable technique (shown in Table 1) from our previous work. The complete matrix for screening was designed using a standard Plackett–Burman factorial design (Table 2). A set of 12 experiments were carried out to determine the effective phenol degradation under different combinations.

Table 1
Plackett–Burman experimental design for screening of important determinants of phenol degradation by *A. faecalis*

Coded factor	Factor	Coded level (−1)	Coded level (+1)
A	Phosphate[a] (g L^{-1})	0.3	0.5
B	Sodium chloride (g L^{-1})	0	0.5
C	Iron sulfate (g L^{-1})	0.35	0.55
D	Sodium molibodate (g L^{-1})	0	0.01
E	Calcium chloride (g L^{-1})	0.05	0.1
F	Magnesium chloride (g L^{-1})	0.1	0.2
G	Manganus sulfate (g L^{-1})	0	0.01
H	Ammonium chloride(g L^{-1})	0	0.3
J	Ammonium nitrate (g L^{-1})	0	0.01
K	Ammonium sulfate (g L^{-1})	0.1	0.3
L	Incubation time (h)	72	120

[a]0.5 g m/L of phosphate ion in 0.5 g of K_2HPO4 and 0.5 g of KH_2PO4 in 1 L medium.

2.6.2. Optimization of key determinants by RSM

The important six key determinants viz. phosphate concentration (A), iron sulfate (C), calcium chloride (E), magnesium chloride (F), ammonium sulfate (K), and incubation time (H), were followed to optimize the objective function of phenol degradation. The response surface approach involving a practical CCD was adopted in the present investigation. A set of 86 experiments including 10 center points were carried out. Each numeric factor is varied over five levels (−1.57, −1, 0, +1, +1.57) i.e. plus and minus alpha (axial point), plus and minus one (factorial points), and zero (center point). The full experimental plan with respect to their actual and coded forms is listed in Table 3. The response values (Y) in each trial were the average of the triplicates.

2.6.3. Statistical analysis and modeling

Analysis of variance (ANOVA) was used for analysis of regression coefficient, prediction equations, and case statistics. The experimental results of RSM were fitted via the response surface regression procedure, using the following second-order polynomial equation.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

In which Y is the predicted response, X_i X_j are independent variables, β_0 is the intercept term, β_i is

Table 2
Plackett–Burman design for eleven variables with actual values, along with the observed phenol concentration

Run no.	A (g/L^{-1})	B (g/L^{-1})	C (g/L^{-1})	D (g/L^{-1})	E (g/L^{-1})	F (g/L^{-1})	G (g/L^{-1})	H (g/L^{-1})	J (g/L^{-1})	K (g/L^{-1})	L (h)	Phenol present ($mg\,L^{-1}$)
1	0.5	0	0.55	0.01	0.05	0.2	0.01	0.3	0	0.1	72	84.01
2	0.3	0	0.35	0	0.05	0.1	0	0	0	0.1	72	67.44
3	0.5	0	0.55	0.01	0.1	0.1	0	0	0.01	0.1	120	91.72
4	0.3	0	0.55	0	0.1	0.2	0	0.3	0.01	0.3	72	85.33
5	0.5	0	0.35	0	0.1	0.1	0.01	0.3	0	0.3	120	91.41
6	0.5	0.5	0.55	0	0.05	0.1	0.01	0	0.01	0.3	72	81.61
7	0.3	0.5	0.55	0	0.1	0.2	0.01	0	0	0.1	120	90.86
8	0.3	0.5	0.55	0.01	0.05	0.1	0	0.3	0	0.3	120	86.84
9	0.5	0.5	0.35	0	0.05	0.2	0	0.3	0.01	0.1	120	89.78
10	0.5	0.5	0.35	0.01	0.1	0.2	0	0	0	0.3	72	88.81
11	0.3	0.5	0.35	0.01	0.1	0.1	0.01	0.3	0.01	0.1	72	67.48
12	0.3	0	0.35	0.01	0.05	0.2	0.01	0	0.01	0.3	120	87.50

Table 3
Experimental range of the six numerical variables studied using practical CCD in terms of actual and coded factors

Coded factor	Factor	Range of variables				
		$-\alpha(-1.57)$	(-1)	(0)	(+1)	$+\alpha(+1.57)$
A	Phosphate	0.24	0.3	0.4	0.5	0.56
C	Iron sulfate	0.29	0.35	0.45	0.55	0.61
E	Calcium chloride	0.04	0.05	0.08	0.1	0.11
F	Magnesium chloride	0.07	0.1	0.15	0.2	0.23
K	Ammonium sulfate	0.04	0.10	0.20	0.3	0.36
L	Incubation time	58.4	72	96	120	133.6

the linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the interaction coefficient.

However, in this study, the independent variables were coded as A, C, E, F, K, and L. Thus, the second-order polynomial equation can be represented as follows

$$\begin{aligned} Y = {} & \beta_0 + \beta_1 A + \beta_2 C + \beta_3 E + \beta_4 F + \beta_5 K + \beta_6 L + \beta_{11} A_2 \\ & + \beta_{22} C^2 + \beta_{33} E^2 + \beta_{44} F^2 + \beta_{55} K^2 + \beta_{66} L^2 + \beta_{12} AC \\ & + \beta_{13} AE + \beta_{14} AF + \beta_{15} AK + \beta_{16} AL + \beta_{23} CE \\ & + \beta_{24} CF + \beta_{25} CK + \beta_{26} CL + \beta_{34} EF + \beta_{35} EK + \beta_{36} EL \\ & + \beta_{45} FK + \beta_{46} FL + \beta_{56} KL \end{aligned}$$

Diagnostics plots and model graphs were obtained using the Design Expert software to understand the effect of variables individually or in combinations and find out their optimum level for phenol degradation. The point prediction method was used in order to optimize the level of each variable for maximum response.

2.6.4. Validation of the experimental model

The statistical model was validated with respect to all the six variables within the design space. A random set of six experimental combinations was used to study phenol degradation in 250 mL shake flasks.

2.7. Biomass estimation

For biomass estimation, the cell pellet was washed twice with distilled water and then dried in a hot air oven. All experiments were done in triplicate. The specific growth rate (μ) has been calculated [27] from

$$\mu = \frac{1}{X}\frac{dX}{dt}$$

3. Results and discussion

3.1. Isolation and identification of phenol-degrading strains

The results of biochemical and microscopical study has been presented in the Table 4. 16S rDNA sequence analysis identified the strain as *A. faecalis* [20,21]. 16S rDNA gene sequencing was submitted in gene bank and gene bank accession number is JF339228.

3.2. Optimization of phenol degradation by A. faecalis

3.2.1. Screening of important physico-chemical determinants

Before optimization, the important factors are screened by Plackett–Burman factorial design. A total of 11 variables were analyzed with regard to their Studentized effects on phenol degradation using the Plackett–Burman design method. Evaluated screening of significant variables for phenol degredation, the corresponding sum square, and percentage of contribution are shown in Table 5. The positive and negative value of studentized effect indicates positive and negative effects of significant variables for phenol degradation. In order to analyze the significant factor effects from the insignificant ones, the larger (in absolute magnitude) effects can be identified from the effects list report. In our study, the six key determinants viz. phosphate (A), iron sulfate (C), calcium chloride (E), magnesium chloride (F), ammonium sulfate (K), and incubation time (L) were picked up as significant variables which have a positive role in phenol degradation.

The optimized simulated media with contained a few inorganic compounds like manganese sulfate, ammonium chloride, ammonium nitrate, sodium chloride, and sodium molybdate which have no significant effect on phenol degradation. Thus, their concentration was kept constant during media preparation. It may be noted that during Plackett–Burman design experiments, mutual interaction of the 11 process variables were not considered.

Table 4
Biochemical and microscopical characteristics of the isolated strain

Characteristics	Isolate
Gram stain	−
Shape	Rods, coccal rods, or cocci
Oxidase	+
Catalase	+
Simmons citrate	+
Motility	+
Urea	−
Nitrate–nitrite reduction	−

Table 5
Estimated effect, corresponding sum square, and percentage of contribution for phenol degredation from Plackett–Burman design experiment

Coded factor	Factor	Studentized effect	Sum square	Percentage of contribution
A	Phosphate	6.98	146.23	18.48
B	Sodium chloride	−0.34	0.34	0.04
C	Iron sulfate	4.66	65.1	8.23
D	Sodium molybodate	−0.01	0	0
E	Calcium chloride	3.07	28.31	3.58
F	Magnesium chloride	6.63	131.94	16.68
G	Magnesium sulfate	−1.18	4.14	0.52
H	Ammonium chloride	−0.52	0.8	0.1
J	Ammonium nitrate	−0.99	2.95	0.37
K	Ammonium sulfate	5.04	76.05	9.61
L	Incubation time	10.57	335.28	42.38

3.2.2. Optimization of the key determinants

In this study, in order to get the shape level of significant factors further optimization technique was applied. In the present case, the RSM has been applied for the optimization of significant factors in phenol degradation to observe the importance of screening factors at different levels. The practical CCD design plan of RSM has been used in the present study to investigate the culture conditions that would support to augment phenol degradation.

The annotated ANOVA analysis and design matrix of the optimization study listed in Table 6(a)–(c). Table 8, show that the model terms A, C, E, F, K, L, AC, AK, CK, EK, FK, KL, A^2, C^2, E^2, F^2, K^2, and L^2 are significant model terms. ("prob > *F*" less than 0.0500). Comparing the result the quadratic model as suggested by the software is presented by Eq. (1). The statistical significance of the second-order polynomial equation was checked by *F*-test (ANOVA). The model *F*-value of 13495.57 implies that the model is

Table 6(a–b)
Regression analysis for phenol degradation by *A. faecalis* for quadratic response surface model fitting (ANOVA)

Source	Sum of squares	*df*	Mean square	Coefficient estimate	Standard error	*F* value	*p*-value Prob >*F*	
(a)								
Model	7066.59	27	261.73	–	–	13495.57	$<10^{-4}$	Significant
Intercept	–	–	–	99.01	0.04	–	–	
A-phosphate	801.11	1	801.11	3.41	0.02	41308.37	$<10^{-4}$	
C-iron sulfate	289.29	1	289.29	2.05	0.02	14917.09	$<10^{-4}$	
E-calsium chloride	303.52	1	303.52	2.10	0.02	15650.81	$<10^{-4}$	
F-magnesium chloride	476.91	1	476.91	2.63	0.02	24591.28	$<10^{-4}$	
K-ammonium sulfate	210.84	1	210.84	1.75	0.02	10871.50	$<10^{-4}$	
L-incubation time	2268.32	1	2268.32	5.74	0.02	116963.2	$<10^{-4}$	
AC	0.25	1	0.25	−0.06	0.02	13.02	0.001	
AE	0.01	1	0.01	−0.01	0.02	0.57	0.453	
AF	0.01	1	0.01	−0.01	0.02	0.60	0.443	
AK	0.00	1	0.00	0.00	0.02	0.03	0.872	
AL	95.01	1	95.01	−1.22	0.02	4899.27	$<10^{-4}$	
CE	0.00	1	0.00	0.00	0.02	0.03	0.872	
CF	0.01	1	0.01	−0.01	0.02	0.29	0.592	
CK	0.00	1	0.00	0.00	0.02	0.01	0.943	
CL	33.70	1	33.70	−0.73	0.02	1737.60	$<10^{-4}$	
EF	0.01	1	0.01	−0.01	0.02	0.39	0.532	
EK	0.00	1	0.00	0.00	0.02	0.03	0.872	
EL	35.02	1	35.02	−0.74	0.02	1805.60	$<10^{-4}$	
FK	0.00	1	0.00	0.00	0.02	0.00	1.0000	
FL	56.03	1	56.03	−0.94	0.02	2888.88	$<10^{-4}$	
KL	24.75	1	24.75	−0.62	0.02	1276.24	$<10^{-4}$	
A^2	101.79	1	101.79	−2.71	0.04	5248.48	$<10^{-4}$	
C^2	73.27	1	73.27	−2.30	0.04	3778.29	$<10^{-4}$	
E^2	72.24	1	72.24	−2.28	0.04	3724.76	$<10^{-4}$	
F^2	76.70	1	76.70	−2.35	0.04	3954.89	$<10^{-4}$	
K^2	73.14	1	73.14	−2.30	0.04	3771.58	$<10^{-4}$	
L^2	139.79	1	139.79	−3.17	0.04	7207.97	$<10^{-4}$	
Residual	1.12	58	0.02					
Lack of fit	1.06	49	0.02			2.80	0.050	Not significant
Pure error	0.07	9	0.01					
Cor. total	7067.71	85						

(b)							
Std. Dev.	0.14	*R*-squared	0.999	CV(%)	0.16	Pred *R*-squared	0.999
Mean	86.91	Adj *R*-squared	0.999	PRESS	3.11	Adeq precision	482.533

significant. It may be mentioned that the probability of obtaining high model *F*-value due to creation of noise is only 0.01%. Moreover "Lack of Fit *F*-value" of 2.8 implies that it is not significant relative to the pure error. Nonsignificant lack of fit indicates a good fitness of model. There is only 5.02% chance that this magnitude of "Lack of fit *F*-value" could occur due to noise. The correlation coefficient (R^2) of polynomial equation was found to be 0.9998 indicating that 99.98% of the variability in the response (phenol degradation) could be explained by the model. So, quadratic model was chosen for this analytical work. The adjusted R^2 value corrects the R^2 value for the sample size and for the number of terms in the model. The adjusted R^2 (0.9998) is also very high that indicates that the model is very significant. The "Pred *R*-Squared" value of 0.9996 is in reasonable agreement with the "Adj *R*-Squared" value of 0.9998. This indicates a good adjustment between the observed and predicted values. "Adeq Precision" measures the

Table 6(c)
Practical CCD matrix for six variables with actual phenol concentration

Run no.	Phosphate (g/L^{-1})	Iron sulfate (g/L^{-1})	Calcium chloride (g/L^{-1})	Magnesium sulfate (g/L^{-1})	Ammonium sulfate (g/L^{-1})	Incubation time (h)	Phenol degradation (%)
1	0.30	0.55	0.05	0.10	0.10	72.00	67.63
2	0.56	0.45	0.08	0.15	0.20	96.00	97.29
3	0.50	0.35	0.05	0.20	0.30	72.00	83.28
4	0.50	0.55	0.10	0.10	0.30	72.00	87.19
5	0.40	0.45	0.08	0.15	0.20	58.40	94.54
6	0.50	0.55	0.10	0.20	0.30	72.00	93.61
7	0.30	0.55	0.10	0.10	0.30	72.00	78.01
8	0.50	0.55	0.05	0.20	0.10	72.00	83.82
9	0.30	0.55	0.10	0.20	0.10	120.00	90.75
10	0.50	0.55	0.05	0.20	0.10	120.00	92.37
11	0.30	0.35	0.10	0.20	0.30	120.00	90.21
12	0.30	0.55	0.05	0.20	0.30	72.00	79.56
13	0.50	0.35	0.10	0.20	0.30	120.00	94.73
14	0.30	0.35	0.10	0.10	0.10	72.00	67.56
15	0.50	0.55	0.05	0.10	0.30	120.00	91.21
16	0.30	0.35	0.05	0.10	0.10	120.00	81.84
17	0.30	0.55	0.10	0.20	0.10	72.00	80.45
18	0.50	0.55	0.05	0.10	0.30	72.00	81.57
19	0.40	0.45	0.08	0.15	0.20	96.00	99.15
20	0.40	0.45	0.08	0.15	0.36	96.00	96.59
21	0.30	0.55	0.05	0.10	0.30	72.00	72.32
22	0.40	0.45	0.04	0.15	0.20	96.00	93.11
23	0.50	0.55	0.05	0.10	0.10	120.00	88.93
24	0.40	0.45	0.08	0.15	0.20	96.00	99.03
25	0.30	0.35	0.10	0.20	0.10	120.00	88.00
26	0.30	0.55	0.10	0.10	0.10	120.00	87.38
27	0.30	0.55	0.10	0.10	0.10	72.00	73.29
28	0.50	0.35	0.05	0.20	0.30	120.00	92.10
29	0.50	0.55	0.10	0.10	0.30	120.00	94.00
30	0.30	0.35	0.05	0.10	0.30	120.00	84.17
31	0.50	0.55	0.05	0.20	0.30	72.00	88.66
32	0.50	0.55	0.10	0.20	0.10	72.00	89.51
33	0.30	0.35	0.05	0.20	0.10	120.00	85.40
34	0.30	0.55	0.05	0.20	0.10	72.00	74.76
35	0.30	0.35	0.10	0.20	0.10	72.00	74.84
36	0.40	0.45	0.11	0.15	0.20	96.00	96.90
37	0.50	0.35	0.10	0.10	0.10	120.00	89.12
38	0.40	0.29	0.08	0.15	0.20	96.00	93.11
39	0.40	0.45	0.08	0.15	0.20	96.00	99.03
40	0.50	0.35	0.10	0.10	0.30	72.00	81.73
41	0.50	0.35	0.05	0.20	0.10	120.00	89.82
42	0.30	0.55	0.10	0.20	0.30	72.00	85.21
43	0.50	0.55	0.10	0.20	0.30	120.00	97.29
44	0.30	0.35	0.05	0.20	0.10	72.00	68.95
45	0.30	0.35	0.05	0.20	0.30	72.00	73.75

(*Continued*)

Table 6c (*continued*)

Run no.	Phosphate (g/L^{-1})	Iron sulfate (g/L^{-1})	Calcium chloride (g/L^{-1})	Magnesium sulfate (g/L^{-1})	Ammonium sulfate (g/L^{-1})	Incubation time (h)	Phenol degradation (%)
46	0.40	0.45	0.08	0.15	0.20	96.00	99.19
47	0.30	0.55	0.05	0.10	0.30	120.00	86.84
48	0.50	0.35	0.05	0.10	0.10	120.00	86.41
49	0.40	0.45	0.08	0.15	0.04	96.00	93.34
50	0.30	0.35	0.10	0.10	0.30	120.00	86.91
51	0.50	0.35	0.10	0.10	0.10	72.00	77.04
52	0.30	0.35	0.10	0.10	0.10	120.00	84.63
53	0.50	0.35	0.05	0.20	0.10	72.00	78.47
54	0.50	0.55	0.10	0.10	0.10	120.00	91.68
55	0.50	0.35	0.05	0.10	0.10	72.00	71.35
56	0.50	0.55	0.10	0.10	0.10	72.00	82.46
57	0.30	0.35	0.05	0.10	0.30	72.00	66.63
58	0.50	0.35	0.10	0.20	0.10	120.00	92.41
59	0.40	0.45	0.08	0.15	0.20	96.00	99.15
60	0.50	0.35	0.10	0.20	0.30	72.00	88.93
61	0.50	0.55	0.10	0.20	0.10	120.00	95.04
62	0.40	0.45	0.08	0.15	0.20	133.56	100.00
63	0.30	0.55	0.05	0.20	0.10	120.00	88.04
64	0.30	0.35	0.05	0.20	0.30	120.00	87.57
65	0.30	0.55	0.05	0.10	0.10	120.00	84.63
66	0.50	0.35	0.05	0.10	0.30	72.00	76.07
67	0.40	0.61	0.08	0.15	0.20	96.00	96.86
68	0.40	0.45	0.08	0.15	0.20	96.00	99.19
69	0.40	0.45	0.08	0.15	0.20	96.00	99.34
70	0.24	0.45	0.08	0.15	0.20	96.00	91.13
71	0.50	0.35	0.10	0.20	0.10	72.00	84.17
72	0.30	0.55	0.05	0.20	0.30	120.00	90.21
73	0.50	0.35	0.05	0.10	0.30	120.00	88.58
74	0.30	0.35	0.10	0.10	0.30	72.00	72.32
75	0.30	0.35	0.10	0.20	0.30	72.00	79.52
76	0.40	0.45	0.08	0.07	0.20	96.00	92.53
77	0.50	0.55	0.05	0.20	0.30	120.00	94.62
78	0.40	0.45	0.08	0.15	0.20	96.00	99.15
79	0.30	0.55	0.10	0.20	0.30	120.00	93.07
80	0.50	0.35	0.10	0.10	0.30	120.00	91.41
81	0.40	0.45	0.08	0.15	0.20	96.00	99.15
82	0.30	0.35	0.05	0.10	0.10	72.00	61.87
83	0.30	0.55	0.10	0.10	0.30	120.00	89.62
84	0.40	0.45	0.08	0.23	0.20	96.00	97.17
85	0.50	0.55	0.05	0.10	0.10	72.00	76.77
86	0.40	0.45	0.08	0.15	0.20	96.00	99.19

signal to noise ratio. A ratio greater than four is desirable. Our ratio of 482.53 indicates an adequate signal. This model can be used to navigate the design space. The coefficient of variation% (CV%) is a measure of residual variation of the data relative to the size of the mean. Usually, the higher the value of CV, the lower is the reliability of experiment. Here, a lower value of CV (0.16%) indicates a greater reliability of the experimental performance. The predicted residual sum of squares (PRESS) is a measure of how well the model

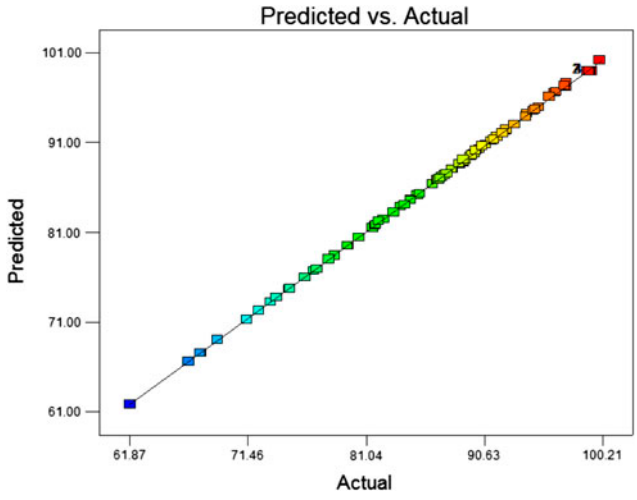

Fig. 1. Diagnostic plots of predicted vs. actual response plot for phenol degradation by *A. faecalis*.

fits each point in the design. The smaller, the PRESS statistic, the better the model fits the data points. Our value of PRESS is 3.11.

An experiment value vs. predicted values (shown in Fig. 1) also represents a high degree of similarity that was observed between the predicted and experimental values. It is evident from Fig. 1 that proposed model satisfies the variance requirement and these also reflect accuracy and applicability of RSM for process optimization of phenol degradation. Perturbation plot in Fig. 2 represents comparison of the

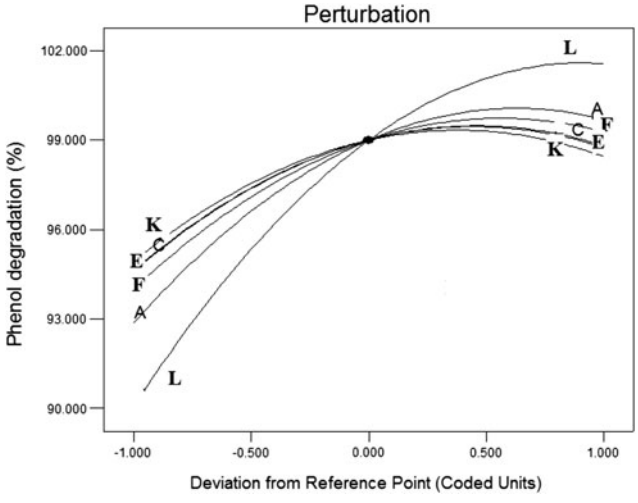

Fig. 2. Perturbation plot of phenol degradation by *A. faecalis* as a function of phosphate concentration (A), iron sulfate concentration (C), calcium chloride concentration (D), magnesium chloride concentration (F), incubation time (H), and ammonium sulfate (J).

effect of culture components at the midpoint (coded 0) in the design space. A curvature curve was found for all factors, which indicate the response is sensitive to all factors.

The special features of the RSM tool is 3D response surface curve, contour plot generation, and point prediction from where we can determine the optimum value of the combination of the important factors for the maximum phenol degradation. The contour plot (Fig. 3(a)–(e)) determines the interaction of the factors and optimum concentration of each component for maximum response. These plots were obtained from the pair-wise combination of independent factors, while keeping other factor at its center point level. From the elliptical contour plot (shown in Fig. 3(a)–3(e)), it is clear that the mutual interaction is prominent among factors. The proposed model predicts maximum phenol degradation from point prediction, at 0.42 g L^{-1} phosphate, 0.46 g L^{-1} iron sulfate, 0.08 g L^{-1} calcium chloride, 0.18 g L^{-1} magnesium chloride, ammonium sulfate 0.25 g L^{-1}, and incubation time 90.8 h. The maximum predictable response was calculated using regression equation by substituting level of factors and was experimentally verified.

The model as given by Eq. (1) was validated for all six variables within the design space. A random set of six production combinations was prepared and tested for phenol degradation. The results are given in Table 7. The experimentally determined production values were in close agreement with the statistically predicted ones, confirming the authenticity of the proposed model. From the validation experiment, result showed that 100% (2,100 mg L^{-1}) phenol degradation can be possible using optimized media composition viz. 0.42 g L^{-1} phosphate, 0.46 g L^{-1} iron sulfate, 0.08 g L^{-1} calcium chloride, 0.18 g L^{-1} magnesium chloride, and ammonium sulfate 0.25 g L^{-1} after 90.8 h incubation.

This has been found to have potential capacities to grow in high phenol containing simulated inorganic media where the only carbon source is phenol. The result indicates that the degradation of phenol is significant by this *A. faecalis*. It can degrade phenol up to 2,100 mg L^{-1}completely in 90 h at pH of 7.4 and temperature of 37˚C in a BOD incubator shaker with 120 rpm. The extent of phenol degradation and tolerance by *A. faecalis* have been found more significant when compared to other micro-organism-mediated phenol degradation like *Pseudomonas Putida, Candida tropicalis* [28], *Candida maltose* [29], and *Trichosporon dulcitum* [30].

Fig. 4 reveals that up to 2,100 mg/L phenol is consumed by the microbes as sole carbon source in the presence of optimized inorganic media

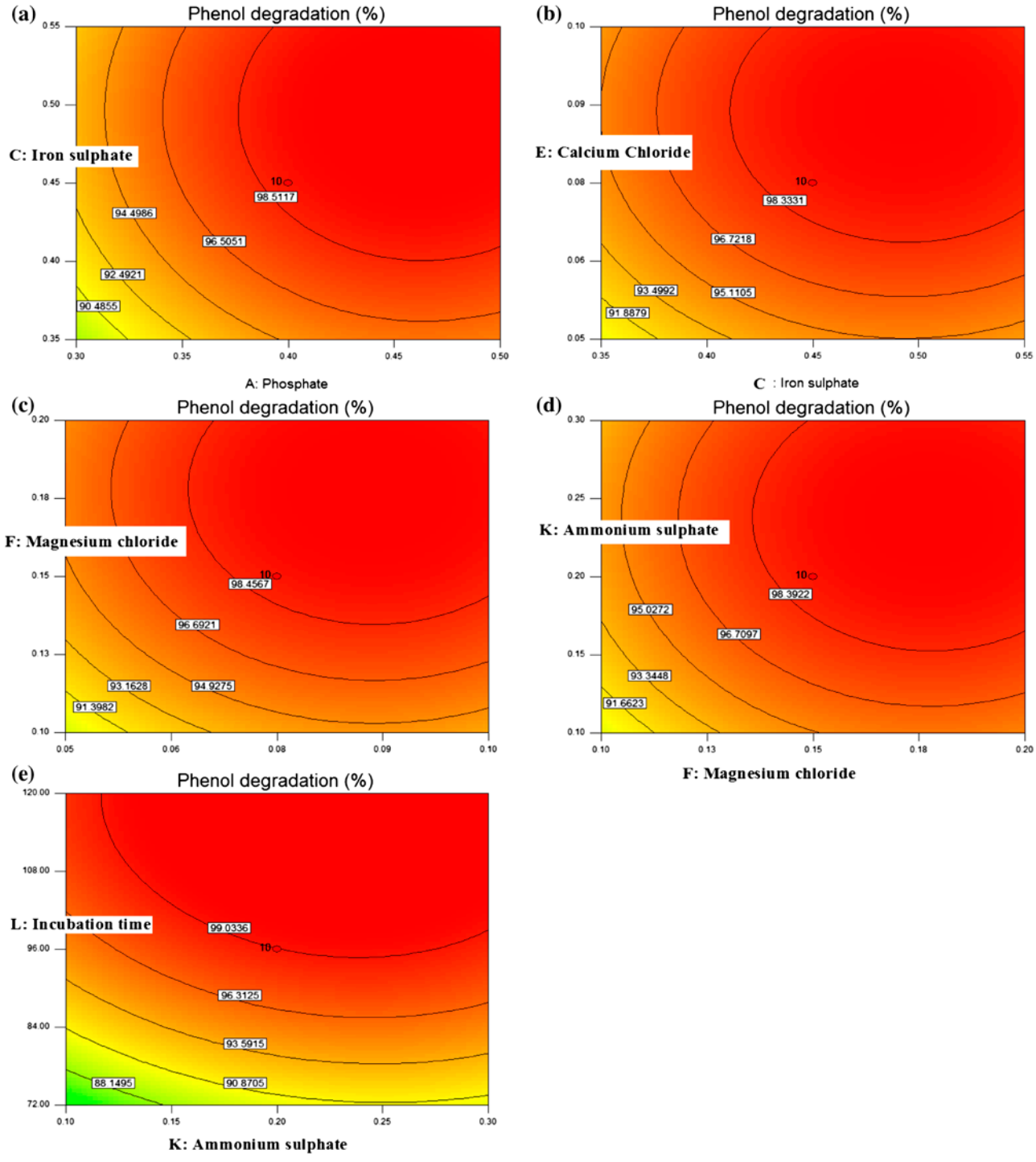

Fig. 3. Contour plot for phenol degradation by *A. faecalis* as a function of (a) of phosphate (A) and iron sulfate (C), (b) iron sulfate (C) and calcium chloride (E), (c) calcium chloride (E) and magnesium chloride (F), (d) magnesium chloride (F) and ammonium sulfate (K) and (e) incubation time (L), and ammonium sulfate (K), when other variables are at zero level.

composition. Thus, specific growth rate has been enhanced. Further, with increasing the phenol concentration the specific growth rate is decreasing due to toxic effect of phenol in its higher concentration. This phenomenon is observed, probably due to inhibitory effect of phenol.

Table 7
Validation of quadratic model within the design space

Number	Phosphate ($g\,L^{-1}$)	Iron sulfate ($g\,L^{-1}$)	Calcium chloride ($g\,L^{-1}$)	Magnesium chloride ($g\,L^{-1}$)	Ammonium sulfate ($g\,L^{-1}$)	Incubation time (h)	Actual response (%)	Predict response (%)
1	0.3	0.45	0.08	0.15	0.18	96	95.92	92.78
2	0.35	0.45	0.1	0.15	0.18	120	99.18	98.57
3	0.35	0.45	0.1	0.15	0.2	84	94.66	92.85
4	0.35	0.45	0.1	0.2	0.2	72	93.16	88.88
5	0.35	0.45	0.1	0.25	0.1	72	78.06	84.22
6	0.42	0.46	0.08	0.18	0.25	90.8	100	100

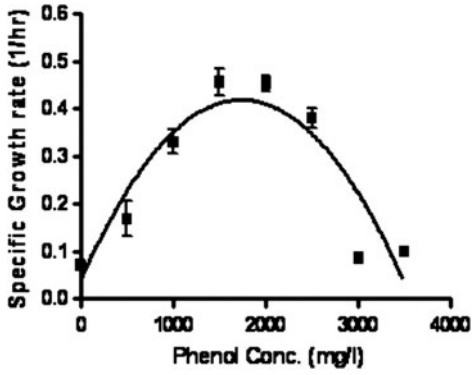

Fig. 4. Specific growth rate (1/h) vs. phenol concentration (mg/L) under optimized medium.

4. Conclusions

The present study focuses on optimization of medium compositions along with incubation time for maximum phenol degradation through microbial fermentation. It is felt that such study is of utmost important for obtaining higher degree of biodegradation of phenol as well to reduce operating cost of the process. Out of the 11 variables investigated using the Plackett–Burman design, it appears that six variables namely phosphate concentration, ferrous sulfate concentration, calcium chloride concentration, magnesium chloride concentration, Incubation time, and ammonium sulfate have significant effects on phenol degradation. Optimization of these six variables has been carried out by using RSM experiments which eventually involved application of CCD. The mathematical analysis has been carried out by standard software and the procedure followed is user friendly. The results found for optimal media composition in *A. faecalis*-mediated high concentration phenol degradation could be suitably used for bioreactor designing for cost effective wastewater treatment plant.

References

[1] Z. Aleksieva, D. Ivanova, T. Godievargova, B. Atanasov, Degradation of some phenol derivatives by *Trichosporon cutaneum* R57, Process Biochem. 37 (2002) 1215–1219.
[2] A. Kumar, S. Kumar, S. Kumar, Biodegradation kinetics of phenol and catechol using *Pseudomonas putida* MTCC 1194, Biochem. Eng. J. 22 (2005) 151–159.
[3] N.M. Arif, S.A. Ahmad, M.A. Syed, M.Y. Shukor, Isolation and characterization of a phenol-degrading Rhodococcus sp. strain AQ5NOL 2 KCTC 11961BP, J. Basic Microbiol. (2012).
[4] L. Wang, Y. Li, P. Yu, Z. Xie, Y. Luo, Y. Lin, Biodegradation of phenol at high concentration by a novel fungal strain *Paecilomyces variotii* JH6, J. Hazard. Mater. 183 (2010) 366–371.
[5] M. Begona Prieto, A. Hidalgo, J.L. Serra, M.J. Llama, Degradation of phenol by *Rhodococcus erythropolis* UPV-1 immobilized on biolite in a packed-bed reactor, J. Biotechnol. 97 (2002) 1–11.
[6] W.J. Middelhoven, Catabolism of benzene compounds by ascomycetous and basidiomycetous yeasts and yeastlike fungi. A literature review and an experimental approach, Antonie Leeuwenhoek 63 (1993) 125–144.
[7] V.L. Santos, V.R. Linardi, Phenol degradation by yeasts isolated from industrial effluents, J. Gen. Appl. Microbiol. 47 (2001) 213–221.
[8] K.T. Semple, R.B. Cain, Biodegradation of phenols by the alga *Ochromonas danica*, Appl. Environ. Microbiol. 62 (1996) 1265–1273.
[9] V.L. Santos, N.M. Heilbuth, D.T. Braga, A.S. Monteiro, V.R. Linardi, Phenol degradation by a Graphium sp. FIB4 isolated from industrial effluents, J. Basic Microbiol. 43 (2003) 238–248.
[10] M. Alexander, Biodegradation and Bioremediation, second ed., Academic Press, San Diego, CA, 1999.
[11] R.L. Greasham, Biotechnology, in: H.J. Rehm, G. Read, A. Puhler, P. Stagler (Eds), Bioprocessing, VCH, New York, NY, pp. 128–139, 1983.
[12] M.A. Bezerra, R.E. Santelli, E.P. Oliveira, L.S. Villar, L.A. Escaleira, Response surface methodology (RSM) as a tool for optimization in analytical chemistry, Talanta 76 (2008) 965–977.
[13] C.M.L. Carvalho, M.L.M. Serralheiro, J.M.S. Cabral, M.R. Airebarros, Application of factorial design to the study of transesterification reactions using cutinase in AOT-reversed micelles, Enzyme Microb. Technol. 21 (1997) 117–123.
[14] J. Li, C. Ma, Y. Ma, Y. Li, W. Zhou, P. Xu, Medium optimization by combination of response surface methodology and desirability function: An application in glutamine production, Appl. Microbiol. Biotechnol. 74 (2007) 563–571.
[15] M.R.S. Srinivas, N. Chand, B.K. Lonsane, Use of Plackett–Burman design for rapid screening of several nitrogen sources, growth/product promoters, minerals and enzyme inducers for the production of alpha-galactosidase by *Aspergillus niger* MRSS 234 in solid state fermentation system, Bioprocess Biosyst. Eng. 10 (1994) 139–144.

[16] Z.J. Xiao, P.H. Liu, J.Y. Qin, P. Xu, Statistical optimization of medium components for enhanced acetoin production from molasses and soybean meal hydrolysate, Appl. Microbiol. Biotechnol. 74 (2007) 61–68.
[17] S.L. Ferreira, R.E. Bruns, E.G. da Silva, W.N. Dos Santos, C.M. Quintella, J.M. David, J.B. de Andrade, M.C. Breitkreitz, I.C. Jardim, B.B. Neto, Statistical designs and response surface techniques for the optimization of chromatographic systems, J. Chromatogr. A 1158 (2007) 2–14.
[18] D. Hank, N. Saidani, A. Namane, A. Hellal, Batch phenol biodegradation study and application of factorial experimental design, J. Eng. Sci. Technol. Rev. 3 (2010) 123–127.
[19] S.E. Agarry, B.O. Solomon, S.K. Layokun, Optimization of process variables for the microbial degradation of phenol by *Pseudomonas aeruginosa* using response surface methodology, Afr. J. Biotechnol. 7 (2008) 2409–2416.
[20] H.A. Berkhoff, G.D. Riddle, Differentiation of Alcaligenes-like bacteria of avian origin and comparison with Alcaligenes spp. reference strains, J. Clin. Microbiol. 19 (1984) 477–481.
[21] H. Salehizadeh, S. Mohammadizad, Microbial enhanced oil recovery using biosurfactant produced by *Alcaligenes faecalis*, Iran. J. Biotechnol. 7 (2009) 216–223.
[22] S.A. Miller, D.D. Dykes, H.F. Polesky, A simple salting out procedure for extracting DNA from human nucleated cells, Nucleic Acids Res. 16 (1988) 1215.
[23] X. Dong, Q. Hong, L. He, X. Jiang, S. Li, Characterization of phenol-degrading bacterial strains isolated from natural soil, Int. Biodeterior. Biodegrad. 62 (2008) 257–262.
[24] Y.L. Guo, C. P., B.X. W., M.Z. G., M. Z., Y. L.Y., J. KE, C. H., Simultaneous removal of Cr(VI) and phenol in consortium culture of *Bacillus sp.* and *Pseudomonas putida MIgula*, Transactions of Nonferrous Meatal Society of China, 18 (2008) 1014–1020.
[25] M.M. Martin, J.A. Perez, F.G. Fernandez, J.L. Sanchez, J. L. Lopez, S.M. Rodriguez, A kinetics study on the biodegradation of synthetic wastewater simulating effluent from an advanced oxidation process using *Pseudomonas putida* CECT 324, J. Hazard. Mater. 151 (2008) 780–788.
[26] R.K. Singh, S. Kumar, S. Kumar, A. Kumar, Biodegradation kinetic studies for the removal of p-cresol from wastewater using gliomastix indicus, Biochem. Eng. J. 40 (2007) 293–303.
[27] S. Jagannath, K.B. Ramachandran, Influence of competing metabolic processes on the molecular weight of hyaluronic acid synthesized by *Streptococcus zooepidemicus*, Biochem. Eng. J. 48 (2010) 148–158.
[28] A.E. Bastos, D.H. Moon, A. Rossi, J.T. Trevors, S.M. Tsai, Salt-tolerant phenol-degrading microorganisms isolated from Amazonian soil samples, Arch. Microbiol. 174 (2000) 346–352.
[29] A. Fialova, E. Boschke, T. Bley, Rapid monitoring of the biodegradation of phenollike compounds by the yeast *Candida maltosa* using BOD measurements, Int. Biodeterior. Biodegrad. 54 (2004) 69–74.
[30] R. Margesin, P.A. Fonteyne, B. Redl, Low-temperature biodegradation of high amounts of phenol by Rhodococcus spp. and basidiomycetous yeasts, Res. Microbiol. 156 (2005) 68–75.