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Evaluation of the phenol biodegradation by *Aspergillus niger*: application of full factorial design methodology

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ABSTRACT

The ability of *Aspergillus niger* to degrade phenol in an aerobic batch culture was investigated under different parameters such as initial concentration of phenol, pH, and temperature. The results showed that *A. niger* can grow using phenol as the sole source of carbon. A high concentration of phenol (1,000 mg/L) was completely degraded after 140 h at $T = 30$°C and pH 5.5. The main and the interactive effects of initial phenol concentration (200–1,000 mg/L), pH (2.5–8.5), and temperature (25–35°C) on the biodegradation of phenol were studied in this work. A full factorial design 2^3 was used to determine the optimum conditions for the phenol biodegradation process. The maximum amount of degraded phenol was 1.32 mg/h, achieved at 200 mg/L of initial phenol concentration, pH 8.5, and $T = 35$°C.

Keywords: Phenol; Biodegradation; Factorial design; Filamentous fungi

1. Introduction

Many chemical industrial processes such as coal refineries, phenol manufacturing, pharmaceuticals, resin, paint and petrochemical industries discharge the phenolic compounds that often contribute to biosphere pollution. They are present at different concentrations in the wastewaters with quantities up to 1 g/L [1]. These compounds have high stability and high toxicity. The Environmental Protection Agency (EPA) ranks them among the most important pollutants [2,3]. Removing phenolic compounds from industrial wastes by biological treatments has proved to be the best promising and economical process. Numerous studies have been carried out with different micro-organisms that are able to degrade phenol [4], such as bacteria [5], yeasts [6], algae [7], and fungi. Fungi micro-organisms have been focused for their important role in the recycling of complex natural substances. Several studies regarding the degradation of phenol and Polycyclic

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Aromatic Hydrocarbon compounds by fungi have been published [8–10]. Santos and Linardi [11] have tested the ability of 15 filamentous fungal strains of the genera *Fusarium* sp., *Aspergillus* sp., *Penicillium* sp., and *Graphium* sp. to degrade phenol. Garcıa et al. [12] studied the removal of phenol compounds from olive mill wastewater aerobically by several fungi such as. *Phanerochaete chrysosporium*, *A. niger*, and *Aspergillus terreus*. Stoilova et al. [13] have studied the ability of the fungus *Aspergillus awamori* to degrade mixtures of some common phenolic compounds. They revealed that *A. awamori* has a great potential for biodegradation of phenol and other related aromatic compounds.

Nowadays, the statistical experiments design is the ideal methodology to optimize and evaluate the factors that influence the biodegradation process [14–16].

The aim of this study was to evaluate the ability of *A. niger* isolated for biodegradation of phenol. The full factorial design was chosen in order to evaluate the influence of the operating variables such as initial concentration of phenol, pH, temperature on phenol biodegradation and their interactions and to establish a mathematical equation of the process. The biodegradation of phenol process by this application is performed for the first time.

2. Materials and methods

2.1. Micro-organism and culture conditions

A. niger LSTE-AH1 was obtained from the "Laboratory of Sciences and Environmental Techniques, Algiers" collection. It was maintained at 4°C on MGYP agar medium (composition g/L: malt extract 3, glucose 10, yeast extract 3, peptone 5, and agar 20) the pH 6.2. The inoculum medium was prepared by inoculating some spores from Petri dishes with a sterile loop in 100 mL of MGYP broth containing (g/L): malt extract 3, glucose 10, yeast extract 3, and peptone 5, in 250 mL Erlenmeyer flasks. The flasks were incubated at 30°C for 72 h. The formed mycelia were then collected by sterile filtration and washed twice with sterile distilled water.

2.2. Phenol biodegradation experiments

For degradation studies, batch mode shake flask experiments were conducted in 250 mL Erlenmeyer flasks containing 150 mL of liquid mineral medium (MSM) (composition g/L: $NaNO_3$ 2, K_2HPO_4 1, $MgSO_4{\cdot}7H_2O$ 0.5, KCl 0.5, $FeSO_4{\cdot}7H_2O$ 0.001) and phenol as the sole source of carbon and energy. The mineral medium (except phenol and $FeSO_4{\cdot}7H_2O$) was autoclaved at 120°C for 20 min for sterilization before using. Phenol and $FeSO_4{\cdot}7H_2O$ were filter sterilized through membranes (pore size of 0.2 μm). Flasks were aseptically inoculated with 2 g (wet weight) of mycelia and incubated in the rotary shaking incubator at 30°C and 120 rpm. The pH of culture medium was 5.5.

2.3. Analytical method

For determining the residual concentration of phenol by spectrophotometric method using aminoantipyrine 1–2 mL of sample was collected at every 2 h interval from the flasks .This method is based on the condensation of 4-aminoantipyrine with phenol in the presence of an oxidizing agent, potassium ferricyanide, in an alkaline medium to give a red complex. The absorbance was read at a 510 nm wavelength using Shimadzu UV–VIS 1240 spectrophotometer [17]. The mycelial biomass was heighted by analytical balance after being filtered from culture medium.

2.4. The factorial design

A 2^3 full factorial design (three factors, each at two levels) was designed to evaluate the importance and the interactions of three independent variables: initial phenol concentration (X_1), pH (X_2), and temperature (X_3).

Each independent variable had two levels: −1 and +1. The variable factors with the coded and actual values are given in Table 1. The low and high levels for the factors were selected according to some preliminary experiments. The factorial design matrix and Y measured in each factorial experiment are shown in Table 2.

A first-order model with interaction terms was chosen to fit the experimental data:

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{12}X_1X_2 + a_{23}X_2X_3 + a_{13}X_1X_3 + a_{123}X_1X_2X_3 \quad (1)$$

The response Y is function of the amount of phenol degraded and the time of the degradation, as follows:

$$Y\,(\mathrm{mg/h}) = \frac{\text{the amount of biodegraded phenol (mg)}}{\text{biodegradation time (hour)}} \quad (2)$$

3. Results and discussion

3.1. The effect of phenol concentration on phenol biodegradation and biomass growth

Phenolic compounds are known to have inhibitory effect on the activity of the biomass. To determine the effect of the initial concentration of phenol on the

Table 1
Factors and levels used in the factorial design

Factors	Low level (−1)	Center (0)	High level (+1)
X_1 C_0 of phenol (mg/L)	200	600	1,000
X_2 pH	2.5	5.5	8.5
X_3 temperature (°C)	25	30	35

Table 2
Design matrix and the results of the 2^3 full factorial design

Experiment	X_1	X_2	X_3	Y (mg/h)	Y predicted
1	−1	1	1	1.3215	1.3373125
2	1	1	−1	0.165	0.1336875
3	−1	−1	−1	0.717	0.6735625
4	1	−1	1	0.0345	0.0381875
5	−1	−1	1	0.5745	0.5708125
6	1	1	1	0.8205	0.8046875
7	−1	1	−1	0.635	0.6663125
8	1	−1	−1	0.0975	0.1409375

phenol degradation and the biomass amount (wet weight), a series of experiments were carried out at different initial concentrations ranging from 100 to 1,000 mg/L. The temperature was fixed at 30°C and the pH of the medium was 5.5.

The time required for phenol degradation increases with the increase in phenol concentration. As it can be seen from Fig. 1, phenol concentrations of 100, 200, 350, 500, and 600 mg/L can be, respectively, degraded by *A. niger* in 40, 54, 73, 100, and 118 h, with an increase in mycelium mass (Fig. 2). On the other hand, at 1,000 mg/L, it takes more than 140 h to achieve the degradation with a negligible biomass growth. Stoilova et al. [13] have reported that *A. awamori* had mineralized phenol at concentrations of 300 mg/L in 60 h, 600 mg/L in 72 h, and 1,000 mg/L in 7–8 d.

The profile of biomass growth with the increase of phenol concentration (Fig. 2) indicates that *A. niger* uses phenol as the sole carbon source to synthesize new cells. The maximum wet weight of mycelium 6.7 g was achieved at 350 mg/L of phenol. Beyond this concentration, the produced biomass decreases. This means that the high concentration of phenol presents an inhibitory effect on the growth of *A. niger* which reflects the toxic effect caused by high concentrations of the pollutant.

3.2. Effect of pH and temperature on phenol degradation

Experiments were carried out to assess the effect of temperature and pH of solution on the biodegradation

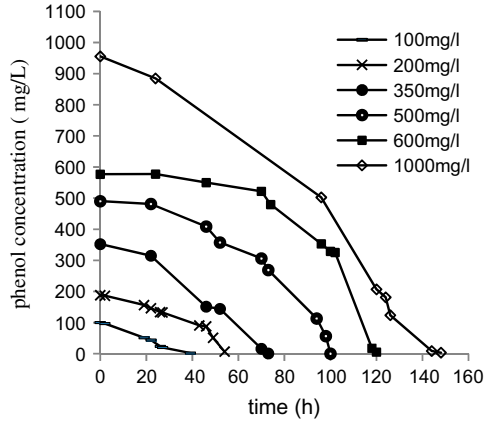

Fig. 1. The effect of the initial phenol concentration on phenol biodegradation.

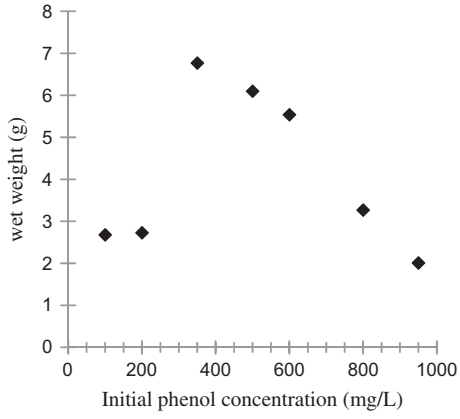

Fig. 2. The effect of the initial phenol concentration on biomass growth.

of phenol. The initial pH of the solution was adjusted using few drops of either HCl or NaOH.

Fig. 3 shows the phenol degradation at the different pH range. It can be seen that 100 mg/L phenol can be completely degraded by *A. niger* after 39 h with a pH of 5.5 and 8.5. More time is required at pH 2.5.

The effect of the temperature on the degradation of phenol was also studied at 25, 30, and 35°C. Under

the same other operating conditions, the best degradation of phenol was observed at 30℃ with a total time degradation of phenol after 39 h (Fig. 4); the time of the degradation was prolonged to 44 h for 25 and 35℃.

3.3. Factorial design evaluation

The experimental matrix along the coded scales is shown in Table 2 with the experimental and predicted responses.

The coded mathematical model for 2^3 factorial designs can be given by Eq. (1).

The response "Y" is a ratio between the amount of phenol degraded and the time of the degradation (mg/h); a_o, a_i, and a_{ij} are the constant, linear, and interaction coefficients of the model, respectively. X_i and X_j (i: 1–3 and j: 1–3) represent the coded independent variables.

The significance of each coefficient in the equation was determined by Students t-test and p-values. F-test indicated that all the factors and interactions considered in the experimental design are statistically significant ($p < 0.05$) at the 95% confidence level.

3.3.1. Student's t-test

The Student's t-test was used to determine the significance of the regression coefficients of the parameters. A large t-value associated with a low p-value ($p < 0.05$) of a variable indicates a high significance of the corresponding model term.

All results obtained with the JMP 7.0 Software are given in Table 3, where t-values, p-values, and coefficients are mentioned.

The results suggest that the initial concentration of phenol, followed by the pH, and temperature are the most significant factors. Only the interaction coefficient between pH and temperature is significant.

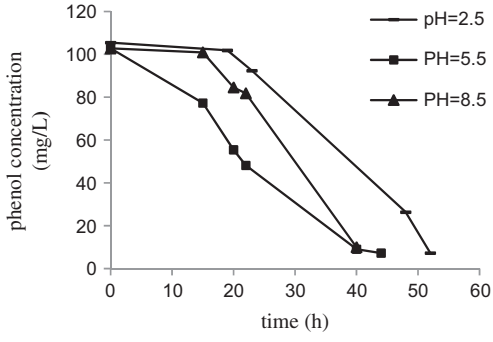

Fig. 3. Effect of pH on phenol biodegradation.

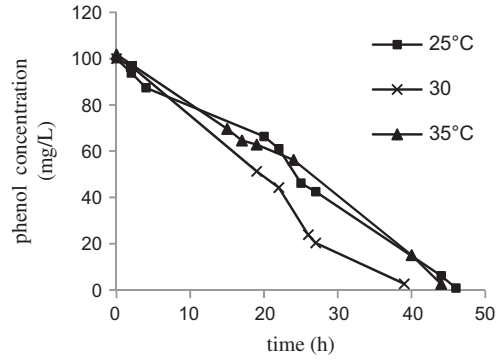

Fig. 4. Effect of temperature on phenol biodegradation.

After elimination of insignificant effects, the empirical model becomes:

$$Y = 0.545 - 0.266X_1 + 0.189X_2 + 0.193X_2X_3 \quad (3)$$

The fit quality of the polynomial model equation was evaluated by the coefficient of determination R^2 and the adjusted determination coefficient R^2 adj.

Table 3
Estimated effects and coefficients

Term	Estimate	t ratio	Prob. > |t|
Intercept	0.5456875	39.51	0.0161[a]
a_1	−0.266313	−19.28	0.0330[a]
a_2	0.1898125	13.74	0.0462[a]
a_3	0.1420625	10.29	0.0617
a_{12}	0.0235625	1.71	0.3375
a_{13}	0.0060625	0.44	0.7367
a_{23}	0.1934375	14.00	0.0454[a]

[a]Significance at 5% level.

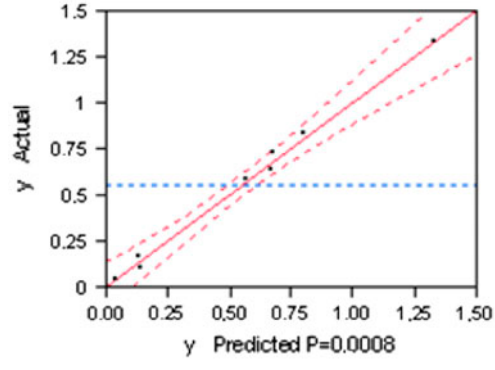

Fig. 5. Observed vs. predicted response plot.

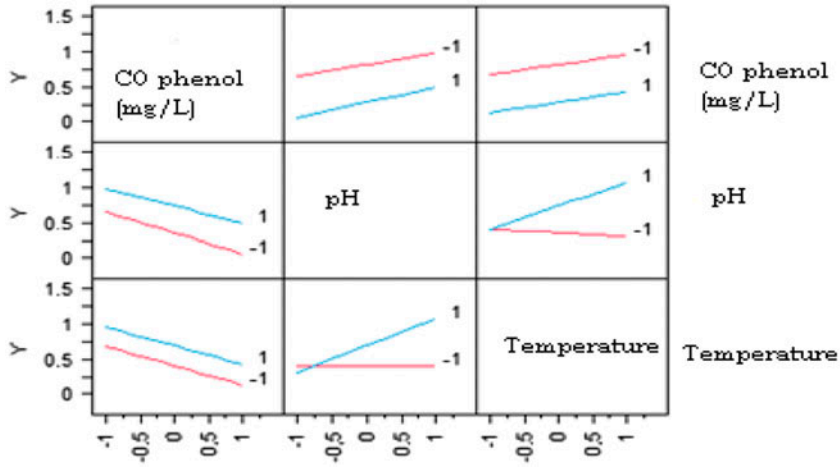

Fig. 6. Interaction effects.

Fig. 5 shows the predicted values versus the experimental values of the degradation of phenol.

The high values for the coefficients of determination R^2 of R^2 adjusted (0.9952 and 0.988) indicate a high level of significance for the model [18].

3.3.2. Main and interaction effects

The main effects represent deviations of the average between the high and the low levels for each factor. When the effect of a factor is positive, Y increases and the factor changes from low to high levels [19].

The effects of the initial concentration of substrate factor is negative ($a_1 = -0.266$), which means that the amount of degraded phenol decreases when the initial concentration of phenol increases from 200 to 1,000 mg/L. However, the effects of pH and temperature factors are positive, which indicates the increase of the response with the two factors increase.

The interaction plot is given in Fig. 6. An interaction is effective when the change in the response from low to high levels of a factor is dependent on the level of a second factor i.e. when the lines do not run parallel.

Interactions a_{12}, a_{13} seem to be insignificant, the lines representing the effects are parallels in the squares of the diagram.

3.3.3. Analysis of variance (ANOVA)

The ANOVA demonstrates that the regression model was highly significant, as is evident from the calculated Fisher's "F" value (157.6701) and a probability p-value of 0.008 (Table 4). A large F-value with a corresponding small p-value indicates a high significance of the respective coefficient [20].

3.3.4. Optimization using the desirability function

The desirability function transforms the values of a response into [0,1], where 0 stands for a non-acceptable value of the response and 1 for values where higher/lower (depending on the direction of the optimization) values of the response have little merit [21].

The desirability function Eq. (4) was used to fund optimum point. There are multiple responses, each function (y_i) provides an individual desirability value (d_i), and the global desirability (D) is the geometric mean of these values [21] ($D = d$ when only one response is considered).

$$d = \begin{cases} 1 & y < y_{\min} \\ \left|\frac{y - y_{\max}}{y_{\min} - y_{\max}}\right|, & y_{\text{imin}} \le y_i \le y_{\text{imax}} \\ 0 & y > y_{\max} \end{cases} \quad (4)$$

where $y_{\min}$ and $y_{\max}$ are, respectively, the minimum and the maximum values of the response.

As shown in Fig. 7, an optimal design with desirability factor of 0.8571 was achieved at the low level

Table 4
Analysis of variance

Source	df	Sum of squares	Mean square	Rapport F
Model	4	1.3164076	0.329102	157.6701
Residues	3	0.0062618	0.002087	Prob. > F
Total	7	1.3226695		0.0008

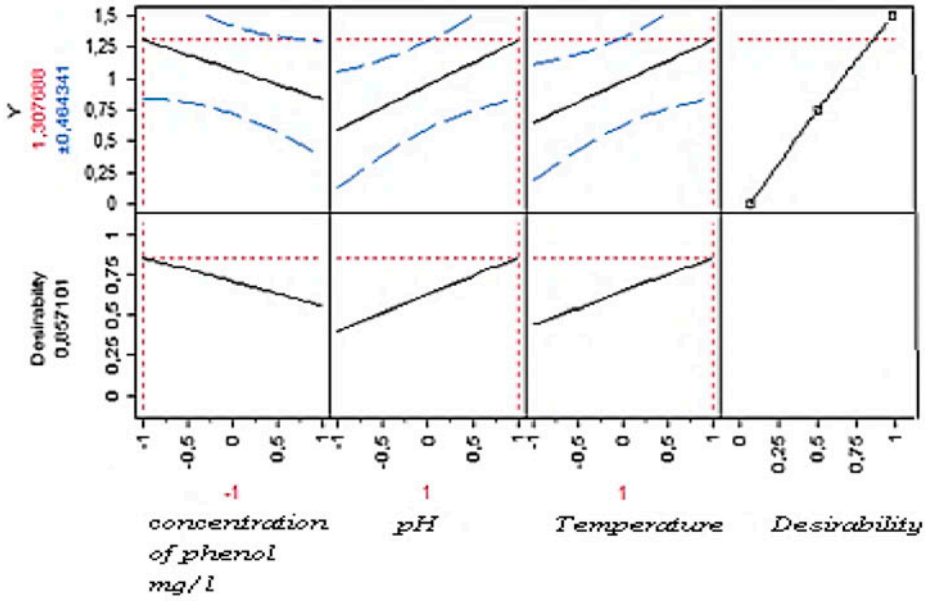

Fig. 7. Main effects.

of the initial phenol concentration (200 mg/L) and the higher levels of pH 8.5 and temperature 35°C.

4. Conclusion

This study indicates the excellent ability of *A. niger* to degrade different concentrations of phenol in batch tests. A full factorial design was carried out to investigate the effects of initial phenol concentration, pH, and temperature on phenol biodegradation. A first-order regression was developed for predicting the response, coefficient R^2 of 99.52%, fitting the statistical model quite well. The effect of the initial concentration of phenol factor has the greatest influence, the second important factor is the interaction between pH and temperature (a_{23}), which was more significant than a_2 (pH) and a_3 (temperature). The maximum rate of phenol elimination was 1.32 mg/h, and this optimum was achieved at 200 mg/L of initial phenol concentration, pH 8.5, and 35°C.

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