Statistical optimisation for improvement of phenol degradation by *Aspergillus niger*

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Abstract
Phenol is an important compound widely used in formulation of dyes, pesticides, in fabrication of plastics, as well as being generated in several industrial effluent other industries. The central composite design (CCD) of RSM was employed to optimize four process parameters namely initial phenol concentration, pH and temperature and inoculum concentration for the removal of phenol. The four independent variables were studied at five different levels and a set of 30 experiments were carried out and the results were analysed by ANOVA.

It was shown that a second order polynomial regression model could properly interpret the experimental data with an R^2 value of 0.9476 and an F-value of 20.07 based on which the maximum degradation of phenol was estimated up to 97% within the range examined.

Keywords: Biodegradation, Phenol, *Aspergillusniger*, Central Composite Design.

Introduction

Phenol is an aromatic organic compound that is also known as carbolic acid and its derivatives are very commonly used or generated in several industries. It was normally found in effluent of gas and coke oven industries, polymeric resin production, petroleum refineries, varnish industries, textile industries because it acts as a key material for the formulation of dye, pharmaceutical drugs, detergents, explosive, polycarbonate and many more. This substance creates a worldwide environmental problem. It has been considered as a priority pollutant by the American Environmental Protection Agency (EPA) as it is highly toxic. On ingestion it is lethal to human. The industries runoff waters will treat aquatic organism and simultaneously reduce the effectiveness of wastewater bio-treatment even at a low level of phenol[1,2].

Degradation of phenol is sensitive and normally affected by physical and cellular factors, which influence growth and enzymes involved However, this technique is time consuming and the materials used increases the cost of the experiment. Besides, this technique is unable to estimate and detect interactions between independent variables and might overlook the optimal settings of factors as only one factor is tested in a batch instead of all simultaneously.

Biodegradation is a promising method to treat phenol containing wastewater, as it is inexpensive, environmental friendly and able to convert toxic substances into harmless products as compared to conventional methods that need high cost and may produce hazardous by-products that can affect the environment. Fortunately, several reports have proved that diverse microorganisms including yeast, bacteria, fungi and algae do have their own mechanism and condition to degrade phenol[3-5]. Degradation is normally affected by physical and cellular factors, which influence growth and enzymes involved.

Among the crucial factors are type of medium, temperature, pH, salinity, oxygen level, compound structure and concentration, nitrogen source and accessibility to inorganic nutrients[6,7]. Hence, in the present investigation, an attempt was made to optimise essential variables that could improve biodegradability of phenol. The common process for optimising a multivariable system normally applies "one variable at a time" approach[8]. With the development of knowledge in statistic and information technology, various statistical softwares for experimental design have been developed as a tool of optimisation to simplify the process and to view the effects and relation of the variables and economic especially for bioreactor design.

Fractional factorial design along with response surface method is common by chosen method. Fractional factorial design is normally applied at first stage for preliminary screening of many factors in order to eliminate insignificant factors for targeted response that will be studied in further optimisation process. These factors can be used in RSM as an experimental design tool to determine optimal condition and to observe mutual interactions of variables by building models, which have been widely used for optimisation in the recent years[9,10].

It is a statistical technique for designing experiment, evaluating the effects of several factors for optimum conditions and building models. RSM minimises the number of experiments, which led to reduces expenses, as it has been reported to be capable of recognising accurate optimum condition of a particular parameter and define interaction between variables[11,12]. Among the experimental design offered are Central Composite Design (CCD), Box-Behnken, 3-levelfactorial, hybrid and one factor. In biological research, CCD is commonly employed as it is well suited for fitting a quadratic surface which is usually efficient for optimisation process with an acceptable number of runs[12,13].

In the present study, statistical optimisation was applied to investigate significant variables between initial phenol concentration, pH, temperature and inoculum concentration to optimise growth and phenol degradation capability of sp. *Aspergillus niger* using RSM.

Material and Methods

Microorganism and its growth conditions: *Aspergillusniger* was obtained from Institute of Microbial Technology, Chandigarh, based on its ability to degrade phenol. Czapek Yeast Extract Agar (CYA) medium was used as the growth medium for *Aspergillus niger*.Czapek concentrate can be stored without sterilization. The precipitate of $Fe(OH)_3$ can be resuspended by shaking well before use.

Procedure for maintenance of cells: The culture *Aspergillus niger* was maintained in the agar media. Agar-Agar was used for the preparation of slants. For growing the media on a large scale, fresh culture was transferred to 100 ml of liquid media containing the Czapek Yeast Extract medium without agar. The media was left for growth of 7 days. The 100ml media was transferred in to a 500 ml media and these cultures was used for further studies.

In all the cases, the media was autoclaved under 121°C at 1.1Kgf/cm^2 guage pressure for 15 minutes and strict asceptic conditions should be maintained. Throughout the experiment precautions were taken while inoculating and transferring the culture.

Preparation of phenol solution: The test solution containing phenol was prepared by diluting the stock solution to the desired concentrations. The concentrations were varied in the range of 100 to 500 mg/l. Stock solution of aqueous phenol was prepared by dissolving the exact quantity (1g) of phenol in double distilled water.

Batch biodegradation experiments: The factors affecting the growth of phenol degradation rate of growing *Aspergillus niger* were examined in 250ml Erlenmeyer flask with 50ml accumulation medium. The accumulation medium was prepared by mixing 25 ml of aqueous phenol solution with 25ml of Czapek Yeast Extract medium.

The pH of the Czapek Yeast Extract medium was adjusted to the desired value by adding acid or alkali solutions. CYA medium was autoclaved separately at 1.1Kgf/cm^{2}guage pressure for 15 min. A known amount of microorganism suspension (10%(v/v)) was added to the accumulation medium and the cultures were grown at 30°C for 7 days on a rotary shaker at 100rpm constant shaking rate.

This shaking frequency supplied the culture with enough oxygen to attain logarithmic growth. For each concentration, a non-inoculated media was served as blank. The dry samples were drawn at predetermined time intervals and analyzed for residual phenol concentration and biomass concentration. The residual concentration in the medium was determined.

To study the effect of agitation, experiments were conducted under shaking and static conditions by keeping the inoculated cultures on a rotary shaker at 100rpm shaking speed and resting conditions. The initial phenol concentration of 400mg/l, pH of 7.3 and at a temperature of 30°C was used for this study. Keeping all other parameters constant, the effect of different phenol concentrations (100mg/l, 200mg/l, 300mg/l, 400mg/l and 500mg/l), temperatures (30^{0}C, 35^{0}C, 40^{0}C and 45^{0}C) and pH (3, 5, 7 and 9) and inoculums size (3%, 5%, 7%, 10% and 13%) on phenol degradation was determined.

Experiments were conducted with 50ml of Czapek Yeast Extract medium containing 30g/l of glucose under no inhibition conditions. To study the type of inhibition, experiments were also conducted with 25ml of Czapek Yeast Extract medium containing 30g/l of dextrose and 25ml of 400mg/l aqueous phenol or O-Cresol solution. The cultures were adjusted with pH 7.3 and inoculated for 7 days at a temperature of 30^{0}C under aerobic conditions. The samples were drawn at pre-determined time intervals to analyze for biomass concentration and residual glucose concentration. The glucose concentration was measured at 540 nm using dinitrosalicylic acid method.

Response Surface Methodology (RSM): The RSM has several classes of designs with its own properties and characteristics. Central composite design (CCD), Box–Behnken design and three-level factorial design are the most popular designs applied by the researchers. A prior knowledge with understanding of the related bioprocesses is necessary for a realistic modeling approach.

The CCD is used to study the effects of the variables towards their responses and subsequently in the optimization studies. This method is suitable for fitting a quadratic surface and it helps to optimize the effective parameters with a minimum number of experiments as well as to analyze the interaction between the parameters. In order to determine the existence of a relationship between the factors and the response variables, the data collected are analyzed in a statistical manner using regression. A regression design is normally employed to model a response as a mathematical function (either known or empirical) of a few continuous factors and good model parameter estimates are desired[14].

The coded values of the process parameters are determined by the following equation:

$$x_i = \frac{X_i - X_o}{\Delta x} \quad (1)$$

where x_i – coded value of the ith variable, X_i – uncoded value of the ith test variable and X_0 – uncoded value of the ith test variable at center point. The regression analysis is performed

to estimate the response function as a second order polynomial:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \quad (2)$$

where Y is the predicted response, β_0 is constant, β_i, β_j, β_{ij} are coefficients estimated from regression. They represent the linear, quadratic and cross products of X_i and X_j on response.

Optimization of process parameters for the removal of phenol using *Aspergillus niger*: The central composite design (CCD) of RSM was employed to optimize four process parameters namely initial phenol concentration, pH, temperature and inoculum concentration for the removal of phenol. The four independent variables were studied at five different levels (Table 1) and a set of 30 experiments were carried out (Table 2) and the results were analyzed by ANOVA (Table 3).

Table 1
Range and levels of process variables

Variables	Code				
	-2	**-1**	**0**	**1**	**2**
Temperature (^{O}C)	27	31	35	39	43
pH	5	5.5	6	6.5	7
Innoculum size (V/V)%	5	7.5	10	12.5	15
Initial Concentration ^{o}C	100	200	300	400	500

Table 2
CCD design matrix with experimental and predicted values for removal of phenol using *Aspergillus niger*

Run No.	Initial Phenol Concentration	pH	Temperature	Inoculum size	Phenol removal efficiency	
					Experimental	**Predicated**
1	-1	-1	-1	-1	72	52.8674
2	1	-1	-1	-1	89	62.4813
3	0	0	0	0	97	74.7874
4	0	0	0	2	83	59.7943
5	0	2	0	0	91	65.4884
6	-1	1	-1	1	77	58.2822
7	0	0	0	0	97	74.7874
8	0	0	0	0	97	74.7874
9	-1	-1	-1	1	64	38.7739
10	1	-1	1	-1	85	60.6155
11	0	0	-2	0	73	52.2536
12	-1	-1	1	-1	86	63.0940
13	1	1	1	-1	77	57.2162
14	0	0	0	0	97	74.7874
15	1	-1	1	1	77	54.8999
16	0	0	0	0	97	74.7874
17	0	0	0	-2	81	60.3179
18	-1	1	1	1	89	70.5088
19	2	0	0	0	84	65.3795
20	-1	1	-1	-1	76	53.0902
21	-1	-1	1	1	79	57.9081
22	1	1	-1	-1	88	65.9896
23	1	1	-1	1	93	70.8960
24	0	0	2	0	86	62.8586
25	-1	1	1	-1	78	56.8977
26	0	-2	0	0	68	49.6238
27	-2	0	0	0	81	55.7327
28	1	1	1	1	95	71.0303
29	0	0	0	0	97	75.0316
30	0	0	0	0	96	75.0316
31	1	-1	-1	1	72	52.8674

The relationship and interrelationship of the variables were determined by fitting the second-order polynomial equation to data obtained from 31 experiments. The response values (Y_1 and Y_2) used in each trial were the average of the duplicates. The second-order regression equations provided the levels of phenol removal as a function of initial phenol concentration, pH temperature and inoculum concentration which can be represented in terms of coded factors as in the following equations:

Removal of Phenol = 97.86 +2.67 * A +3.83 * B +2.42 * C +0.083 * D +0.50 * A * B -3.38 * A * C +0.25 * A * D -1.37 * B * C +4.50 * B * D +1.87 * C * D -3.53 * A^2 - 4.2 * B^2 -4.28 * C^2 -3.65 * D^2----------1

A, *B*, *C* and *D* are the coded values of initial phenol concentration, pH temperature and inoculum concentration respectively. ANOVA for the response surface is shown in table 3.

To test the fit of the model equation, the regression-based determination coefficient R^2 was evaluated, which is the proportion of variation in the response attributed to the model rather than to random error. The closer are the values of R^2 to 1, the better the model would explain the variability between the experimental and the model predicted values. The coefficients of determination (R^2) for phenol removal are calculated as (R^2=0.9476 which are nearly equal to 1) 94.76 % variability of the response and only about 5.24% of the total variation cannot be explained by the models.

The predicted R^2 value of phenol removal was 69.97 % and has a reasonable agreement with the adjusted R^2 value of 90.18 %. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Adeq precision of 16.942 indicates an adequate signal. These models can be used to navigate the design space.

The statistical significance of Eq. (1) was checked by *F*- test and the Analysis of Variance (ANOVA) for the response surface quadratic model is shown in table 3. The results demonstrated that the model is highly significant and is evident from Fischer's *F*- test with a low probability value (*P* model > *F* less than 0.05). Model coefficients estimated by regression analysis for each variable are shown in table 3. The significance of each coefficient was determined by *t*-values and *P*-values. The larger is the magnitude of *t*-test value and smaller is the *P*-value indicates the high significance of the corresponding coefficient.

In the present work, the linear effects of *A*, *B* and *C* the interactive effects of *BD* and squared effects of A^2, B^2, C^2 and D^2 are significant model terms for phenol removal.

Table 3
Analysis of variance (ANOVA) for phenol removal

Source	Coefficient factor	Sum of squares	DF	F	P > F
Model	2618.08	14	187.01	20.68	< 0.0001
A	170.67	1	170.67	18.87	0.0005
B	352.67	1	352.67	39	< 0.0001
C	140.17	1	140.17	15.5	0.0012
D	0.17	1	0.17	0.018	0.8937
AB	4	1	4	0.44	0.5155
AC	182.25	1	182.25	20.15	0.0004
AD	1	1	1	0.11	0.7438
BC	30.25	1	30.25	3.35	0.0861
BD	324	1	324	35.83	< 0.0001
CD	56.25	1	56.25	6.22	0.024
A2	355.68	1	355.68	39.33	< 0.0001
B2	523.04	1	523.04	57.84	< 0.0001
C2	523.04	1	523.04	57.84	< 0.0001
D2	381.34	1	381.34	42.17	< 0.0001
Residual	144.69	16	9.04		
Lack of Fit	143.83	10	14.38	100.68	< 0.0001
Pure Error	0.86	6	0.14		
Cor Total	2762.77	30			

Std. Dev.-3.01, R-Squared- 0.9476, Mean- 85.68, Adj R-Squared- 0.9018, C.V. %- 3.51, Pred R-Squared- 0.6997, PRESS- 829.65, Adeq Precision-16.942

To test the fit of the model equation, the regression-based determination coefficient R^2 was evaluated. The nearer are the values of R^2 to 1, the model would explain better for variability of experimental values to the predicted values. The above models can be used to predict the within the limits of the experimental factors.

Fig. 1 shows that the actual response values agree well with the predicted response values of phenol removal. The interaction effect of the variables on phenol removal was investigated by plotting the 3D response surfaces with the vertical (Z) axis representing removal efficiency (response) yield and two horizontal axes representing the coded levels of two explanatory factors, while maintaining other variables at their median levels shown in fig. 2-7.

Effect of initial phenol concentration: The effect of initial phenol concentration was vital role in the degradation process. It was observed from the figs 2-4. The removal efficiency of phenol increases with increase in initial phenol concentration up to 150mg/l and there after decreases with further increase in initial phenol concentration. The rapid decrease of RE suggested that substrate inhibition might occur. Substrate inhibition is well known to predominate at a higher phenol concentration owing to its toxicity to cell.

Effect of temperature: Fig. 3, 5 and 7 show the effect of temperature on removal efficiency of phenol. Increase in temperature favors removal efficiency of phenol till 30°C. Incubation temperature plays an important role in the metabolic activities of microorganism. A temperature range starting from 27 to 43°C was investigated and among these, nearly 30°C was optimized for the best growth of *Aspergillus niger*. Any change, either increase or decrease in temperature resulted in the gradual decrease in removal efficiency of phenol. A higher temperature alters the cell membrane composition and stimulates protein catabolism, thus, causes the cell death. Optimum temperature recorded for maximum phenol removal was at 30°C.

Effect of initial pH: The experiment was conducted at different intial pH (5-7). It was observed in fig. which shows the effect of initial pH on removal of phenol. The removal efficieny of phenol increases with increase in initial pH up to 6 and thereafter phenol removal decreases with further increase in initial pH.

Effect of inoculum concentration: Higher number of microorganism restrict microbial activity due to nutrient limitations whereas a lower amount of inoculation causes lower number of cells in the production medium thus consuming the lesser phenol. The fig. 4 shows the effect of inoculum concentration on phenol removal. The removal efficiency of phenol increases with increase in inoculum concentration up to 12 g/l and thereafter removal efficiency decreases with further increase in inoculum concentration.

The parity plot (Figure 1) showed a satisfactory correlation between the experimental and predicted values (obtained from eq. 1) of percentage phenol degradation, wherein, the points cluster around the diagonal line indicating the optimal fit of the model, since the deviation between the experimental and predicted values was minimal.

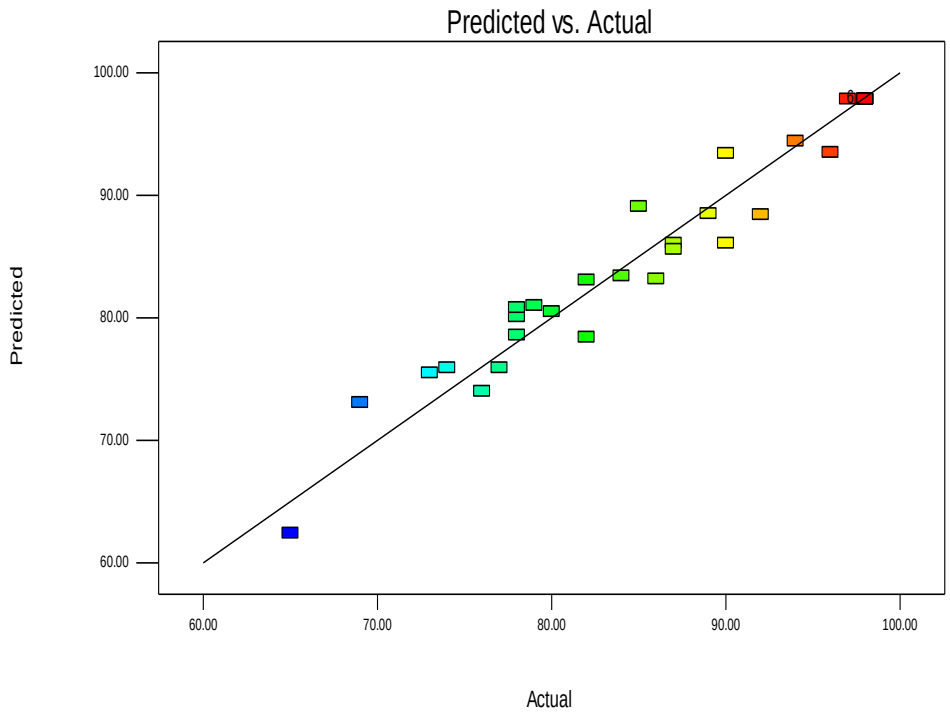

Figure 1: Parity plot showing the distribution of experimental vs. predicted values of percentage phenol degradation.

Optimum condition: The optimum conditions for the maximum removal of phenol were determined by response surface analysis and also estimated by optimizer tool using statistical software package "Design Expert 7.1.5". The optimum conditions are initial phenol concentration – 125 mg/l, initial pH – 5.5, temperature – 31 ^{o}C and inoculum concentration – 13g/l.

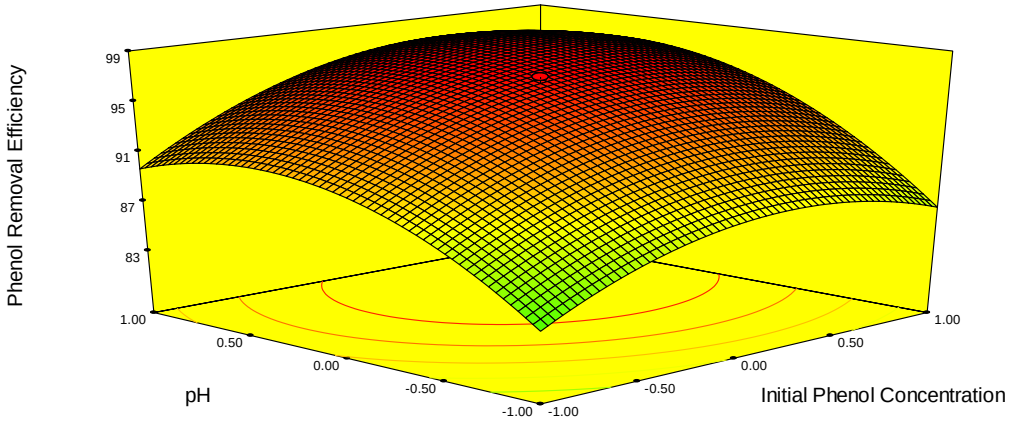

Figure 2: 3D plot shoes the effect of pH and initial phenol concentration on removal efficiency of phenol using *Aspergillus niger*

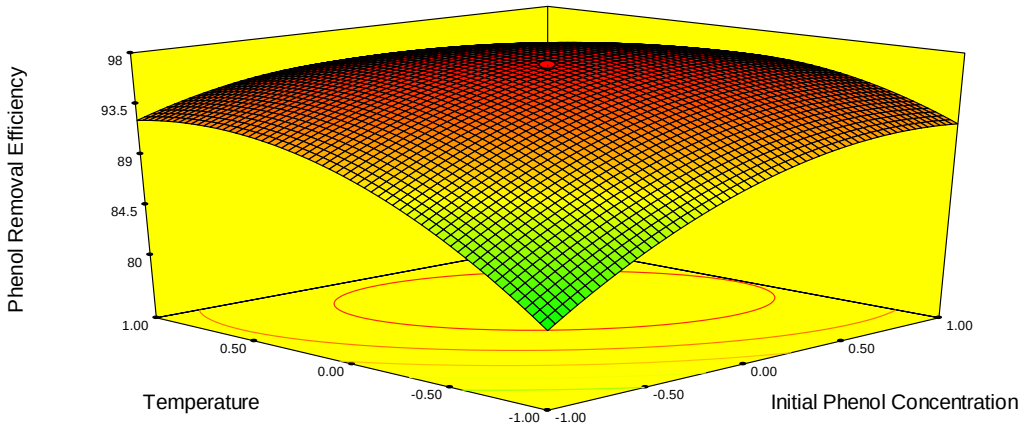

Figure 3: 3D plot shoes the effect of temperature and initial phenol concentration on removal efficiency of phenol using *Aspergillus niger*

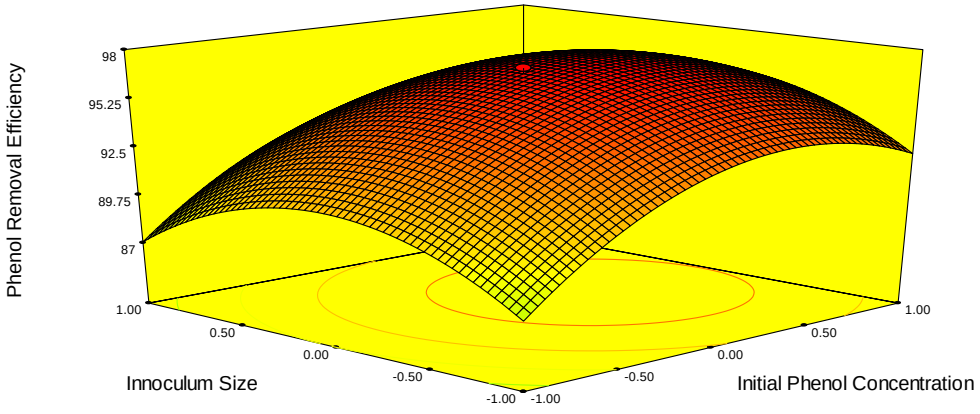

Figure 4: 3D plot shoes the effect of temperature and initial phenol concentration on removal efficiency of phenol using *Aspergillus niger*

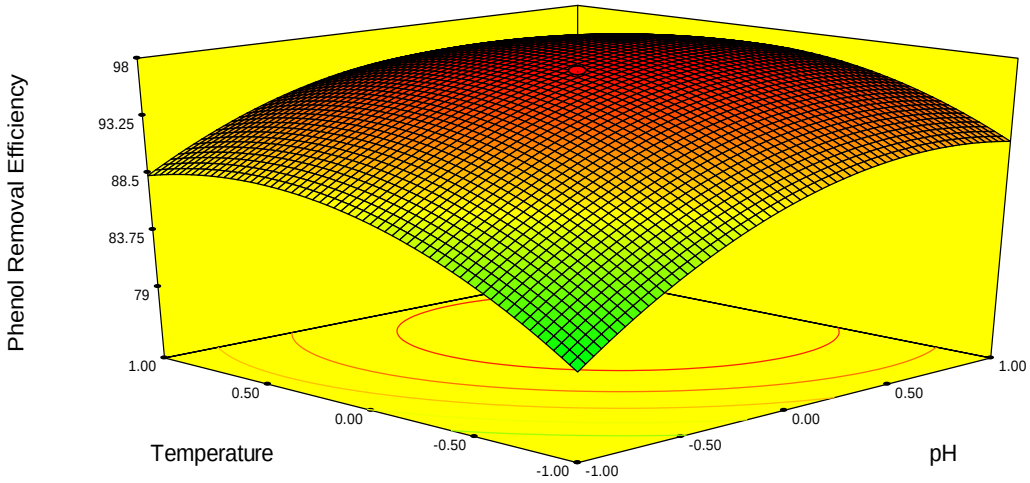

Figure 5: 3D plot shoes the effect of temperature and pH on removal efficiency of phenol using *Aspergillus niger*

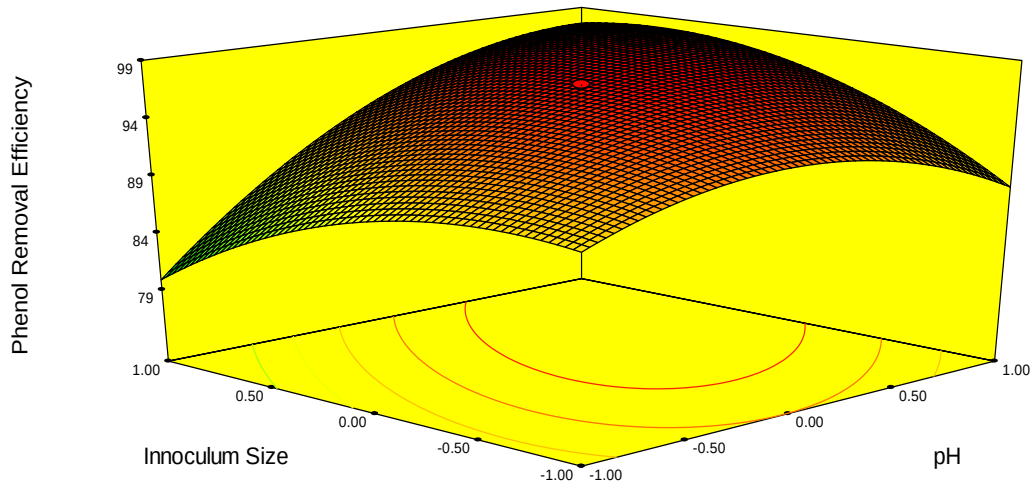

Figure 6: 3D plot shoes the effect of innoculum size and pH on removal efficiency of phenol using *Aspergillus niger*

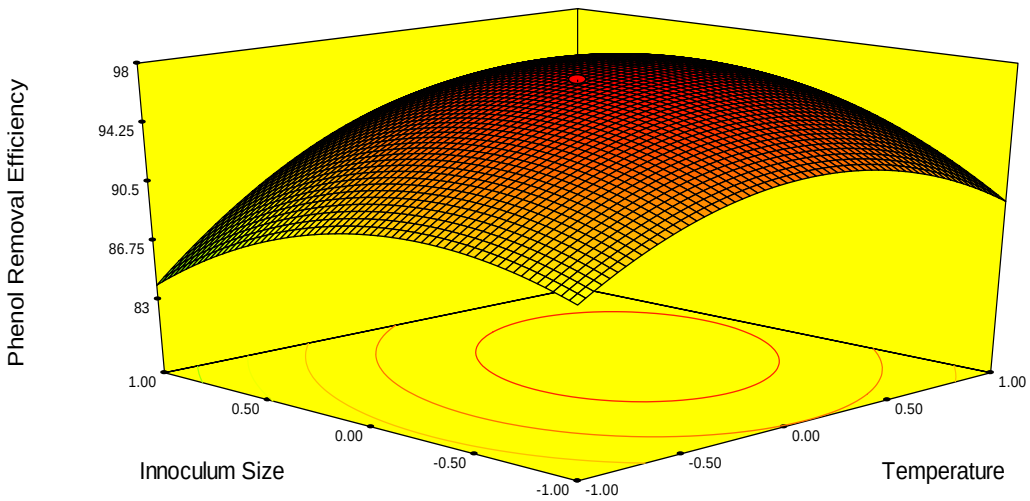

Figure 7: 3D plot shoes the effect of temperature and innoculum size on removal efficiency of phenol using *Aspergillus niger*

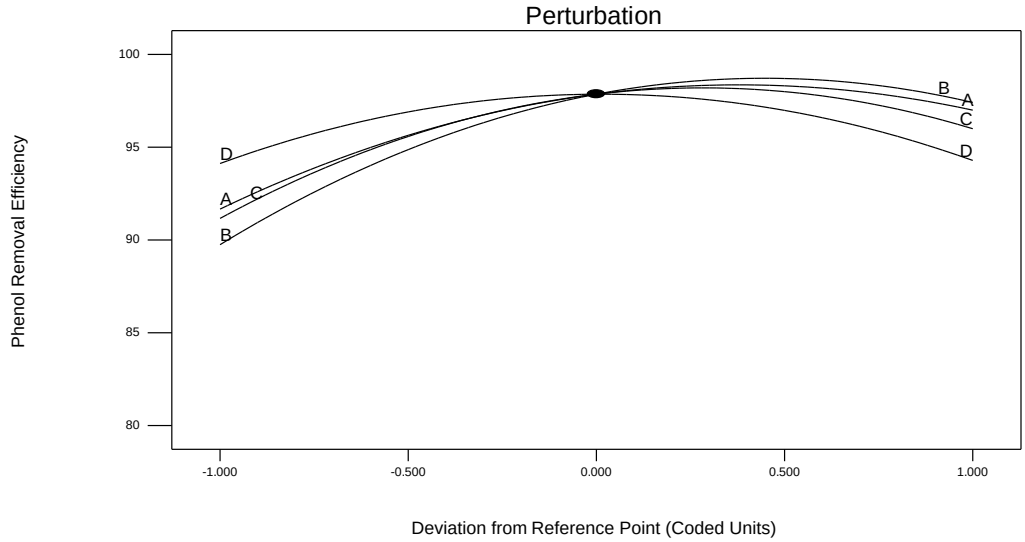

Figure 8: Perturbation plot for the Removal Efficiency of phenol using *Aspergillus niger*

The perturbation plot of the factors used in this study was shown in fig. 8. Although all factors showed significant effects, the steep curve was the perturbation curve of agitation speed and temperature (A and B curve) compared to other factors. Thus, it can be concluded that agitation speed and temperature were the most significant factors that contributed to the removal of phenol. The innoculum size showed the least prominent change compared to the other factors, but it still showed a significant effect for removal Efficiency of phenol. It was clear from the perturbation plots that the most significant factor on removal efficiency of phenol, was agitation speed followed by temperature, pH and innoculum size.

Validation of the experimental model: Validation of the experimental model was done by carrying out the batch experiment under optimal operating conditions. The experiments were done in triplicate and the results were compared. The removal efficiency of phenol obtained from experiments was very close to the actual response predicted by the regression model, which proved the validity of the model. At these optimized conditions, the maximum removal efficiency was found to be 96%.

Conclusion

From the above study it was concluded that as the phenol is one of the major effluents of so many chemical industries and as it is causing lethal effect to the human system. so, it has to be treated to control its toxic effects. So, we have chosen a simple, cost effective method known as biodegradation to degrade the phenol and other effluents to protect the environment. In the present study we used one fungal culture as a biosorbant to degrade the phenol and we fixed the optimum parameters for the maximum degradation of phenol.

From the above study it was concluded that the phenol degradation by *Aspergillus Niger* was the optimum at initial phenol concentration – 125 mg/l, initial pH – 5.5, temperature – 31^{o}C and inoculum concentration – 13g/l.

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