Statistical optimization of process variables by response surface methodology to enhance phenol degradation by *Pseudomonas putida* (NCIM 2102)

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ABSTRACT

Removal efficiency of phenol from aqueous solutions was determined using Pseudomonas putida (NCIM 2102). Experiments were made as a function of pH (4 - 9), temperature (28°C - 36°C), and agitation speed (100 - 200 rpm). Optimization of these three process parameters for phenol degradation was studied. Statistically designed experiments using response surface methodology was used to get more information about the significant effects and the interactions between these three parameters. A 2^3 full-factorial central composite design was employed for experimental design and for analysis of the results. A second order polynomial regression model, has been developed using the experimental data. It was found that the degrading potential of *P. putida* (NCIM 2102) was strongly affected by the variations in pH, temperature and agitation speed. The experimental values were in good agreement with the predicted values and the correlation coefficient was found to be 0.9871. The optimum process conditions for maximizing phenol degradation were recognized as follows: pH (7.49), temperature (29.99°C), and agitation speed (138.89) rpm.

Keywords: Biodegradation of Phenol; *P. putida* (NCIM 2102); Central Composite Design; Correlation Coefficient

1. INTRODUCTION

Nowadays, contamination of the environment by hazardous and toxic chemicals is being considered as one of the major problems facing by the industrialized nations [1]. Phenol and its higher molecular homologues are important environmental pollutants [2], which attracted public attention due to its presence in ground water, rivers and drinking waters [3]. It was also listed as a priority pollutant by the USEPA [4,5]. Acute exposure to phenol and its quinone analogues is known to cause skin irritation, kidney damage, gastrointestinal discomfort and headaches. Aesthetically, this compound also poses a nuisance with detectable taste and odors in water at concentrations as low as 1 - 8 mg·l^{-1} [6]. Most of the countries specify the maximum allowable concentration of phenol in the effluent streams to be less than 1 mg·l^{-1} [5]. Phenol and phenolic compounds of high concentrations can be found in aqueous effluents from various industries such as oil refineries, petrochemical plants, ceramic plants, steel plants, coal conversion processes, phenol resin industries and pharmaceutical industries [7]. Due to its toxicity, hazardous character and an increasing social concern on environment, the discharge of such compounds in the environment has become more restrict. Phenolic wastes are treated by several physico-chemical methods like ozonization, adsorption, reverse osmosis, electrolytic oxidation, H_2O_2, photocatalysis etc. [8]. But all the above methods have their own limitations [3,9]. Hence the realization of the high cost of phenol waste treatment by physico-chemical methods has opened the door for the investigation of new methods. Biological treatment an alternative method is versatile, inexpensive [10,11], receiving the most attention due to its environmentally friendly approach due to their high biodegradation abilities. If properly designed and operated, biological processes can realize total oxidation of organic matter so that there can be no sludge's that must be eradicated as a result of treatment [12].

Microbial degradation of phenol has been actively

studied and these studies have shown that the phenol can be aerobically degraded by wide variety of fungi and bacterial cultures such as *Candida tropicalis* [13-15], *Acinetobacter calcoaceticus* [16], *Alcaligenes eutrophus* [17,18], *P.putida* [19,20] and *Burkholderia cepacia* G4 [21,22]. It has been demonstrated that treatment of small volumes of toxic compounds at the point of emission using specific microbial strains allows a higher control over the process and higher removal efficiencies [23]. Thus optimization of process variables is recognized to be an essential aspect of successful fermentation. Temperature exerts an important regulatory influence on the rate of metabolism. The effects of oxygen supply vary from species to species. 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 [24].

Our main goals were to find out 1) the effect of pH; 2) the effect of temperature; 3) the effect of agitation speed on the biodegradation of phenol by *P. putida* (NCIM 2102) 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.

2. DESIGN OF EXPERIMENTS

Response surface methodology is an empirical modelization technique derived to the evaluation of the relationship of a set of controlled experimental factors and observed results. It requires a prior knowledge of the process to achieve statistical model [25-28]. Basically this optimization of process involves three major steps, which are, performing the statistically designed experiments, estimating the coefficients in a mathematical model and predicting the response and checking the adequacy of the model.

$$Y = f(X_1, X_2, X_3, \cdots, X_k) \tag{1}$$

The true relationship between Y and X_k may be complicated and, in most cases, it is unknown, however, a second-degree quadratic polynomial can be used to represent the function in the range of interest;

$$Y = R_0 + \sum_{i=1}^{k} R_i X_i + \sum_{i=1}^{k} R_{ii} X_i^2 + \sum_{i=1,i<j}^{k-1} \sum_{j=2}^{k} R_{ij} X_i X_j + \varepsilon \tag{2}$$

where $X_1, X_2, X_3, \cdots, X_k$ are the input variables which affect the response Y, R_0, R_i, R_{ii} and R_{ij} ($i = 1 - k, j = 1 - k$) are the known parameters, ε is the random error. A second-order model is designed such that variance of Y is constant for all points equidistant from the centre of the design;

$$X_i = \left(\frac{X_i - X_0}{\Delta X_i} \right) \tag{3}$$

where X_i is the coded value, X_0 is the actual value at the center point and ΔX_i is the step change value. The parameters and their values (in brackets) were three levels, like pH (4 - 9), temperature (28°C, 30°C, 32°C, 34°C, 36°C), agitation speed (100, 120, 140, 160, 180, 200 rpm), at constant phenol concentration 0.100 g·l^{-1}. This also enabled the identification of significant effects of interactions for the batch studies. In system involving three significant independent variables X_1, X_2, and X_3, the mathematical relationship of the response of these variables can be approximated by quadratic (second degree) polynomial equation;

$$y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_{11} x_1^2 + b_{22} x_2^2 + b_{33} x_3^2 + b_{12} x_1 x_2 + b_{13} x_1 x_3 + b_{23} x_2 x_3 \tag{4}$$

where Y is the predicted value, b_0 is the constant, X_1 is the pH, X_2 is the temperature, X_3 is the agitation speed, b_1, b_2 and b_3 are linear coefficients, b_{12}, b_{23} and b_{13} are cross product coefficients and b_{11}, b_{22} and b_{33} are quadratic coefficients. The low, middle and high levels of each variable were designated as –1, 0, and +1 respectively, as given in **Table 1**. A total of 20 runs were necessary to estimate the coefficients of the model using multiple linear regressions. The design of experiments was carried out for analysis using the Statistica 6.0 version.

3. MATERIALS AND METHODS

3.1. Microorganism

The microorganism *P.putida* (NCIM 2102) was procured from culture collection NCL, Pune. The microorganism was maintained on beef extract: 1.0 g·l^{-1}; yeast extract: 2.0 g·l^{-1}; peptone: 5.0 g·l^{-1}; sodium chloride: 5.0 g·l^{-1} and agar: 20.0 g·l^{-1}. The medium was adjusted to pH 7.0 by 1 N sodium hydroxide. It was stored at 4°C ± 1°C for further use.

Table 1. Optimization of medium components and the conditions of fermentation for the degradation of phenol by *P.putida* (NCIM 2102).

Variables	Coded levels				
	–1.682	–1	0	1	1.682
pH (X_1)	5.32	6	7	8	8.68
Temperature (°C), (X_2)	26.64	28	30	32	33.36
Agitation Speed (rpm), (X_3)	123.18	130	140	150	156.82

3.2. Optimization Studies

The physical parameters namely pH: (4 - 9 g·l^{-1}); temperature: (28˚C, 30˚C, 32˚C, 34˚C, 36˚C) and agitation speed: (100, 120, 140, 160, 180, 200 rpm) at constant 0.100 g·l^{-1} phenol concentration were studied in the minimal medium composed of dihydrogen potassium phosphate: 1.5 g·l^{-1}; potassium dihydrogen phosphate: 0.5 g·l^{-1}; ammonium sulfate: 0.5 g·l^{-1}; sodium chloride: 0.5 g·l^{-1}; sodium sulfate: 3.0 g·l^{-1}; yeast extract: 2.0 g·l^{-1}; glucose: 0.5 g·l^{-1}; ferrous sulfate: 0.002 g·l^{-1} and calcium chloride: 0.002 g·l^{-1} by *P.putida* (NCIM 2102) for the maximum degradation of phenol. Samples were withdrawn at regular intervals for phenol determination. From the above experiments the range of pH, temperature, and agitation speed were chosen for further optimization of the design.

3.3. Design of Experiments

To optimize the range of experiments the 2^3 full-factorial Central Composite Design (CCD) was applied. The range and the levels of the process variables under study are given in **Table 1**: pH (6 - 8), temperature (28˚C, 30˚C and 32˚C) and agitation speed (130, 140 and 150 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.

3.4. Estimation of Phenol

Phenol was determined quantitatively by the Spectrophotometric method (DR/4000 V, Hach) using 4-amino antipyrine as the color reagent (λ_{max}: 500 nm) according to standard methods of analysis [29].

4. RESULTS AND DISCUSSION

P.putida (NCIM 2102) was inoculated into minimal salt medium at different pH, temperature and agitation speed.

The maximum percentage of phenol degradation was observed at pH (7), temperature (30˚C) and agitation speed (140 rpm) as shown in **Figures 1-3**. The optimum values of (**Figures 1-3**), pH (6 - 8), temperature (28˚C, 30˚C and 32˚C) and agitation speed (130, 140 and 150 rpm) were determined using CCD. Using the results of the experiments, the following second order polynomial equation giving the percentage phenol degradation as a function of pH (X_1), temperature (X_2, ˚C) and agitation speed (X_3, rpm) was obtained by using Statistica 6.0 version.

$$y = -918.346 + 22.952X_1\,29.584X_2 + 6.962X_3$$
$$- 1.794X_1^2 - 0.595X_2^2 - 0.029X_3^2 \qquad (5)$$
$$+ 0.155X_1X_2 + 0.036X_2X_3 - 0.005X_1X_3$$

Table 2. The CCD matrix employed for the optimization of medium components and the conditions of fermentation.

| Experiment number	pH		Temperature		Agitation Speed	
	Code	Value	Code	Value (˚C)	Code	Value (rpm)
1	−1	6	−1	28	−1	130
2	−1	6	−1	28	+1	150
3	−1	6	+1	32	−1	130
4	−1	6	+1	32	+1	150
5	+1	8	−1	28	−1	130
6	+1	8	−1	28	+1	150
7	+1	8	+1	32	−1	130
8	+1	8	+1	32	+1	150
9	−1.682	5.32	0	30	0	140
10	+1.682	8.68	0	30	0	140
11	0	7	−1.682	26.64	0	140
12	0	7	+1.682	33.36	0	140
13	0	7	0	30	−1.682	123.18
14	0	7	0	30	+1.682	156.82
15	0	7	0	30	0	140
16	0	7	0	30	0	140
17	0	7	0	30	0	140
18	0	7	0	30	0	140
19	0	7	0	30	0	140
20	0	7	0	30	0	140
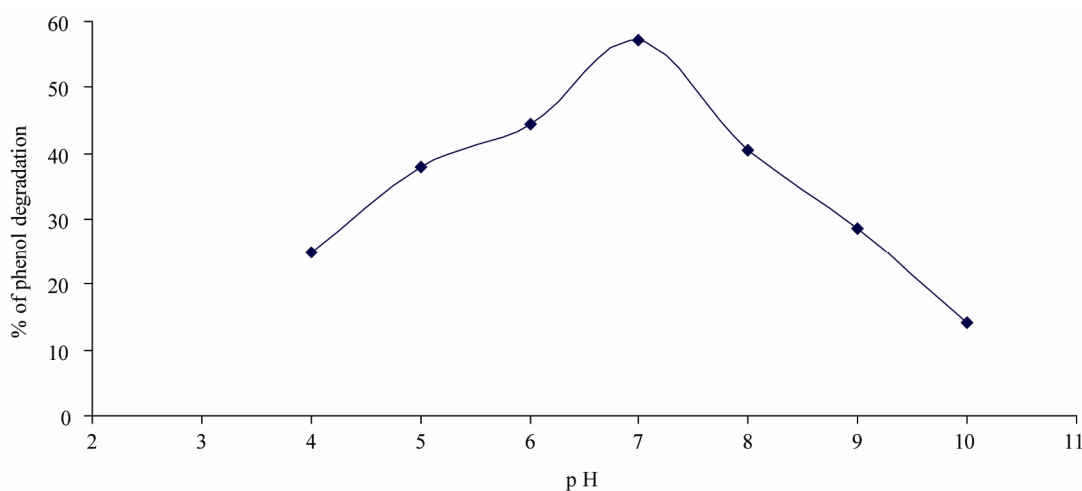
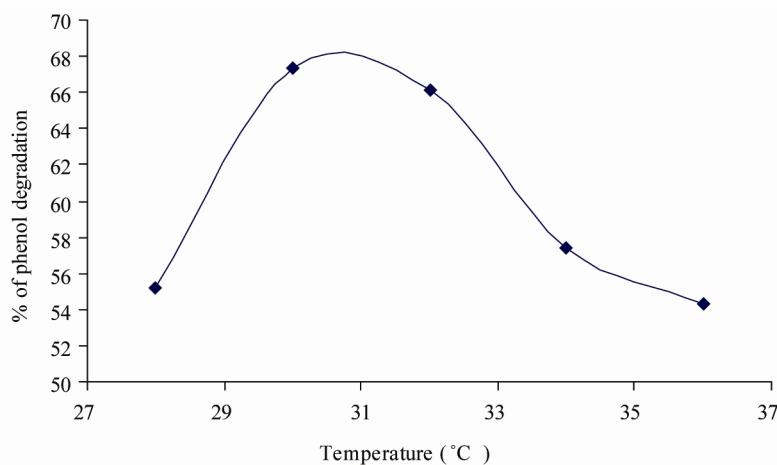**Figure 1.** Optimum pH for phenol degradation.

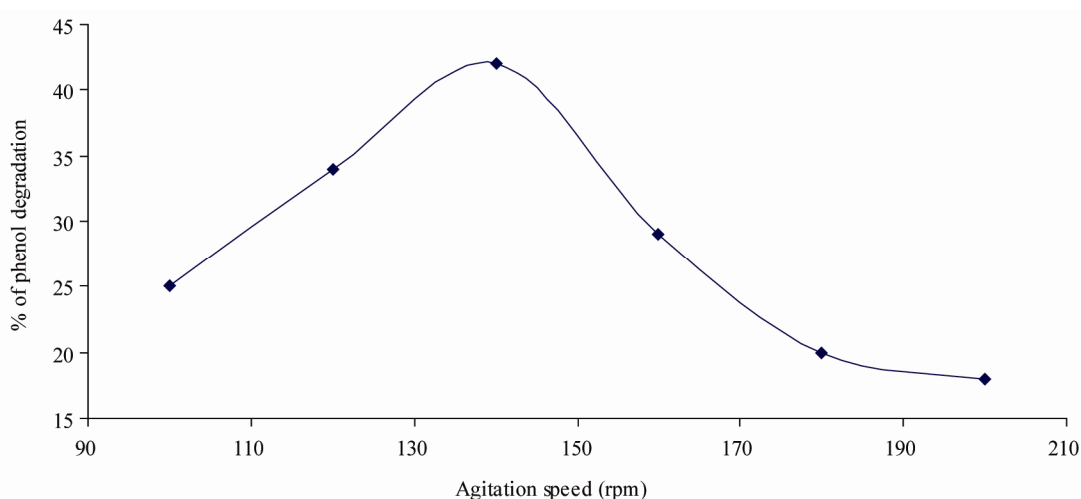**Figure 2.** Optimum temperature for phenol degradation.

Figure 3. Optimum agitation speed for phenol deradation.

The predicted values of percentage phenol degradation using the above equation are given in **Table 3** along with experimental values.

The coefficients of the regression model (**Eq.5**) calculated are listed in **Table 4**, in which they contain three linear, three quadratic and three interaction terms and one block term. The significance of each coefficient in **Eq.5** was determined by student's *t*-test and *p*-values which were also listed in **Table 4**. The larger the magnitude of the t-value and smaller the p-value, the more significant is the corresponding coefficient. This implies that the linear and quadratic effects of pH, temperature and agitation speed were highly significant as is evident from their respective p-values. The interaction effect of temperature and agitation speed was found to be significant ($p \leq 0.05$). The remaining two interaction terms *i.e.* pH × temperature and agitation speed × pH were found to be insignificant ($p > 0.05$) which were also presented in **Table 4**.

The parity plot (**Figure 4**) showed a satisfactory correlation between the experimental and predicted values (obtained from **Eq.5**) of percentage phenol degradation, wherein, the points cluster around the diagonal line which indicated the optimal fit of the model, since the deviation between the experimental and predicted values was minimal.

The results of the second order response surface model fitting in the form of Analysis of Variance (ANOVA) were given in **Table 5**. It is required to test the significance and adequacy of the model. The Fisher variance ratio, the *F*-value ($= S^2_r/S^2_e$), is a statistically valid measure of how well the factors describe the variation in the data about its mean. The greater the *F*-value is from unity, the more certain it is that the factors explain adequately the variation in the data about its mean, and the estimated factor effects are real. The ANOVA of the regression

Table 3. CCD matrix showing real values along with the experimental values of percentage phenol degradation.

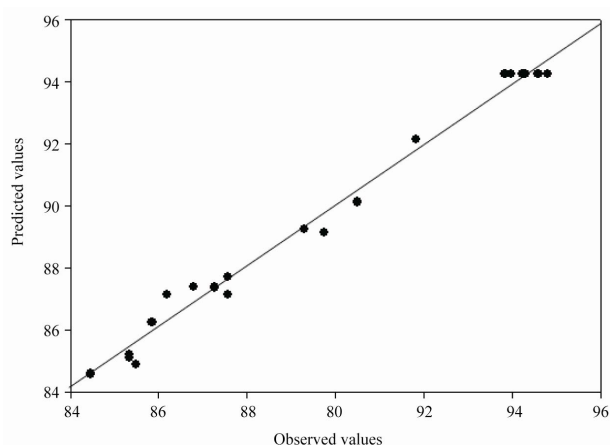| Run	X_1	X_2	X_3	% Phenol degradation	
				Experimental	Predicted
1	6	28	130	87.57	87.1215
2	6	28	150	84.47	84.5813
3	6	32	130	85.49	84.8932
4	6	32	150	85.34	85.2030
5	8	28	130	90.48	90.1140
6	8	28	150	87.27	87.3638
7	8	32	130	89.74	89.1257
8	8	32	150	89.28	89.2255
9	5.32	30	140	85.85	86.2451
10	8.68	30	140	91.82	92.1377
11	7	26.64	140	87.57	87.6903
12	7	33.36	140	86.79	87.3824
13	7	30	123.18	86.18	87.1417
14	7	30	156.82	85.34	85.0893
15	7	30	140	94.21	94.2541
16	7	30	140	93.96	94.2541
17	7	30	140	94.28	94.2541
18	7	30	140	94.58	94.2541
19	7	30	140	93.83	94.2541
20	7	30	140	94.79	94.2541

Table 4. Coefficients, *t*-statistics and significance probability of the model.

Term	Coefficient	Value	Standard error of coefficient	*t*-value	*p*-value
Constant	b_0	–918.346	71.99615	–12.7555	0.000000*
pH	b_1	22.952	4.73834	4.8438	0.000678*
Temperature	b_2	29.584	2.81038	10.5267	0.000001*
Agitation speed	b_3	6.962	0.54787	12.7072	0.000000*
pH × pH	b_{11}	–1.794	0.15349	–11.6864	0.000000*
Temperature × temperature	b_{22}	–0.595	0.03837	–15.5066	0.000000*
Agitation speed × agitation speed	b_{33}	–0.029	0.00153	–18.7779	0.000000*
pH × temperature	b_{12}	0.155	0.10284	1.5072	0.162673
Temperature × agitation speed	b_{23}	0.036	0.01028	3.4642	0.006080*
Agitation speed × pH	b_{31}	–0.005	0.02057	–0.2553	0.803698
				* Significant ($p \leq 0.05$)	

model demonstrates that the model is highly significant, as is evident from the Fisher's *F*-test ($F_{\text{model}} = 85.54974$) and a very low probability value ($P_{\text{model}} > F = 0.000000$).

The goodness of the fit of the model was checked by

Figure 4. Parity plot showing the distribution of experimental vs. predicted values of percentage phenol degradation.

Table 5. ANOVA for the entire quadratic model.

Source of variation	Sum of squares (SS)	Degree of freedom (d.f.)	Mean squares (MS)	*F*-value	Probe > F
Regression	260.5647	9	28.95164	85.54974	0.000000
Residual	3.3842	10	0.33842		
Total	263.9489	19			

$R^2 = 0.98717863$; Adjusted $R^2 = 0.97563939$

the determination coefficient (R^2). The R^2 value provides a measure of how much variability in the observed response values can be explained by the experimental variables and their interactions. The R^2 value is always between 0 and 1. The closer the R^2 value is to 1, the stronger the model is and the better it predicts the response. In this case, the value of the determination coefficient ($R^2 = 0.9871$) indicates that 98.71% of the variability in the response could be explained by the model. In addition, the value of the adjusted determination coefficient (Adj $R^2 = 0.9756$) is also very high to advocate for a high significance of the model.

Figures 5-7 represent the contour plots for the optimization of physical conditions of percentage phenol degradation. The effect of the pH and temperature on the percentage phenol degradation was shown in **Figure 5**. An increase in the pH with temperature up to the optimum point increased the percentage phenol degradation to a maximum level and a further increase in the pH with temperature the trend is reversed.

The interaction effect of the temperature and agitation speed on the percentage phenol degradation is shown in **Figure 6**. An increase in the temperature with agitation speed increased the percentage phenol degradation gradu-
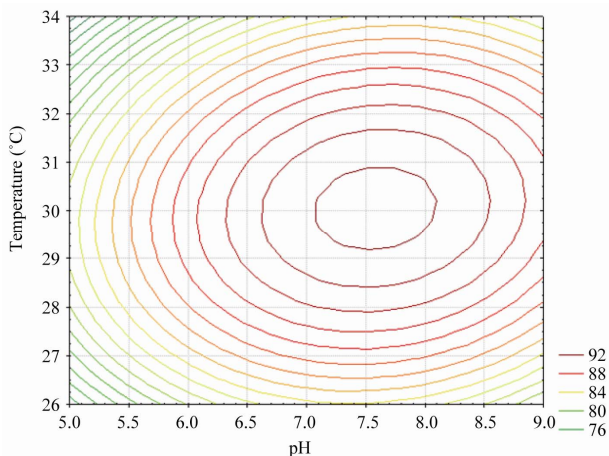
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Figure 5. Contour plot of pH vs. temperature on % phenol degradation (agitation speed was kept constant at 140 rpm).
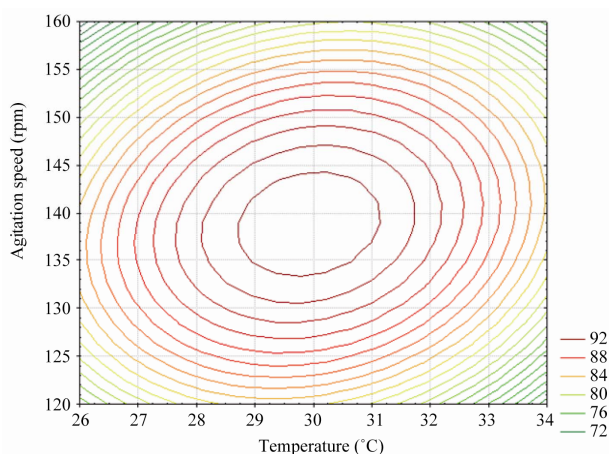

Figure 6. Contour plot of temperature vs. agitation speed on % phenol degradation (pH was kept constant at 7).
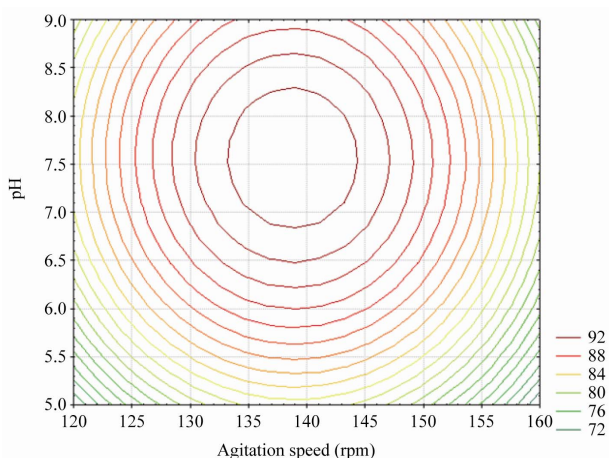

Figure7. Contour plot of agitation speed vs. pH on % phenol degradation (temperature was kept constant at 30°C).

ally but at a higher agitation speed and temperature the

trend is reversed. The optimum for maximum percentage phenol degradation lies near the center point of the temperature and agitation speed.

A similar effect on the response was observed for the agitation speed at any level of the pH. An increase in the agitation speed with pH up to the optimum point increased the percentage phenol degradation to maximum level and a further increase in the agitation speed with pH decreased the percentage phenol degradation as shown in **Figure 7**. Therefore, an optimum was observed near the central value of pH, temperature and agitation speed. The optimum physical conditions for percentage phenol degradation can be attained at the 7.49 of pH, 29.99°C of temperature and 138.89 rpm agitation speed. At these optimum physical conditions maximum percentage phenol degradation of 95.12 was obtained.

5. CONCLUSIONS

The true functional relationship between the dependent variable (pH, temperature and agitation speed) and maximum percentage of phenol biodegradation have been studied. The response surface methodology using 2^3 full-factorial composite design was adopted to optimize these process parameters for the microbial degradation of phenol by *P.putida* (NCIM 2102). The optimum conditions for degradation of phenol by *P.putida* (NCIM 2102) were as follows: pH 7.49, temperature 29.99°C and agitation speed138.89 rpm were investigated. A quadratic model was obtained for this design Statistica 6.0 version. The model employed provided good quality of predictions for the above variables in terms of effective phenol degradation and good correlation coefficient 0.9871 was obtained. By this model, we can predict the response for the above variables at any time

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