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Full Length Research Paper

Statistical optimization of cultural conditions by response surface methodology for phenol degradation by a novel *Aspergillus flavus* isolate

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Phenol is a hydrocarbon compound that highly pollutes the environment. *Aspergillus flavus* having high ability to degrade phenol was isolated. The fungus fully degraded phenol concentration of 100 mgl^{-1} in 72 h, 300 mgl^{-1} in 96 h, 500 mgl^{-1} in 120 h, 700 mgl^{-1} in 240 h, while 900 and 1000 mgl^{-1} needed more than 240 h. On the other hand, 2000 and 3000 mgl^{-1} was lethal to the fungal growth. Statistical designs of the multi-factorial experiment consisting of two serial designs (Plackett-Burman and Box-Behnken) were applied to optimize medium components and growth conditions to raise the fungus potency for phenol degradation and to reduce required time. The fungus achieved 100% (of 500 mgl^{-1}) phenol degradation in 99 h, after application of Plackett-Burman design. The design reduced required time for phenol degradation from 120 to 99 h. And after application of Box-Behnken design, the required time to complete phenol degradation became 97 h instead of 99 h. So the statistical programs raised the fungus efficiency by 20% and reduced required time to complete phenol degradation from 120 to 97 h. These results were applied for the bioremediation of the crude sewage containing phenol concentration of 0.7 mgl^{-1}, which was obtained from the main track of Makkah sewage, where *A. flavus* completed phenol degradation with optimized conditions in four hours. This efficiency proved the ability of this fungus to remove the phenolic compounds from pollution.

Key words: Statistical design, phenol degradation, *Aspergillus flavus*.

INTRODUCTION

Phenol is a hydrocarbon compound that is highly toxic, carcinogenic and mutagenic; it is responsible for environmental pollution while resisting biodegradation (IPCS, 1994; ATSDR, 1998; HazDat, 1998; Colin et al., 1999; Wattiau, 2002; Gondal et al., 2005). It is included in manufactured products like antiseptics and disinfectants, pesticides and paints (TARC, 1989; ANZECC, 1992; WHO, 1994; ATSDR, 2005). It spills and discharges to the environment at high concentrations (TRI96, 1998). The efficient removal of this compound from environment is of great practical significance for environmental protection. Traditionally, phenol is removed by physico-chemical methods, but recently developed biodegradation techniques have the potential to mineralize this toxic compound completely at low processing costs (Bux et al., 1999; Zumriye, 1999), and this makes for rare possibility of secondary pollution (Wang et al., 2000).

Many microorganisms are found to be capable of degrading phenol, especially bacteria (Jiang et al., 2007; Yang and Lee, 2007; Wei et al., 2007) and some yeasts (Bergauer et al., 2005; Yan et al., 2006). But the using of fungi for degradation of phenol is relatively an untouched area.

The optimization of the medium and growth conditions for phenol degradation is of primary importance in the development of the bioprocess. In general, optimization studies involving the one-factor at a time approach are not tedious, but tend to overlook the effects of interacting factors and might lead to misinterpretations of the results. On the other hand, statistical planned experiments effectively solve such problems; minimize the error in determining the effect of parameters and the results are achieved in an economical manner (Abdel-Fattah et al., 2007).

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Response surface methodology, which is supported by software, is an empirical modelization technique derived for 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. Basically, this optimization process involves three major steps: estimating the coefficients in a mathematical model, predicting the response and checking the adequacy of the model (Annadurai et al., 2008).

The present study aimed to isolate local microorganism, having the capability of degrading phenol, as a sole source of carbon and energy and that can optimize its cultural conditions using statistical designs for maximal phenol degradation.

MATERIALS AND METHODS

Microorganism

Aspergillus flavus was isolated from polluted soil at cars reparation's area in Makkah, Saudi Arabia. Identification was done mainly on the basis of cultural and morphological characteristics (Frey et al., 1979; Watanabe, 2002; CBS, 2006).

Inoculums and maintenance

Disc of 8 mm diameter of *A. flavus* grown for 5 days at Sabouraud dextrose agar gl^{-1} (10 mycological peptone, 40 dextrose, 15 agar and pH 5.6 ± 0.2) was used to inoculate 250 ml conical flask containing 100 ml of mineral medium phenol (MMP) mgl^{-1} (400 K_2HPO_4 , 200 KH_2PO_4 , 400 $(NH_4)_2SO4$, 100 NaCl , 200 $MgSO_4$. $7H_2O$, 10 $MnSO_4$. H_2O, 20 $FeSO_4$. $7H_2O$, 10 $NaMoO_4$. $2H_2O$, pH6) (Yan et al., 2005), and phenol concentration of 500 mgl^{-1}; it was incubated at 30 ± 2°C for 120 h with shaking at 150 rpm. Thereafter, 1 ml (2.75×10^4 conidia/ml) was used as inoculum in assays of phenol degradation. Sabouraud dextrose agar slants were used for fungal maintenance, where they were grown for 3 days at 30°C. Then stocks were kept in the refrigerator.

Analytical methods

The residual phenol concentration in the culture medium was estimated colorimetrically using 4-aminoantipyrene (Lacoste et al., 1959) at 492 nm. All experiments were replicated three times and the mean values obtained are reported. The control was a conical flask containing a medium without inoculation undergone under the same experimental conditions. All chemicals were highly pure and obtained from Sigma-Aldrich.

Phenol degradation assay

The efficiency of the fungus to degrade phenol was carried out in 250 ml conical flask containing 100 ml of MMP supplemented with 500 mgl^{-1} phenol and incubated at 30 ± 2°C under shaking (150 rpm) for 120 h. Thereafter, the residual phenol in culture medium was estimated at different time intervals.

Phenol concentration

The effect of phenol concentration (100, 300, 500, 700, 900, 1000, 2000 and 3000 mgl^{-1}) on degradation by *A. flavus* was carried out using 250 ml conical flask containing 100 ml of MMP and incubated at 30 ± 2°C under shaking (150 rpm).

Statistical optimization

Plackett-Burman design

As a preliminary optimization experiment, various medium components and environmental factors have been evaluated, based on the Plackett-Burman factorial design. Each factor was examined in two levels: (-1) for a low level and (1) for a high level (Plackett and Burman, 1946). Table 1 shows the factors under investigation as well as levels of each factor used in the experimental design. The Plackett-Burman experimental design is based on the first order model:

$$Y = \beta_o + \sum \beta_1 X_i$$

Where Y is the response (phenol degradation), β_o is the model intercepts, β_1 is the linear coefficient and X_i is the level of independent variable. In the present study, eleven assigned variables were screened in thirteen experimental designs. All experiments were carried out in triplicates and average of phenol degradation results were taken as the response, and then three chosen variables that have the highest effect on the response were treated as significant variables.

The obtained results from Plackett-Burman design were confirmed in an experiment, where estimation of the residual phenol was done at different time intervals.

Box-Behnken design

In order to describe the nature of the response in the experimental region, a Box-Behnken design (Box and Behnken, 1960) was applied. Table 2 represents the design matrix of a nine trials experiment. Using this design, factors of highest confidence levels were prescribed into three levels coded -1, 0 and 1 for low, middle and high concentrations (or values), respectively. For predicting the optimal point, a second order polynomial function was fitted to correlate relationship between independent variables and response (phenol degradation). For three factors the equation is:

$$Y = \beta_o+\beta_1X_1+ \beta_2 X_2+ \beta_3X_3+ \beta_{12}X_1X_2+ \beta_{13}X_1X_3+ \beta_{23}X_2X_3+ \beta_{11}X_1^2+ \beta_{22}X_2^2+ \beta_{33}X_3^2$$

Where Y is the predicted response, β_o is the model constant, X_1, X_2 and X_3 are independent variables, β_1, β_2 and β_3 are linear coefficients, β_{12}, β_{13} and β_{23} are cross product coefficients and β_{11}, β_{22} and β_{33} are the quadratic coefficients.

Experiments were performed in triplicates and mean values are given. Microsoft Excel was used for the regression analysis of the experimental data obtained. The fitting quality of the polynomial model equation was expressed by the coefficient of determination R^2. The optimal value of phenol degradation was estimated using the solver function of microsoft excel tools.

The obtained results from Box-Behnken design were confirmed in an experiment, where the residual phenol was estimated at different time intervals.

Application of the final obtained results

Bioremediation of sewage, polluted with 0.7 mgl^{-1} concentration of phenol by application of the final obtained results, was carried out.

Table 1. Plackett-Burman experimental design for elucidation of factors affecting phenol degradation.

Trial	Independent variables (gl^{-1})											Phenol Deg. (%)
	K2	NH	Na	Mg	Mn	Fe	Mo	P	IS	MS	Ag	
1	(1) 1	(-1) 0.1	(1) 0.2	(-1) 0.05	(1) 0.0	(-1) 0.0	(1) 0.03	7	2.0	50	200	72
2	(1) 1	(1) 0.8	(-1) 0.0	(1) 0.5	(1) 0.0	(-1) 0.0	(-1) 0.0	7	2.0	150	100	82.4
3	(-1) 0.2	(1) 0.8	(1) 0.2	(-1) 0.05	(1) 0.03	(-1) 0.0	(-1) 0.0	5	2.0	150	200	57.8
4	(1) 1	(-1) 0.1	(1) 0.2	(1) 0.5	(-1) 0.0	(1) 0.05	(-1) 0.0	5	0.5	150	200	71.6
5	(1) 1	(1) 0.8	(1) 0.0	(1) 0.5	(1) 0.03	(-1) 0.0	(1) 0.03	5	0.5	50	200	100
6	(1) 1	(1) 0.8	(1) 0.2	(-1) 0.05	(1) 0.03	(1) 0.05	(-1) 0.0	7	0.5	50	100	96.2
7	(-1) 0.2	(1) 0.8	(1) 0.2	(1) 0.5	(1) 0.0	(1) 0.05	(1) 0.03	5	2.0	50	100	59
8	(-1) 0.2	(-1) 0.1	(1) 0.2	(1) 0.5	(1) 0.3	(-1) 0.0	(1) 0.03	7	0.5	150	100	23
9	(-1) 0.2	(-1) 0.1	(1) 0.0	(1) 0.5	(1) 0.3	(1) 0.05	(-1) 0.0	7	2.0	50	200	32
10	(1) 1	(-1) 0.1	(1) 0.0	(-1) 0.05	(1) 0.03	(1) 0.05	(1) 0.03	5	2.0	150	100	70
11	(-1) 0.2	(1) 0.8	(1) 0.0	(-1) 0.05	(1) 0.0	(1) 0.05	(1) 0.03	7	0.5	150	200	47
12	(-1) 0.2	(-1) 0.1	(1) 0.0	(-1) 0.05	(1) 0.0	(-1) 0.0	(-1) 0.0	5	0.5	50	100	7
13	(0) 0.6	(0) 0.4	(0) 0.1	(0) 0.2	(0) 0.01	(0) 0.02	(0) 0.01	6	1.0	100	150	87.4

K_2HPO_4 (K2), $(NH_4)_2SO_4$ (NH), NaCl (Na), $MgSO_4$. $7H_2O$ (Mg), $MnSO_4$. H_2O (Mn), $FeSO_4$. $7H_2O$ (Fe), $NaMoO_4$. $2H_2O$ (Mo), pH (P), inoculum size (IS), medium size (MS), agitation (Ag).

Table 2. Box-Behnken design for three significant independent variables.

Trial	Variables			Consumption (%)
	X_1 (gl^{-1})	X_2 (gl^{-1})	X_3 (rpm)	
1	(-1) 0.5	(-1) 0.4	(-1) 100	87.8
2	(-1) 0.5	(0) 0.8	(1) 300	90
3	(-1) 0.5	(1) 1.6	(0) 200	90.4
4	(0) 1	(-1) 0.4	(1) 300	94
5	(0) 1	(0) 0.8	(0) 200	97.8
6	(0) 1	(1) 1.6	(-1) 100	93.8
7	(1) 2	(-1) 0.4	(0) 200	100
8	(1) 2	(0) 0.8	(-1) 100	98.4
9	(1) 2	(1) 1.6	(1) 300	96.2

K_2HPO_4 (X_1), $(NH_4)_2SO_4$ (X_2), Agitation (X_3)

RESULTS AND DISCUSSION

Isolation and identification of fungus

One fungus that was able to grow and degrade phenol as the sole carbon and energy source was isolated from polluted soil at cars reparation's area in Makkah-Saudi Arabia. Identification was done based on macroscopic characters and microscopic characteristics, where it was found to belong to *A. flavus* according to Frey et al. (1979), Watanabe, (2002), CBS, (2006). And according to available literatures, this is the first time this fungus has been reported to possess phenol-degradative ability.

Degradation of phenol by *A. flavus*

A. flavus showed high potency for phenol degradation at concentration of 500 mgl^{-1} by 100% at 120 h. The genus, *Aspergillus* contains more than 200 species. Their ability to degrade phenol was known for *A. awamori* (Shyam et al., 2005; Stoilova et al., 2006), *A. fumigatus* (Jones et al., 1985, 1993, 1994 and 1995), *A. niger* (Garcia et al., 2000; Hegde et al., 2006) and *A. terreus* (Borja et al., 1993; Garcia et al., 1997; Garcia et al., 2000) which showed high efficiency to degrade phenol. However, *A. flavus*, super competency was not reported.

The effect of degradation on phenol concentration

Figure 1 showed that phenol concentrations of 100, 300, 500 and 700 mgl^{-1} can be degraded by *A. flavus* in 72, 96, 120 and 240 h, respectively. While at 900 and 1000 mgl^{-1} there was weak fungal growth and more than 240 h

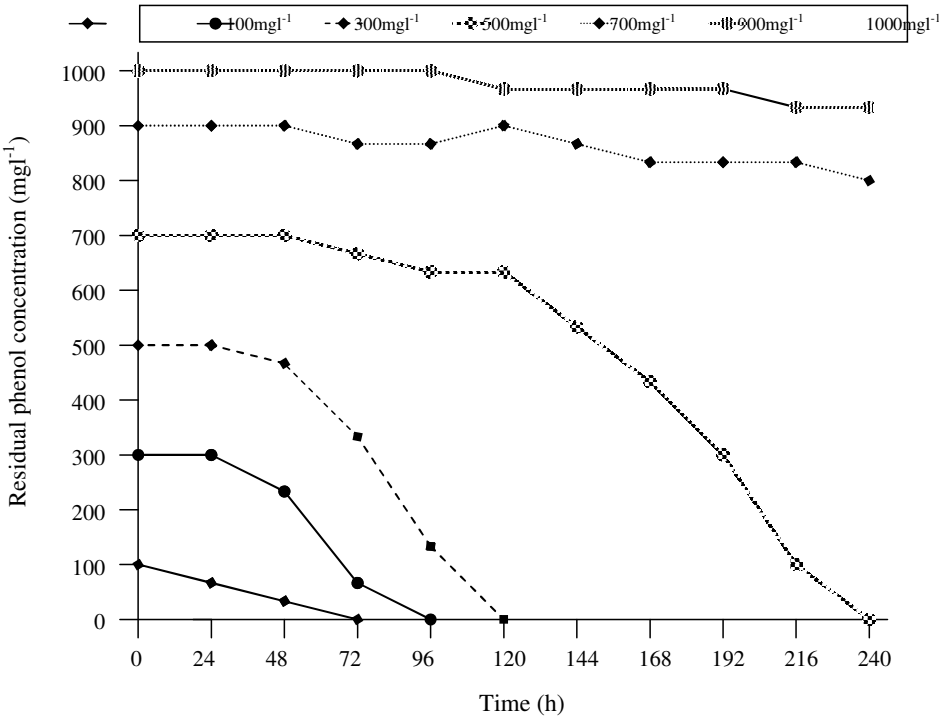

Figure 1. The residual concentrations of phenol (mgl^{-1}) after different incubation periods of degradation by*A. flavus.*

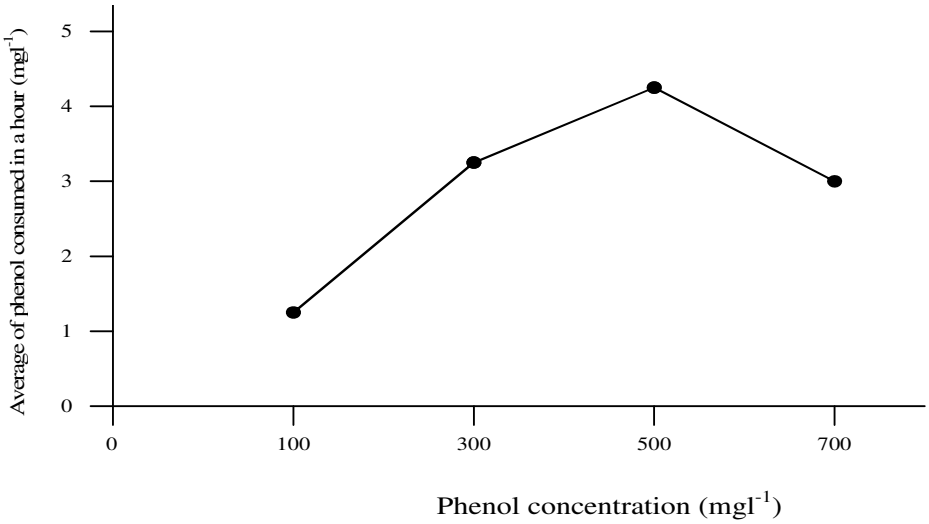

Figure 2. Quantity of phenol consumed per an hour by *A. flavus* under effect of different phenol concentrations.

was needed for their full degradation. No growth was observed at 2000 and 3000 mgl^{-1}. At 100, 300 and 500 mgl^{-1}, vegetative fungal growth (mycelium) was detected and at 700 mgl^{-1} both mycelium and conidia were detected. At 900 and 1000 mgl^{-1} fungal sporulation dominated.

This finding may be interpreted that conidia are more constant than vegetative structures in fungi. Also, data about the physiological activity of the spores of other fungi capable of organic compounds transformation have been published (Kojuharova et al., 2000). Similar observations were reported using *A. awamori* (Stoilova et al., 2006). Figure 2 indicated that phenol concentration of 500 mgl^{-1} is optimum for degradation by *A. flavus* (4.166 mgh^{-1}).

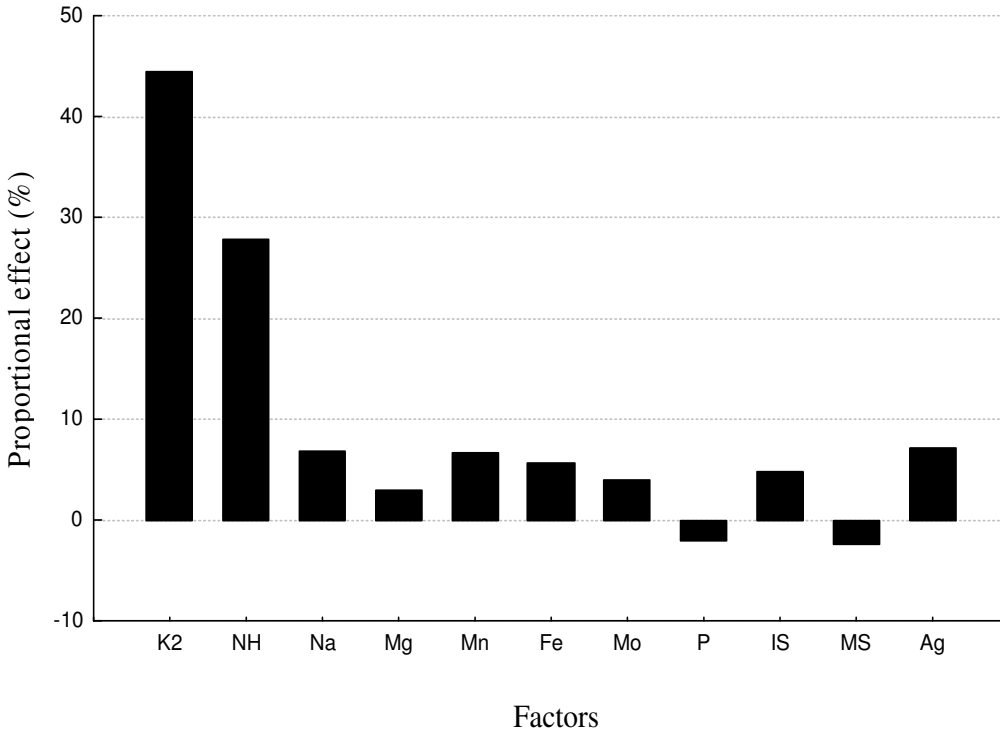

Figure 3. Proportional effect for applied factors in Plackett-Burman design.

Evaluating the significance of the cultural conditions by Plackett-Burman design

Plackett-Burman design was applied to estimate the significance of culture conditions in phenol degradation by *A. flavus*. Table 1 represents the design matrix together with response observations. The results indicated that levels of factors at trial 5 were better. The main effect for each variable was estimated and the results presented graphically in Figure 3 revealed that, the three factors which were more effective were dipotassium hydrogen phosphate by positive effect of 44.4%, ammonium sulphate by 27.8%, and agitation by 7.1%. The main effect of both pH and medium size/flask was negative -2.1 and -2.3%, respectively. Other factors have positive main effect that ranged from 3 - 6.9%.

According to the results the obtained medium formula which was predicted to be near optimum was (mgl^{-1}): 1.0 K_2HPO_4, 0.8 $(NH_4)_2SO_4$, 0.0 NaCl, 0.5 $MgSO_4$. $7H_2O$, 0.03 $MnSO_4$. H_2O, 0.05 $FeSO_4$. $7H_2O$, 0.03 $NaMoO_4$. $2H_2O$, pH 5, inoculum size 0.5 ml, medium size 50 ml/ 250 ml conical flask, agitation 200 rpm, phenol conc. 0.5, and incubation temperature of 30°C for 100.18 h.

A confirmatory experiment was conducted to verify the predicted results, where the result of the new levels of cultural conditions that indicated phenol degradation percentage (100%) of 500 mgl^{-1} was achieved in 99 h. Thus, the time of degradation reduced 17.5% as compared to basal conditions (before optimization process).

Optimization of the factors affecting phenol degradation by using Box-Behnken design

In this part further optimization of significant variables including K_2HPO_4, $(NH_4)_2SO_4$ and agitation by applied response surface methodology via Box-Behnken design (Box and Behnken, 1960) were performed. Table 2 shows the design matrix; nine trials with their response observations which follow formula (mgl^{-1}): 0.2 NaCl, 0.5 $MgSO_4$. $7H_2O$, 0.03 $MnSO_4$. H_2O, 0.05 $FeSO_4$. $7H_2O$, 0.03 $NaMoO_4$. $2H_2O$, pH5 , inoculum size 2 ml, medium size 50 ml, agitation 200 rpm, phenol conc. 0.5, temperature of 30°C and incubation period of 99 h.

Trial 7 was the best where it verified highest percentage (100%) of phenol degradation (500 mgl^{-1}) in 97 h. So, the required time for phenol degradation was reduced from 120 h in basal conditions to 99 h after primary optimization via Plakett-Burman design, to 97 h by Box-Behnken design. Figures 4, 5 and 6 show graphically the relationship and interaction between independent variables (K_2HPO_4, $(NH_4)_2SO_4$ and agitation) and response (phenol degradation percentage).

Moreover, a confirmatory experiment was carried out to verify the predicted results using the basal conditions as control. When the optimized conditions obtained from the second optimization by Box-Behnken design was applied, the results showed 100% of degradation of phenol of 500 mgl^{-1} in 97 h instead of 99 h after primary optimization by Plackett-Burman design, and instead 120 h before the

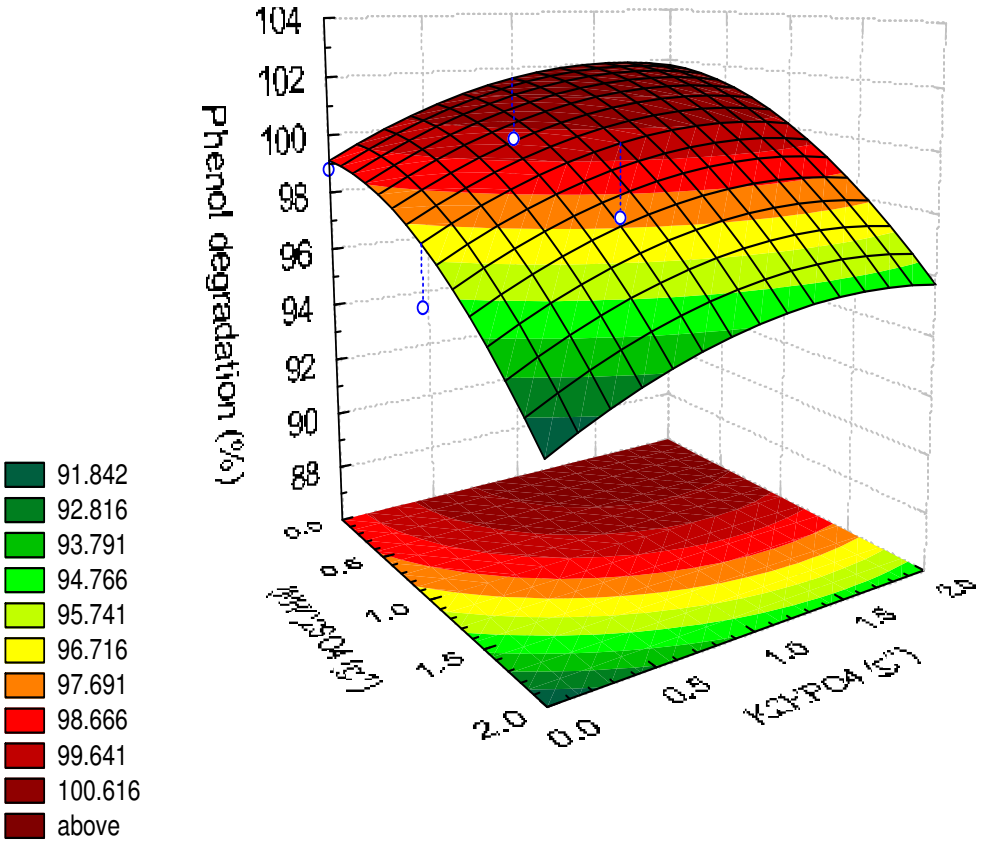

Figure 4. Interaction K_2HPO_4 with $(NH_4)_2SO_4$ with respect to phenol degradation percentage based on Box-Behnken experiments results.

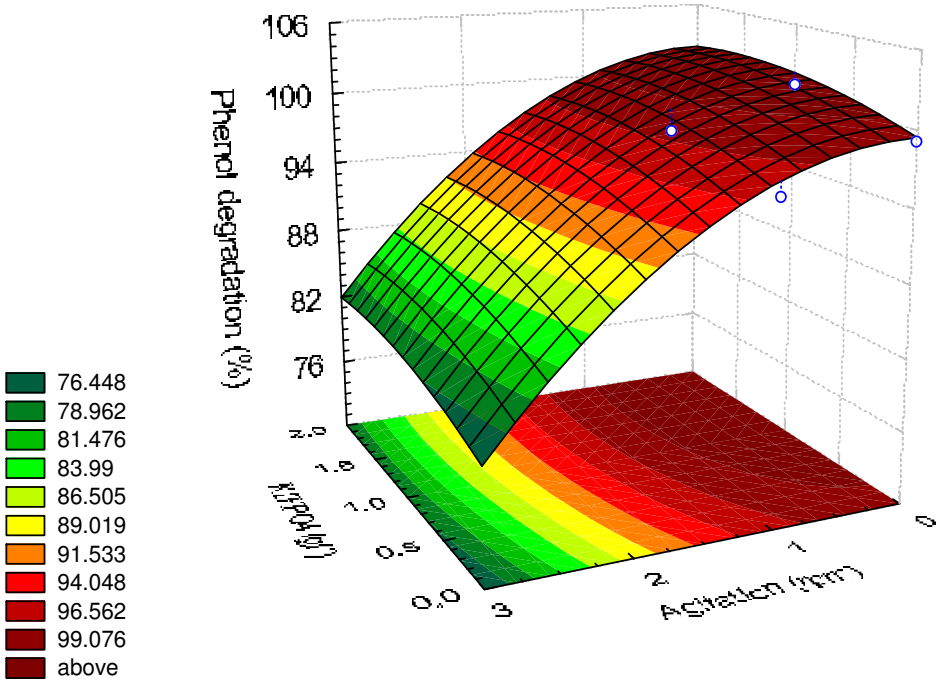

Figure 5. Interaction K_2HPO_4 with Agitation with respect to phenol degradation percentage based on Box-Behnken experiments results.

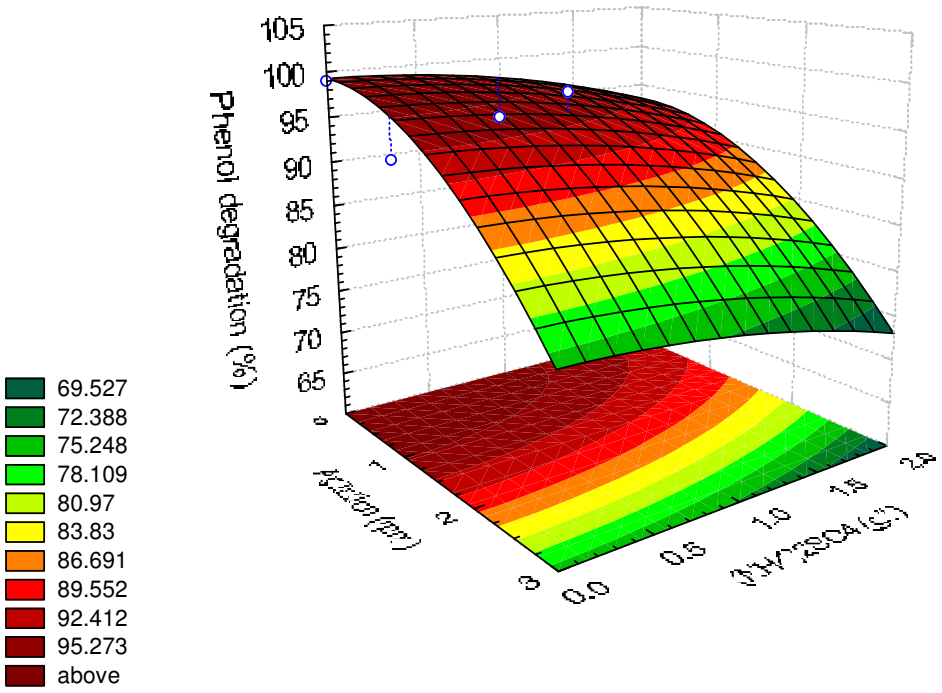

Figure 6. Interaction Agitation with $(NH_4)_2SO_4$ with respect to phenol degradation percentage based on Box-Behnken experiments results.

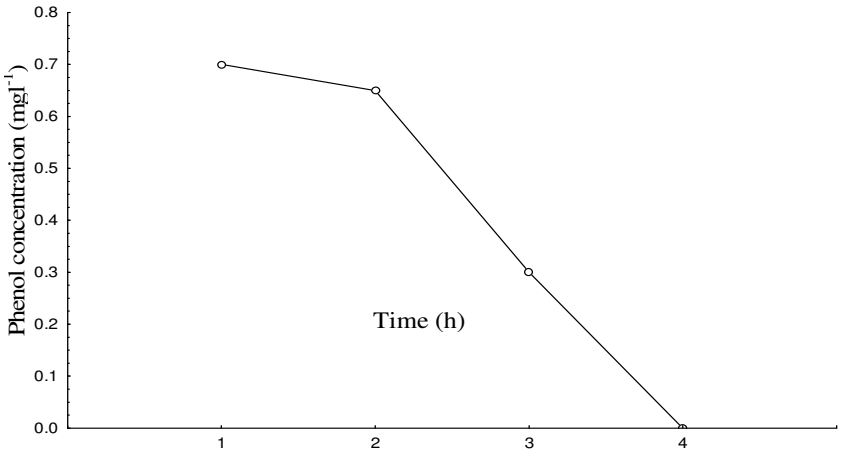

Figure 7. Result of phenol degradation by *A. flavus* in crude polluted sewage.

optimization. From that, the required time to full degradation of phenol at concentration of 500 mgl^{-1} by *A. flavus* reduced by 23 h after completing whole optimization.

So, *A. flavus* for first time showed high ability and efficiency on phenol as a sole source of carbon and energy by 100% degradation of 500 mgl^{-1} in 97 h and by average 5.15 mgh^{-1}. It was better than fungus *Penicllium chrysogenum* that could degrade 300 mgl^{-1} of phenol in 100 h (Leitao et al., 2007) and exceeded the strain LA2 of *Aspergillus* sp. which verified average 3.297 mgl^{-1} of phenol degradation (Santos and Linardi, 2004).

Degradation of phenol polluted sewage

The optimized cultural conditions obtained were applied to bioremediate crude sewage polluted with phenol at concentration of 0.7 mgl^{-1}, which was obtained from the main track of sewage network of Makkah City. *A. flavus* could degrade the whole phenol content, which is present in the crude sewage, only after four hours as shown in Figure 7. This finding is promising as compared to those reported by Stover and Kincannon (1983) who bioremediated only 30% of phenol polluted sewage after 8 h.

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REFERENCES

Abdel-Fattah YR, El-Helow ER, Ghanem KM, Lotfy WA (2007). Application of factorial designs for optimization of avicelase production by a thermophilic *Geobacillus* isolate, J. Microbiol. 2: 13-23.

Annadurai G, Ling LY, Lee JF (2008). Statistical optimization of medium components and growth conditions by response surface methodology to enhance phenol degradation by *Pseudomonas putida*. J. Hazar. Mate. 151: 171-178.

ANZECC (1992). Australian and New Zealand Environment and Conservation Council, Australian water quality guidelines for fresh and marine water.

ATSDR (1998). Agency for Toxic Substances and Disease Registry, Toxicologyical profile for phenol, Atlanta GA.: U.S. Department of Health and Services, Public health service.

ATSDR (2005). Agency for Toxic Substances and Disease Registry, Toxicologyical profile for phenol, Atlanta, Coorgia, p. 111.

Bergauer P, Fonteyne PA, Nolard N (2005). Biodegradation of phenol and phenol-related compounds by psychrophilic and cold-tolerant alpine yeasts, chemosphere, 59: 909-918.

Borja R, Garrido SE, Martinez L, Ramos-Comenzana A, Martin A (1993). Kinetic study of anaerobic digestion of olive mill waste-water previously fermented with *Aspergillus terreus*, Process Biochem. 28: 397-404.

Box GEP, Behnken DW (1960). Some new three level designs for the study of quantitative variables, Technometrics, 2: 455-475.

Bux F, Akkinson B, Kasan K (1999). Zinc biosorption by waste activated and digested sludges. Water Sci. Technol. 39: 127-130.

CBS (2006). Centraal Bureauvoor Schimmelcultures, Identification common species of *Aspergillus*, Available at: www.cbs.knaw.

Colin F, Bassi A, Gijzen M (1999). Removal of aqueous phenol and 2-chlorophenol with purified soybean peroxidase and raw soybean hulls, J. Chem. Technol. Biotechnol. 74: 303-309.

Frey D, Oldfield RJ, Bridger RC (1979). Color atlas of pathogenic fungi, Printed by Smeets-Weert, Holland, pp. 92-93.

Garcia I, Bonilla JL, Jimenez PR, Ramos E (1997). Biodegradation of phenols compounds in vinasse using *Aspergillus terreus* and *Geotrichum candidum*, Water Res. 31: 8-11.

Garcia I, Pena, PR, Venceslade JL, Martin A, Santos MA, Gomez ER (2000). Removal of phenol compounds from olive mill wastewater using *Phanerochaete chrysosporium*, *Aspergillus niger*, *Asprgillus terreus* and *Geotrichum candidum*, Process Biochem. 35: 751-758.

Gondal MA, Hameed A, Yamani ZH, Seddigi Z (2005). Laser-induced photocatalysis and its applications in petrochemicals, Fuel cells and phenol degradation, Proccedings of 15th, Saudi-Japan Joint Symposium, Dhahran, Saudi Arabia.

HazDat (1998). Hazardous substance database, Agency for toxic substances and disease registry, Atlanta, GA.

Hegde S, Kavitha S, Varadaraj MC, Muralikrishna G (2006). Degradation of cereal bran polysaccharide-phenolic acid complexes by *Aspergillus niger* CFR 1105, J. Food Chem. 96: 14-19.

IPCS (1994). Phenol, Environmental health criteria document No. 161.

Jiang Y, Jianping W, Bai J, Jia X, Hu Z (2007). Biodegradation of phenol at high initial concentration by *Alcaligenes faecalis*, J. Hazar. Mat. 12: 321-328.

Jones KH, Trudgill PW, Hopper DJ (1993). Metabolism of *p*-cresol by the fungus *Aspergillus fumigatus*, Appl. Environ. Microbial. 59: 1125-1130.

Jones KH, Trudgill PW, Hopper DJ (1994). 4-Ethylphenol metabolism by *Aspergillus fumigatus*, Appl. Environ. Microbiol. 60: 1978-1983.

Jones KH, Trudgill PW, Hopper DJ (1995). Metabolism of phenolic compounds by *Aspergillus fumigatus*, Appl. Environ Microbiol. 66: 112-123.

Jones KH, Trudgill PW, Hopper DJ (1985). Evidence of two pathways for the metabolism of phenol by *Aspergillus fumigatus*, Arch. Microbiol. 163: 176-181.

Kojuharova L, Gargova S, Bahchevanska S (2000). Biotransformation, In: Biotechnology Industries, Publ. UFT Plovdiv, pp. 335-343.

Lacoste RJ, Venable SH, Stone JC (1959). Modified 4-aminoantipyrene colorimetric method for phenols. Applications to an acrylic monomer. Anal. Chem. 31: 1246-1249.

Leitao AL, Duarte MP, Oliveira JS (2007). Degradation of phenol by a halotolerant strain of *Penicillium chrysogenum*, Int. Biodeteri. Biodegrad. 59: 220-225.

Plackett RL, Burman JP (1946). The design of optimum multifactorial experiments, Biometrika, 33: 305-325.

Santos VL, Linardiu VR (2004). Biodegradation of phenol by a filamentous fungi isolated feom industrial effluents-identification and degradation potential, Process Biochem. 39: 1001-1006.

Shyam SP, Daniel DK, Krastanov AI (2005). Determination of the most significant factors affecting the degradation of phenol using immobilized *Aspergillus awamori* cells, J. Agric. Sci. 11: 61-67.

Stoilova I, Krastanov A, Stanchev V, Daniel D, Gerginova M, Alexieva Z (2006). Biodegradation of high amounts of phenol, catechol, 2, 4-dichlorophenol and 2, 6-dimethoxyphenol by *Aspergillus awamori* cells, Enz. Microbial Technol. 39: 1036-1041.

Stover EL, Kincannon DF (1983). Biological treat ability of specific organic compounds found in chemical industry wastewaters, J. Water Pollut. Control. Fed. 55: 97-109.

TARC (1989). Thailand Accident Research Center: Phenol and pollution, TARC, 115.

TRI96 (1998). Toxic chemical release inventory, National Toxicology information program, Bethesda, MD.

Wang CC, Lee CM, Lu CJ, Chuang MS, Huang CZ (2000). Biodegradation of 2,4,6-trichlorophenol in the presence of primary substrate by immobilized pure culture bacteria, Chemosphere, 41: 1873-1879.

Watanabe T (2002). Pictorial atlas of soil and seed fungi, CRC Press, 2nd Ed, pp. 190-196.

Wattiau P (2002). Microbial aspects in bioremediation of soil polluted by polyaromatic hydrocarbons, Focus Biotechnol. 3: 1-22.

Wei G, Yu J, Zhu Y, Chen W, Wang L (2007). Characterization of phenol degradation by *Rhizobium* sp. CCNWTB 701 isolated from *Astragalus chrysopteru* in mining tailing region, J. Hazar. Mat. 22: 85-91.

WHO (1994). IPCS Environmental health criteria for phenol first draft prepared by Ms G. K. Montizan, Published by WHO, Printed in Finland.

Yan J, Jianping W, Hongmei L, Suliang Y, Zongding H (2005). The biodegradation of phenol at high initial concentration by the yeast *Candida tropicalis*, J. Biochem. Eng. 24: 243-247.

Yan J, Jianping W, Jing B, Daoquan W, Zongding H (2006). Phenol biodegradation by the yeast *Candida tropicalis* in presence of *m*-cresol, J. Biochem. Eng. 29: 227-234.

Yang CF, Lee CM (2007). Enrichment, isolation, and characterization of phenol-degrading *Pseudomonas resinovorans* strain P-1 and *Brevibacillus* sp. strain P-6, Inter. Biodeteri. Biodegrad. 59: 206-210.

Zumriye A, Derya A, Elif R, Burcin K (1999). Simultaneous biosorption of phenol and nickel from binary mixtures onto dried aerobic activated sludge. Process. Biochem. 35: 301-308.