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Full Length Research Paper

Optimization of process variables for the microbial degradation of phenol by *Pseudomonas aeruginosa* using response surface methodology

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Removal efficiency of phenol from aqueous solutions was measured using a freely suspended monoculture of indigenous *Pseudomonas aeruginosa.* Experiments were performed as a function of temperature (25– 45°C), aeration (1.0 – 3.5 vvm) and agitation (200 – 600 rpm). Optimization of these three process parameters for phenol biodegradation was studied. Statistically designed experiments using response surface methodology was used to get more information about the significant effects and the interactions between the three parameters. A 2^3 full-factorial central composite designed followed by multistage Monte-Carlo optimization technique was employed for experimental design and analysis of the results. The optimum process conditions for maximizing phenol degradation (removal) were recognized as follows: temperature 30.1°C, aeration 3.0 vvm, and agitation 301 rpm. Maximum removal efficiency of phenol was achieved (94.5%) at the optimum process conditions.

Key words: *Pseudomonas aeruginosa*, phenol, biodegradation, regression model, statistical optimization.

INTRODUCTION

Phenol and its derivatives are the basic structural unit in a wide variety of synthetic organic compounds (Annadurai et al., 2000). It is an organic, aromatic compound that occurs naturally in the environment (Prpich and Daugulis, 2005), but is more commonly produced artificially from industrial activities such as petroleum processing, plastic manufacturing, resin production, pesticide production, steel manufacturing and the production of paints and varnish (Mahadevaswamy et al., 1997; Banyopadhyay et al., 1998). This aromatic compound is water-soluble and highly mobile (Collins and Daugulis, 1997) and as such waste waters generated from these industrial activities contain high concentrations of phenolic compounds (Chang et al., 1998) which eventually may reach down to streams, rivers, lakes, and soil, which represent a serious ecological problem due to their widespread use and occurrence throughout the environment (Fava et al., 1995).

Phenol is a listed priority pollutant by the U.S. Environmental Protection Agency (EPA, 1979) and is considered to be a toxic compound by the Agency for Toxic substances and Disease Registry (ATSDR, 2003). The adverse effects of phenol on health are well documented (Calabrese and Kenyon, 1991) and death among adults has been reported with ingestion of phenol ranging from 1 to 32 g (Prpich and Daugulis, 2005). The low volatility of phenol and its affinity for water make oral consumption of contaminated water the greatest risk to humans (Prpich and Daugulis, 2005).

A variety of techniques have been used for the removal of phenol from industrial effluents and contaminated waters with bioremediation receiving the most attention due to its environmentally friendly, its, ability to completely mineralize toxic organic compounds and of low-cost (Kobayashi and Rittman, 1982; Prpich and Daugulis, 2005). Microbial degradation of phenol has been actively

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Table 1. Experimental range and the levels of the variables.

Dependent variable	- α	- 1	0	+ 1	+ α
Temperature (X_1), ^oC	21.6	25	30	35	38.4
Aeration (X_2), vvm	2.16	2.5	3.0	3.5	3.84
Agitation (X3), rpm	132	200	300	400	568

studied and these studies have shown that phenol can be aerobically degraded by wide variety of fungi and bacteria cultures such as *Candida tropicalis* (Ruiz-ordaz et al., 2001; Chang et al., 1998; Ruiz-ordaz et al., 1998), *Acinetobacter calcoaceticus* (Paller et al., 1995), *Alcaligenes eutrophus* (Hughes et al.,1984; Leonard and Lindley, 1998), *Pseudomonas putida* (Hill and Robinson, 1975; Kotturi et al., 1991; Nikakhtari and Hill, 2006) and *Burkholderia cepacia* G4 (Folsom et al., 1990; Solomon et al.,1994).

It has been demonstrated that treatment of small volumes of toxic compounds at the point of emission using specific microbial strains and better bioreactors allows a higher control over the process and higher removal efficiencies (Schroder et al., 1997). Thus optimization of process variables is recognized to be an essential aspect of successful fermentation (Sivakumar, 1995). The high dependence of enzymatic activity and cellular maintenance requirements on temperature makes it an important quantity.

Temperature exerts an important regulatory influence on the rate of metabolism (Ghosh and Swaminathan, 2003). The effects of oxygen supply vary from species to species (Onken and Liefke, 1989). In addition, oxygen mass transfer is aided by agitation rates that create turbulence and shear forces in the cultivation system causing significant influences on the growth rate and product formation (Hoq et al., 1995).

This work examines the effect of temperature, aeration, and agitation on the degradation of phenol by local strains of *Pseudomonas aeruginosa* using both the classical method of optimization that involves varying the level of one parameter at a time over a certain range while holding the rest of the variables constant and statistical optimization technique for multivariable effect.

MATERIALS AND METHODS

Microorganism

The microorganism *P. aeruginosa* being an indigenous strain isolated from an oil-polluted area of the Niger-Delta region of Nigeria was procured from the Department of Microbiology, Obafemi Awolowo University, Ile-ife, Nigeria. The microorganism was maintained on nutrient agar slant and stored at 4 ± 1^oC for further use.

Culture medium and inoculum preparation

The mineral salt medium used was modified from the one suggested by Bettman and Rehm (1984). The medium had the following composition per litre: 700 ml deionized water, 100 ml buffer solution A, 100 ml trace elements solution B, 50 ml solution C and 50 ml solution D. Compositions of each solution were as follows: Buffer solution A composition; K_2HPO_4 1.0 g , KH_2PO_4 0.5 g, $(NH_4)_2SO_4$ 0.5 g, deionized water 100 ml. Trace element solution B composition; NaCl 0.5 g, $CaCl_2$ 0.02 g , $MnSO_4$ 0.02 g, $CuSO_4.5H_2O$ 0.02 g, H_3BO_3 0.01 g, deionized water 50 ml. Solution C composition; $MgSO_4.7H_2O$ 0.5 g, deionized water 50 ml. Solution D composition; $FeSO_4$ 0.02 g, molybdenum powder 0.02 g, deionized water 50 ml. To prevent the precipitation of $CaSO_4$ and $MgSO_4$ in storage, the water, buffer solution A, trace elements solution B, solution C and solution D were autoclaved at 121^oC for 15 min. After cooling, all the solutions were then mixed together and kept as stock solution from which known quantities were taken for the cultivation of the microorganisms

A primary culture was prepared by transferring two loops full of microorganisms from an agar slant culture into 100 ml of feed medium containing 20 ml of mineral salt medium and 80 ml of 50 mg phenol solution in a 250 ml Erlenmeyer conical flask. This was then incubated in a NewBrunwick gyratory shaker (G25-R model, N.J. U.S.A) for 48 h at a temperature of 30^oC and agitated with a speed of 120 rpm. Thereafter, 10 ml of the primary culture was transferred into another 100 ml of feed medium in a 250 ml Erlenmeyer conical flask and the incubation process was repeated. This was the secondary culture that was used as the inoculum for the degradation studies as this ensures that the organisms had fully adapted to growth on the phenol as sole source of carbon and energy.

Optimization studies

To optimize the range of experimentation for the 2^3 full-factorial central composite design, the following experiments were carried out in a NewBrunswick Microferm Twin Bioreactor (PH – 22 model, N.J., U.S.A) with 4 litres working volume. 800 ml of the autoclaved mineral salt medium and 3 litres of phenol solution (100 mg/l) were measured into the bioreactor vessel and 200 ml of the inoculum was introduced asceptically to make up 4 litres of working volume. The bioreactor was operated for 48 h at different temperature (25, 30, 35, 40 and 45^oC); aeration (1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 vvm); and agitation rates (200, 300, 400, 500 and 600 rpm). Culture broth was withdrawn at every 6 h for phenol determination. From the above experiments, the range of temperature, aeration and agitation rates were chosen for further optimization of the design.

Design of experiments

The range and the levels of the process variables under study are given in Table 1: temperature (25, 30 and 35^oC); aeration (2.5, 3.0 and 3.5 vvm) and agitation (200, 300 and 400 rpm) which served as critical variables X_1, X_2, and X_3, respectively. The actual design of experiments is given in Table 2 for each experiment that was carried out in a NewBrunswick Microferm Twin Bioreactor containing 800 ml mineral salt medium, 3 litres of phenol solution (100 mg/l) and 200 ml of inoculum which was operated for 48 h. Samples were withdrawn after 48 h for phenol determination.

Phenol determination

The under graded phenol was estimated quantitatively by the spectrophotometric method using 4 – amino antipyrene as colour indicator (Yang and Humphrey, 1975) at an obsorbance of 510 nm.

Response surface methodology and statistical optimization

A full – factorial central composite design using response surface

Table 2. Coded and uncoded full-factorial central composite design for the three independent variables.

Experiment number	Temperature		Aeration		Agitation	
	Code	Value(^oC)	Code	Value(vvm)	Code	Value(rpm)
1	-1	25	-1	2.5	-1	200
2	+1	35	-1	2.5	-1	200
3	-1	25	+1	3.5	-1	200
4	+1	35	+1	3.5	-1	200
5	-1	25	-1	2.5	+1	400
6	+1	35	-1	2.5	+1	400
7	-1	25	+1	3.5	+1	400
8	+1	35	+1	3.5	+1	400
9	-1.682	21.6	0	3.0	0	300
10	+1.682	38.4	0	3.0	0	300
11	0	30	-1.682	2.16	0	300
12	0	30	+1.682	3.84	0	300
13	0	30	0	3.0	-1.682	132
14	0	30	0	3.0	+1.682	568
15	0	30	0	3.0	0	300
16	0	30	0	3.0	0	300
17	0	30	0	3.0	0	300
18	0	30	0	3.0	0	300
19	0	30	0	3.0	0	300
20	0	30	0	3.0	0	300

methodology (Khuri and Cornell, 1987; Montgomery, 1991; Ghosh and Swaminathan, 2003) is a powerful tool for understanding complex processes. The full-factorial central composite design consists of (a) a complete 2^k factorial design where k(= 3) is the number of tests variables, (b) n_o center points ($n_o > 1$) and (c) two axial points on the axis of each design variables at a distance of α ($\alpha = 2^k/4 = 1.682$ for k = 3) from the design center (Khuri and Cornell, 1987; Ghosh and Swaminathan, 2003). Hence, the total number of design points is $N = 2^k + 2k + n_o$, and these data are fitted in a second order polynomial model (Ghosh and Swaminathan, 2003). An orthogonal 2^3 full-factorial central composite design with six replicates ($n_o = 6$) at the central point, all in duplicates resulting in total of 20 experiments were used to optimize the chosen key variables that have effect on phenol microbial degradation are given in Table 1. The variables were coded according to the equation 1 (Box and Behnken, 1960; Box and Draper, 1959; Annadurai et al., 2000).

$$X_i = (x_i - x_o)/\Delta x_i \quad (1)$$

Where X_i, x_i and x_o are the coded value, uncoded value and the value at the center point respectively of the i^{th} test variable and Δx_i is the step change value. The full experimental design in coded and uncoded form is given in Table 2. The behaviour of the system was explained by the following second order polynomial equation:

$$Y = \beta_o + \sum \beta_i X_i + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_{ij} \quad (2)$$

Where Y = predicted response, β_o = offset term, β_i = linear effects, β_{ii} = quadratic effects, and β_{ij} = interaction effects. X_i (i = 1, 2, 3) and X_j (j = 1, 2, 3)($i \neq j$) are the process variables.

The graphical representation of these equations are called response surfaces which was used to describe the individual and cumulative effects of the test process variables on the response and to determine the mutual interactions between the test variables and their subsequent effect on the response (Ghosh and Swaminathan, 2003).

A statistical program package, Design Expert (Start-Ease Inc, Minneapolis, MN) was used for regression analysis of the data obtained and to estimate the coefficient of the polynomial equation.

RESULTS AND DISCUSSION

The effect of aeration rate, agitation rate and temperature on phenol degradation by indigenous (local strains) *Pseudomonas aeruginosa* is as shown in Figures 1, 2 and 3, respectively. From Figure 1 it could be seen that the rate of degradation (percent degradation) increased with increase in aeration up till an aeration rate of 3.0 vvm, above which it decreased. In this work, it was observed that biomass concentration increased up till an aeration rate of 3.0 vvm above that it decreased. The increase in degradation rate may be due to the fact that as aeration rate increases more dissolved oxygen (i.e. higher mass transfer) was made available for the metabolism of the organism while the decrease may be due to toxic effect of the excess air (oxygen). Oboirien et al. (2005) and Hannaford and Kuek (1999) also gave a similar report that biodegradation rate increased with increase in aeration rate when they considered an aeration rate of 1.5 to 2.5 vvm and 4 to 16 vvm, respectively. Oboirien and his co-workers used freely suspended *P. aeruginosa* NCIB 950 and a NewBruns-

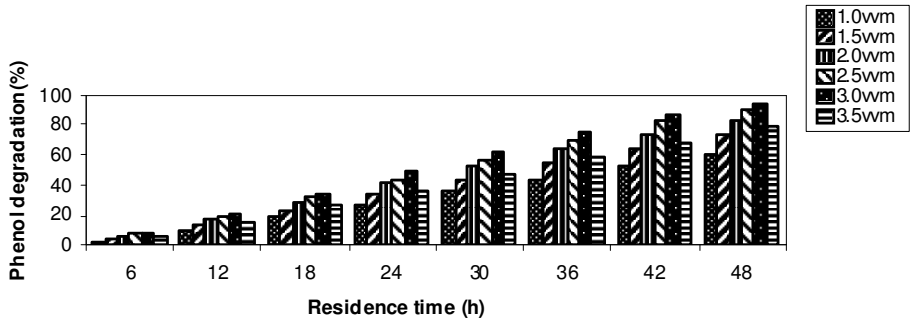

Figure 1. Effect of aeration rate on phenol degradation by *P. aeruginosa*.

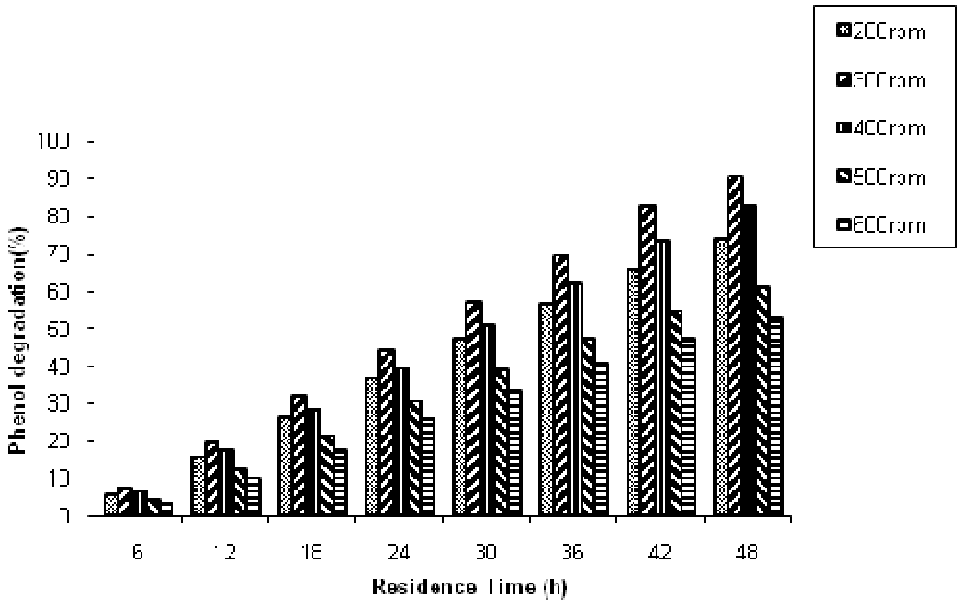

Figure 2. Effect of agitation rate on degradation of phenol by *P. aeruginosa*.

wick Microferm Twin Fermentor for their studies, while Hannaford and Kuek used an immobilized *Pseudomonas putida* ATCC 11172 and a bubble column reactor for their studies. Collins and Daugulis (1997) reported that aeration rate greater than 0.5 vvm produced excessive foaming and led to solvent and cell losses. In this work, no foaming was observed and this is in agreement with the observation of Oboirien et al. (2005).

From Figure 2, it could be seen that the rate of phenol degradation increased from an agitation rate of 200 to 400 rpm and above 400 rpm the rate decreased. In this work, it was observed that the biomass concentration increased up till an agitation rate of 400 rpm above that there was a decrease in biomass concentration. Sobczuk et al. (2006) reported a similar observation. The increase in biodegradation rate may be due to adequate high mass transfer thus allowing more oxygen to be dissolved and made available for the metabolism of the organism. While, the decrease may be due to higher shear stress effect thus leading to cell loss or lower biomass concentration (Hoq et al., 1995). However, the optimum agitation rate was found to be 300 rpm at which the percent degradation was highest.

From Figure 3, it could be seen that the phenol degradation rate (percent degradation) increased with increase in temperature up to 40°C above which, it decreased. It was observed that the biomass concentration increased up till 40°C above that it decreased. The decrease in the rate of degradation may be due to decrease in the effective reactivity of the multi-enzyme complex system within the cell (Bandyopadhyay et al., 1998). A similar observation was reported by Bandyopadhyay et al. (1998) that biodegradation rate of phenol by Pseudomonas putida MTCC 1194 increased with increase in temperature from 15 to 30°C above which it decreased. However, from the same Figure 3, it could be observed

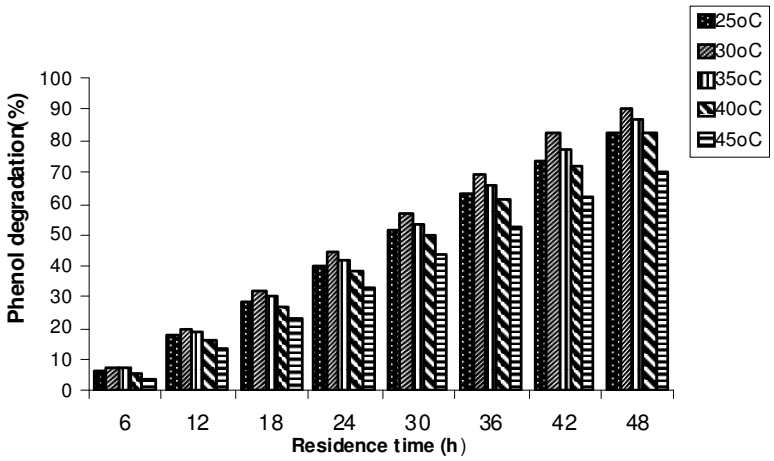

Figure 3. Effect of temperature on phenol degradation by *P. aeruginosa*.

Table 3. Experimental and theoretically predicted values for degradation of phenol by *Pseudomonas aeruginosa*.

Run	Experimental value %	Predicted value %
1	66.4	71.3
2	70.2	74.1
3	60.7	65.8
4	62.3	67.9
5	77.6	76.4
6	80.4	79.8
7	71.1	71.6
8	74.7	74.3
9	82.5	79.2
10	86.8	83.8
11	84.1	82.1
12	76.9	72.8
13	67.6	58.1
14	64.3	67.7
15	94.2	94.5
16	94.4	94.5
17	94.5	94.5
18	94.6	94.5
19	94.5	94.5
20	94.6	94.5

that the highest percent degradation was at a temperature of 30°C and thus represent the optimum temperature.

Optimization of temperature, aeration and agitation using response surface methodology

The quantitative description of the process variables (physical condition) effects on phenol microbial degradation was performed. The optimum level of aeration, agitation, and temperature are as shown in Figures 1, 2, and 3. Aeration (2.5, 3.0 3.5 vvm), agitation (200, 300, 400 rpm) and temperature (25, 30, 35°C) were optimized using the 2^3 full factorial central composite design of experiments. Response surface methodology is an empirical modeling technique involved in the evaluation of the relationship of a set of controlled experimental factors and observed results (Annadurai et al., 2000). The average percent phenol degraded at each fermentation run is summarized and presented in Table 3 along with predicted values. The experimental data were fitted to a second order polynomial regression model containing 3 linear, 3 quadratic and 3 interaction terms using the same experimental design software. The regression equation obtained after analysis of variance gives the level of degradation of phenol as a function of the different process variables: aeration, agitation, and temperature. All terms regardless of their significance are included in the following equation:

$$Y = 94.49 + 1.39X_1 - 2.79X_2 + 2.86X_3 - 4.60X_1^2 - 6.04X_2^2 - 11.19X_3^2 - 0.18X_1X_2 + 0.13X_1X_3 + 0.18X_2X_3 \quad (3)$$

Where X_1, X_2 and X_3 represent coded values of temperature, aeration and agitation respectively and Y is the response variable (maximum degradation of phenol)

The optimum coded and uncoded value was obtained by solving equation 3 analytically. The optimum coded and uncoded value of temperature was found to be 0.02 and 30.1°C, respectively, while for aeration, it was obtained as –0.01 and 3.0 vvm, respectively, and for agitation, it was found to be 0.01 and 301 rpm respectively at maximum percentage of phenol degradation 94.6%. The optimum temperature for the biodegradation of phenol has generally been reported to be 30°C (Bandyopadhyay et al., 1998; Polymenakou and Stephanou, 2005).

The analysis of variance (ANOVA) of the regression

Table 4. Regression analysis for the degradation of phenol by *P.aeruginosa,* quadratic response surface model fitting (ANOVA).

Source	Sum of squares	DF	Mean square	F_{-value}	P_{-value}
Model	24.76	9	2.75	6.71	0.0001
Residual (error)	4.1	10	0.41		
Correlation total	28.86	19			
R^2 = 0.8579	Adj R^2 = 0.7300				

Table 5. Coefficient of the model for *P. aeruginosa.*

Variables	Coefficient value	Standard error	t-value	p-value
β_0	94.49	2.61	36.20	-
β_1	1.39	1.73	0.80	0.096
β_2	-2.79	1.73	-1.61	0.154
β_3	2.86	1.73	1.65	0.127
β_{11}	-4.60	1.31	-3.51	0.004
β_{22}	-6.04	1.31	-4.61	0.001
β_{33}	-11.19	1.31	-8.55	0.000
β_{12}	-0.18	2.25	-0.08	0.910
β_{13}	0.13	2.25	0.06	0.142
β_{23}	0.18	2.25	0.08	0.146

model demonstrates that the model is highly significant (Table 4), as is evident from Fisher F_{test} (F_{model} = 6.71) and a very low failure probability (P = 0.0001). Moreover, the computed F value ($F_{0.01(9,10)}$ = 6.71) was greater than the tabular F value (F = 4.94) at the 1% level indicating that the treatment differences are highly significant. The value of R (= 0.9262) indicates a high degree of correlation between the observed value and predicted values. The value of the determination coefficient (R^2 = 0.8579) being a measure of goodness of fit to the model indicates that only about 14% of the total variations are not explained by the model. The coefficient of variation (CV) indicates the degree of precision with which the treatment is compared. Usually the higher the value of CV, the lower is the reliability of the experiment (Ghosh and Swaminathan, 2003). Here, the low value of CV (= 8.04%) indicates a greater reliability of the experiment performed.

The coefficient of the model (parameter estimation) and the corresponding P-values (Table 5) suggest that among the test variables, linear and quadratic effect of aeration and agitation are highly significant. The quadratic effect of temperature was more pronounced than linear effect. These observations can be interpreted as a consequence of proportional relationship between the variables and phenol degradation. The mutual effects of temperature, aeration and agitation are of equal significance. This data analysis also substantiates the inference that can be drawn from three-dimensional contour plots (3-D graphics) as shown in Figures 4, 5, and 6, respectively. The

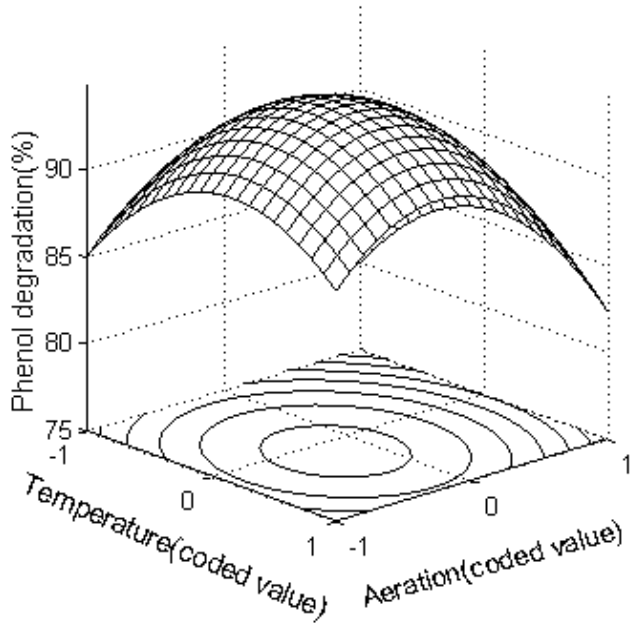

Figure 4. Phenol degradation by *P. aeruginosa* on 3-D graphics for response surface optimization vs temperature and aeration.

interactions amongst temperature, aeration and agitation are quite prominent from the elliptical nature of the respective contour plots. These Figures also suggest the optimum range of the process variables.

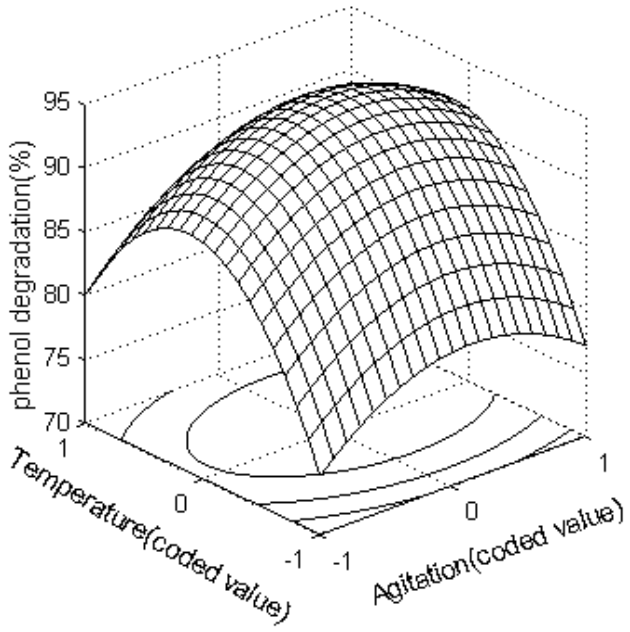

Figure 5. Phenol degradation by *P. aeruginosa* on 3-D graphics for response surface optimization vs temperature and agitation.

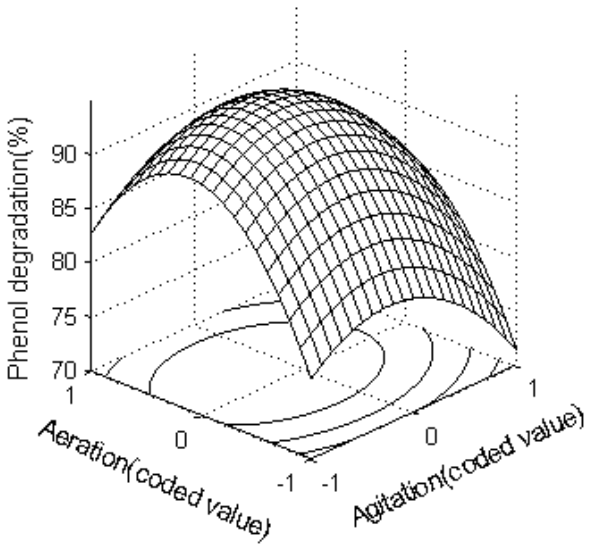

Figure 6. Phenol degradation by *P. aeruginosa* on 3-D graphics for response surface optimization vs aeration and agitation.

Factor plot

The factor effect function plot (Figure 7) was used to assess the effect of each factor graphically. From the trace plot as shown in Figure 7, it can be seen that each of the three variables used in the present study has its individual effect on phenol biodegradation *P. aeruginosa.*

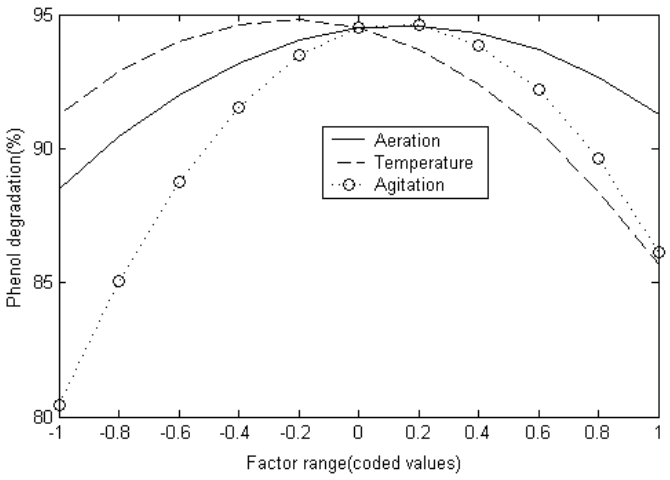

Figure 7. Factor plot representing the individual variables effect on phenol degradation of *P. aeruginosa.*

Gradual increase in temperature, aeration and agitation rates from low level (coded value –1) to a higher level (coded value +1) resulted in both increase and decrease of phenol degradation. Moreover, it is also to be noted from Figure 7 that over the range of agitation (200 to 400 rpm) the phenol degradation changed in a wide range, which was not the case for temperature and aeration. This clearly indicates that keeping temperature and aeration at the optimum level a change in agitation affects the process more severely than done otherwise. The optimum values were found by solving the regression equation analytically. The highest phenol degradation that can be achieved according to the model prediction under the optimal experimental conditions is 94.5%. The experimental results indicated a degradation of about 94.6% under the optimal process conditions. This confirms the closeness of the model to the experimental results.

Conclusions

The present study shows the potential of the isolated indigenous *P. aeruginosa* for phenol wastewater treatment. The performance of the indigenous strain in biodegradation of phenol in the nutrient medium is excellent. The response surface methodology using 2^3 full-factorial composite design was adopted to optimize the process variables like temperature, aeration and agitation for the microbial degradation of phenol using a mechanically agitated bioreactor. The design generated may be used for designing a treatment plant for phenol waste effluents where collection can be achieved on a large scale. The statistical analyses and the closeness of the experimental results and model predictions show the reliability of the regression model.

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