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# Optimization of chloroxylenol degradation by *Aspergillus niger* using Plackett-Burman design and response surface methodology

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## Abstract

*Chloroxylenol is a very toxic phenolic derivative and it represents potential hazard towards human health and to the environment. Aspergillus niger, local isolate, is an efficient fungus to degrade 99.72% of 2mg/L of chloroxylenol after 7days of fermentation. It also has a high capacity to degrade 91.83% of higher chloroxylenol concentration of 20mg/L after 6days of incubation on mineral medium amended with 2g/L of glucose. Statistical experimental designs were used to optimize the process of chloroxylenol degradation by the fungus. The most important factors influencing chloroxylenol degradation, as identified by a two-level Plackett-Burman design with 11 variables, were NaCl, $(NH_4)_2SO_4$, and inoculums size. Response surface analysis was adopted to further investigate the mutual interactions between these variables and to identify their optimal values that would generate maximum chloroxylenol degradation. Under the optimized medium compositions and culture conditions, A.niger degraded completely (100%) chloroxylenol (20mg/L) after 134.6hr of fermentation. The predicted values of Plackett-Burman conditions and response surface methodology were further verified by validation experiments. The excellent correlation between predicted and experimental values confirmed the validity and practicability of this statistical optimum strategy. Optimal conditions obtained in this work laid to a solid foundation for further use of A.niger in treatment of high strength chloroxylenol polluted effluents. So, the optimized conditions were applied to bioremediate crude sewage containing 27.8mg/L of chloroxylenol by A.niger. The fungus efficiently degraded chloroxylenol after 8days of fermentation.*

**Key words:** Chloroxylenol degradation; *Aspergillus niger*, Statistical optimization; Plackett-Burman design; Response surface methodology.

## Introduction

The compound 4-chloro-3,5-dimethylphenol, chloroxylenol, is a phenolic derivative, the key halophenol used in many antiseptic or disinfectant formulations ( Bruch, 1996). It has unique antiseptic properties and is very effective topical antimicrobial agent against the common infectious germs, where it acts as a disruptor of the proton gradient of the cell membrane necessary for the bacteria to produce ATP, whose deficiency leads to the death from starvation (Wilson and Mowad, 2007). It also changes the permeability of the cell walls of microorganisms and hindering their biological processes. It oxidizes the cell structure, which retards the passage of nutrients through the cell wall, resulting in a loss of normal enzyme activity and cell death (Kim et al, 2002). Owing to its large production as well as various applications and presence in many products and formulations, its compounds can be discharged directly or via urban sewage systems into the aquatic systems. Chloroxylenol as a biocide represents potential hazard towards human health and welfare, and can have adverse impacts on the ecological environment (Yamano et al, 2004; Kupper et al, 2006). Hence efforts are now focused on the removal of these compounds from aqueous solutions by adsorption (Kestioglu et al, 2005), coagulation (Zhang et al, 2004), or oxidation (Song et al, 2009). All these methods have significant limitations and disadvantages. Bioremediation has

received the most attention, because it is friendly to environment, inexpensive and can potentially turn a toxic material into a harmless product (Prpich and Daugulis, 2005). Bioremediation technique could potentially degrade cholorophenols to innocuous products of $CO_2$, $H_2O$ and chlorine (Murialdo et al, 2003). Microbial degradation of cholorophenols has been reported by many workers (Tuomela et al, 1999; Reddy and Gold, 2000; Cortex et al, 2002; Murialdo et al, 2003).

The ability of microorganisms to degrade pollutants and growth of cells are strongly influenced by nutritional and environmental parameters. However, as far as we know, there is no knowledge about nutritional and environmental requirements for chloroxylenol degradation by *A.niger*. Therefore, it is necessary to design an appropriate process for maximizing the degradation efficiency of chloroxylenol by *A.niger*.

Statistical experimental designs such as Plackett-Burman and response surface methodology (RSM) (Kennedy and Krouse, 1999) can collectively optimize all the affecting parameters to eliminate the limitations of a single-factor optimization process. Plackett-Burman design provides a fast and effective way to identify the important factors among a large number of variables, thereby, saving time and maintaining convincing information on each parameter (Abdel-Fattah et al, 2005). RSM, which includes factorial design and regression analyses, helps in evaluating the important factors, building models to study the interactions between the variables or desirable responses (Ghanem et al, 2010). Plackett-Burman design and RSM have been successfully employed to optimize some bioprocesses (Lotfy et al, 2007; Mohana et al, 2008; Ghanem et al, 2011; 2012). Recently, statistical optimization designs for phenol degradation have been reported (Agarry et al, 2008; Annadurai et al, 2008; Ghanem et al, 2009; Zhou et al, 2011). But there is no report focusing on biodegradation of chloroxylenol by *A.niger*.

The present study aimed to degrade chloroxylenol by local isolate of *A.niger* as influenced by fermentation medium, level of chloroxylenol, and fermentation period. Thereafter, a Plackett-Burman design and RSM are used to optimize medium compositions and culture conditions for maximizing chloroxylenol degradation by *A.niger*.

## Materials and method

### Microorganism

*Aspergillus niger* was isolated from sewage polluted soil at the lake of Sewage Disposal, Jeddah, Saudi Arabia, using Sabouraud dextrose agar. Identification was done on the basis of cultural and morphological characteristics (Frey et al, 1979; Watanabe, 2002; CBS, 2006).

### Inoculum and cultivation

*A.niger* was maintained on Sabouraud dextrose agar (SDA), where the fungus was grown for 5days at 30$^o$C. The stocks were kept in the refrigerator and subcultures at monthly intervals were done. Spores suspension of *A.niger* was prepared by washing 5days old culture slants with sterilized saline solution (0.9% NaCl) and shaking vigorously for 1min. Spores were counted by a haemocytometer to adjust the count approximately to $5x10^6$ spores/ml. Basal medium used in chloroxylenol degradation included (g/L): glucose, 2.0; $(NH_4)_2SO_4$, 0.5; $(NH_4)NO_3$, 1.0; $MgSO_4.7H_2O$, 0.5; $K_2HPO_4$. 1.0; $KH_2PO_4$, 0.5; NaCl, 0.5; $CaCl_2$, 0.02; trace elements solution, 2ml and pH6 (Ghanem et al, 2009). Trace elements solution included (g/L): $FeSO_4$, 0.1; $ZnSO_4$. 0.1; $KAl(SO_4)_2$ ,0.01; $NaMo_2O_4$, 0.01; $CoCl_2$, 0.1; $CuSO_4$, 0.01 and $H_3BO_3$, 0.01.

Batch mode shake flask experiments were conducted in 250ml Erlenmeyer flasks containing 50ml of the basal medium. Flasks were inoculated with standard inoculums ($5x10^6$ spores/ml) and incubated in shaking incubator (180rpm) at 30$^o$C. After degradation for 168hr (7days), the

fermentation media were centrifuged at 7000xg for 20min in a cooling centrifuge and supernatants were used to measure concentrations of residual chloroxylenol. It was determined quantitatively by the spectrophotometric method using 4-aminoantipyrine as color indicator with maximum absorbance of 510nm according to the standard methods (APHA, 1998).

All experiments were performed in triplicates and the average of the three independent experiments was taken as the result.

**Effect of chloroxylenol (CDP) concentration**

The effect of chloroxylenol concentration (2-20mg/L) on degradation activity of *A.niger* was carried out using 250ml Erlenmeyer flasks containing 50ml of basal medium and incubated at 30$^{o}$C for 168hr.

**Effect of fermentation media**

The quantity of ingredients of the biodegradation medium in favor of chloroxylenol degradation by *A.niger* were tested using seven different media as follows (g/L): I) basal medium (Ghanem et al, 2009); II) $MgSO_4.7H_2O$, 0.5; $K_2HPO_4$, 2.3; $KH_2PO_4$, 11.8; $CuSO_4$, 0.05; NaCl, 0.05; $(NH_4)Cl$, 0.25; $MnSO_4$, 0.01, $FeSO_4$, 0.1, $ZnSO_4$, 0.01 (Cai et al, 2007); III) $K_2HPO_4$, 1.0; $(NH_4)_2SO_4$, 1.0; $MgSO_4.7H_2O$, 0.2; $FeCl_3.6H_2O$, 0.033; NaCl, 0.1; $CaCl_2$, 0.1 (modified Leitao et al, 2007); IV) $K_2HPO_4$, 3.4; $KH_2PO_4$, 4.3; $MgSO_4.7H_2O$, 0.3; $(NH_4)_2SO_4$, 1.0; yeast extract, 0.05; $MnCl.4H_2O$,1.0; $FeSO_4.7H_2O$, 0.6; $CaCl_2.H_2O$, 2.6; $NaMoO_4.2H_2O$, 6.0; trace elements solution, 5ml (modified Santos and Linardi, 2004); V) $K_2HPO_4$, 1.0; yeast extract, 2; sucrose, 0.2; $NaNO_3$, 30.0; KCl, 35.0; $MgSO_4.7H_2O$, 5.0; $ZnSO_4.7H_2O$, 0.1, $CuSO_4.7H_4O$, 0.05; 1ml of Czapek's concentrated solution (modified Pitt, 1973); VI) $NaNO_3$, 3.0; $K_2HPO_4$, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.01; sucrose, 0.2 (Czapek's broth); VII) $K_2HPO_4$, 0.4; $KH_2PO_4$, 0.2; $(NH_4)_2SO_4$, 0.4; NaCl, 1.0; $MgSO_4.7H_2O$, 0.2; $MnSO_4$, 0.01; $FeSO_4.7H_2O$, 0.02; $NaMoO_4.H_2O$, 0.01 (Yan et al, 2005). After degradation period of 168hr the chloroxylenol concentrations were determined.

**Time course degradation of 20mg/L of chloroxylenol (CDP)**

In order to minimize the degradation period for chloroxylenol (20mg/L), *A.niger* was inoculated into 50ml aliquots of the basal medium (best medium) and incubated for different incubation periods. Thereafter, growth and concentrations of chloroxylenol were determined at 24hr intervals, after 48hr of growth, up to 240hr.

**Experimental designs**

The basal medium composition, quantities of inoculums and culture conditions were used for statistical optimization designs of Plackett-Burman and RSM.

**Plackett-Burman design**

It is an efficient way to identify the important factors among a large number of variables (Stanbury et al, 1986). It was used to screen the important variables that significantly influenced chloroxylenol degradation. In this study, a 13-run Plackett-Burman design was applied to evaluate eleven factors. Each variable was examined at two levels: -1 for the low level, and +1 for the high level (Table 2). All trails were performed in duplicates and the averages of degradation observation results were treated as responses. The main effect of each variable (Table 2) was determined with the following equation:

$$E_{xi} = (\Sigma M_{i+} - \Sigma M_{i-})/N$$

Where $E_{xi}$ is the variable main effect, $M_{i+}$ and $M_{i-}$ are the chloroxylenol degradation percentages in trails where the independent variable (xi) was present in high and low concentrations, respectively, and N is the number of trails divided by 2. The main effect figure with a positive sign indicates that high concentration of this variable is nearer to optimum and a negative sign indicates that the low concentration of this variable is nearer to optimum. Using Microsoft Excel, statistical *t*-values for equal unpaired sample (Table 2) were calculated for determination of variable significance.

**Table 1.** Effect of different fermentation media on the efficiency of *Aspergillus niger* to degrade cholorxylenol (1000µg/50ml) within 7 days of degradation.

| Medium | Final pH | Dry weight (mg/50ml) | Biodegraded CDP (µg/50ml) | Biodegradatn. ( % ) | Biodegradatn. rate (µg/day) | Biodegradatn. efficiency ( % ) |
|---|---|---|---|---|---|---|
| I (Basal) | 5.5 | 126 | 907.70 | 90.77 | 129.67 | 100 |
| II | 6.0 | 80 | 693.13 | 69.31 | 99.02 | 76.36 |
| III | 5.9 | 80 | 736.13 | 73.61 | 105.16 | 81.10 |
| IV | 6.0 | 60 | 536.88 | 53.69 | 76.70 | 59.14 |
| V | 6.0 | 90 | 889.23 | 88.92 | 127.03 | 97.96 |
| VI | 5.5 | 70 | 663.13 | 66.31 | 94.73 | 73.05 |
| VII | 5.9 | 60 | 786.88 | 78.68 | 112.41 | 86.68 |

$$\text{Biodegradation (\%)} = \frac{\text{Degraded chloroxylenol (µg)}}{\text{Initial chloroxylenol (µg)}} \times 100$$

$$\text{Biodegradation Rate (µg/day)} = \frac{\text{Degraded chloroxylenol (µg)}}{\text{Fermentation period (day)}}$$

$$\text{Biodegradation efficiency (\%)} = \frac{\text{Degradation rate of test(µg/day)}}{\text{Highest degradation rate(µg/day)}} \times 100$$

**Table 2.** Factors examined as independent variables affecting chloroxylenol degradation, their levels and their main effects in the Plackett-Burman experiment.

| Factor | Symbol | Level | | | Main effect (%) | *t*-value (at 5% signif.) |
|---|---|---|---|---|---|---|
| | | -1 | 0 | +1 | | |
| Glucose (g/L) | G | 0.0 | 2 | 4 | -4.17 | -0.65 |
| Trace elements solution (ml/L) | T | 0.0 | 2 | 4 | -4.35 | -0.68 |
| $(NH_4)_2SO_4$ (g/L) | N | 0.1 | 0.5 | 0.9 | 6.48 | 1.02 |
| $(NH_4)NO_3$ (g/L) | NN | 0.5 | 1 | 1.5 | -0.35 | -0.05 |
| $MgSO_4$. $7H_2O$ (g/L) | Mg | 0.1 | 0.5 | 0.9 | -5.82 | -0.91 |
| $K_2HPO_4$ (g/L) | $K_2$ | 0.2 | 1 | 1.8 | -5.60 | -0.88 |
| $K H_2PO_4$ (g/L) | K | 0.1 | 0.5 | 0.9 | -5.79 | -0.91 |
| NaCl (g/L) | Na | 0.1 | 0.5 | 0.9 | -9.02 | -1.42 |
| $CaCl_2$ (g/L) | Ca | 0.0 | 0.02 | 0.04 | -2.49 | -0.39 |
| Inoculums size (ml/L) | IS | 0.2 | 0.5 | 0.8 | 6.02 | 0.94 |
| Agitation (rpm) | Ag | 110 | 180 | 250 | 0.75 | 0.11 |

**Response surface methodology (RSM)**

In order to describe the nature of response surface in the experimental region and to elucidate the optimal concentrations of the most significant independent variables, a Box-Behnken design (Box and Behnken, 1960) was applied, which is a RSM. As presented in Table 4, factors of highest confidence levels namely; NaCl (A), $(NH_4)_2SO_4$ (B), and inoculums size

(C) were tested in three levels (low, basal, and high) coded (-1, 0, and +1). According to the applied, nine chloroxylenol treatment combinations were executed. For predicting the optimal point, the following second order polynomial model was fitted to correlate relationship between independent variables and response:

$Y=b_0+b_1A+b_2B+b_3C+b_{12}AB+b_{13}AC+b_{23}BC+b_{11}A^2+b_{22}B^2+b_{33}C^2$

Where, Y is the dependent variable (chloroxylenol degradation %), A, B and C are the levels of the independent variables; $b_0$ is regression coefficient at the center point; $b_1$, $b_2$ and $b_3$ are linear coefficients; $b_{12}$, $b_{13}$ and $b_{23}$ are the second order interaction coefficients; and $b_{11}$, $b_{22}$ and $b_{33}$ are quadratic coefficients. The values of the coefficients were calculated using Microcal Origin 4.1 software and the optimum concentrations were predicted using Microsoft Excel 2000. The quality of the fit of the polynomial model equation was expressed by coefficient of determination, $R^2$. The optimal value of chloroxylenol degradation was estimated using the solver function of Microsoft Excel tool. Three-dimensional graphical representations were also constructed using Statistica 6.1 software, in order to reflect the effects as well as the interactions of independent variables on the objective.

**Bioremediation of chloroxylenol polluted sewage**

The optimized cultural conditions, after RSM, were applied to bioremediate crude domestic sewage polluted with chloroxylenol at a concentration of 27.8mg/L. Where, the optimized medium components were dissolved in sewage water having 27.8mg/L of chloroxylenol, and after sterilization the medium inoculated and incubated under the optimized culture conditions. Thereafter, residual chloroxylenol was estimated after for 6, 7, and 8 day.

**Results and discussion**

**Degradation of different concentrations of chloroxylenol**

Screening experiment for degradation of 2mg/L of chloroxylenol by locally isolated fungi indicated that *A.niger* degraded 99.72%, while *A.terreus* and *A.versicolor* only degraded 55.62 and 45.62%, respectively. While both *Penicillium corylophilum* and *P.chrysogenum* failed to degrade chloroxylenol.

The efficiency of *A.niger* to degrade different concentrations of chloroxylenol (Fig.1) showed that as the concentration of chloroxylenol increased ten times (2-20mg/L) the degradation efficiency showed only less than 9% decrease (99.72-90.77%), while the degradation rate (µg chloroxylenol /day) was increased more than nine times. These data indicate the high efficiency of *A.niger* to degrade lower and higher levels of chloroxylenol in polluted water. The higher toxicity of lower concentrations of chlorophenols than higher levels of phenol to microorganisms was reported (Ba-Abbad et al, 2012).The articles concerned with bioremediation of chloroxylenol are rare, due to its high toxicity, and hence no data to compare with the degradation efficiency by *A.niger*. Therefore, it is safe to conclude that the fungus efficiency can be satisfactory compared with that recorded with phenol degradation. So, as *P.chrysogenum* could degrade 100mg phenol/L (Leitao et al, 2007) two species of the same genus (*P.corylophilum* and *P.chrysogenum*) failed to degrade 2mg/L of chloroxylenol in our work. It was found that *A.terreus* degraded 1200mg phenol/L (Garcia et al, 2000) and this species degraded only 55.62% of 2mg/L of chloroxylenol. It was reported that increasing the concentration of a phenolic compound may led to a toxic effect or decreasing the available oxygen, and water potential of the medium of the microorganism, as well as, lowering the contact between the organism and nutrients (Suflita, 1989). Moore-Landeker (1996) indicated that microorganisms differ between each others in tolerance of higher levels of toxic materials. So, it is safe to decide that *A.niger* can tolerate higher concentrations of a very toxic compound (chloroxylenol) and can degrade it efficiently.

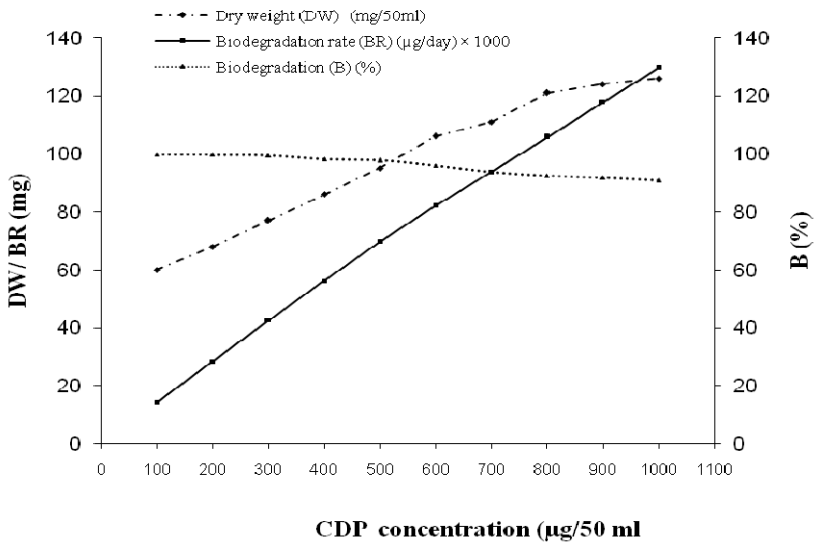


**Fig.1.** Efficiency of *A.niger* to degrade different concentrations of chloroxylenol (CDP).

**Effect of degradation medium**

The results (Table 1) indicated that formulation of media (I, V), that have chloroxylenol as a second carbon and energy source, fortified *A.niger* by nutrients, qualitatively and quantitatively, in favor the formation of highly active chloroxylenol degrade enzymes and growth. Also, the ingredients of the basal medium (I) was the best for maximum degradation (90.77%) and fungal growth yields (252mg/100ml medium). The data indicated that no correlation between fungal growth outputs and its degradation efficiency. Thus, the same growth yields was recorded from media (II, III, 160mg/100ml) and also from media (IV, VII, 120mg/100ml) and they were accompanied with varied degradation efficiencies (69.31, 73.61%, and 53.69, 78.68%, respectively). The priority of the basal medium (I) for *A.niger* growth and chloroxylenol degradation, may be due to its content of glucose, sufficient and balanced amounts of carbon, nitrogen, phosphorous and other minerals. In this respect, it was reported that the addition of non-toxic substances as glucose activates cell viability and degradation process (Topp and Hanson, 1988) as well glucose supports cell densities and microbial growth that increased its degradation activity (Loh and Wang, 1998). Suitable and balanced amount of carbon: nitrogen: phosphorus ratios activate hydrocarbons degradation (Horowitz and Atlas, 1980).

**Time course study of chloroxylenol degradation**

The results (Fig. 2) indicated that the highest chloroxylenol degradation (91.83%) and the high growth yields (240mg/100ml) were achieved after 144hr (6days) of fermentation. This indicates that the highest degrading enzymes production was achieved at the late stages of logarithmic growth phase. It was reported that the highest production of enzymes including hydrolytic enzymes takes place at the accelerated growth phase of the microorganism (Ghanem et al, 2011). In accordance with our findings, some workers reported that as phenol concentration increased, within its non-toxic level, the fermentation period for biodegrading increased as well (did not exceed the logarithmic phase of microbial growth, followed by a constant degradation activity after that) (Stoilova et al, 2006; Leitao et al, 2007; Ghanem et al, 2009).

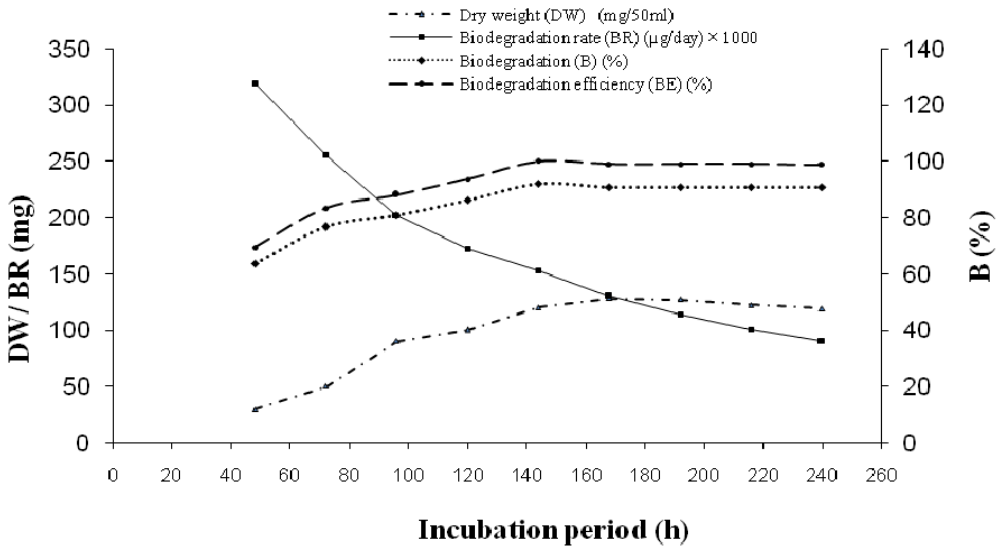


**Fig. 2.** Effect of incubation period on the efficiency of *A.niger* to degrade chloroxylenol (CDP).

**Screening of important variables using Plackett-Burman design**

The data listed in Table 3 indicated a wide variation in chloroxylenol degradation, from 74.19 to 99.81%, in the 13 trails. The variation suggested that the optimization process was important for improving the degradation efficiency of chloroxylenol. The results revealed that the levels of factors at trail (4) were the best (99.81%) for chloroxylenol degradation. Analysis of the regression coefficients and the *t*-values of 11 factors (Table 2) showed that glucose, trace elements solution, $(NH_4)NO_3$, $MgSO_4.7H_2O$, $K_2HPO_4$, $KH_2PO_4$, NaCl, and $CaCl_2$ had negative main effects on chloroxylenol degradation, whereas $(NH_4)_2SO_4$, inoculums size and agitation had positive main effects. It was clear that the most significant three factors in chloroxylenol degradation were NaCl, $(NH_4)_2SO_4$, and inoculums size. In accordance that NaCl (salinity) at its lower concentration (0.1g/L) appeared to be optimum, Shiaris (1989) found that there is a positive relationship between salinity decrease and rate of hydrocarbons degradation. While, $(NH_4)_2SO_4$ at its higher level (0.9g/L) proved to be optimum, as a source of nitrogen assimilated by the organism to biosynthesize amino acids, proteins, enzymes, nucleic acids and others (Moore-Landecker, 1996). However, inoculums size, as the source of the degradative enzymes, had a positive effect in its higher level (0.8ml= $4x10^6$ spores). The importance of inoculums size for degradation of phenol compound was reported (Zhou et al, 2011). The predicted medium composition and culture conditions to be near optimum, which resulted from application of Plackett-Burman statistical design was (g/L): $(NH_4)_2SO_4$, 0.9; $(NH_4)NO_3$, 1.5; $MgSO_4.7H_2O$, 0.1; $K_2HPO_4$. 0.2; $KH_2PO_4$, 0.1; NaCl, 0.1; trace elements solution, 4ml; pH6; inoculums size, 0.8ml; chloroxylenol, 20mg and agitation, 250rpm at $30^oC$ for 144hr.

A confirmatory experiment was conducted to verify the predicted Plackett-Burman conditions. The results of both confirmatory test and that predicted are congruent (99.81% degradation after 144hr) indicating the validity and efficiency of Plackett-Burman design, where the degradation percentage and degradation rate (µg /day) increased by about 8.7%.

**Optimization by response surface methodology (RSM)**

In order to approach the optimum response region of chloroxylenol degradation, significant independent variables (NaCl, $(NH_4)_2SO_4$, and inoculums size) were further explored by

applying RSM, each at three levels according to Box and Behnken (1960). This was done in order to study the interactions between them and also to determine their optimal levels. The design matrix of the coded variables together with the experimental results of chloroxylenol degradation was represented in Table 4. The optimal levels of the three examined independent variables as predicted from the model, trail 8, were (g/L): NaCl, 0.2; $(NH_4)_2SO_4$, 0.9 and inoculums size, 0.2ml. Under these conditions, 100% of chloroxylenol degradation after only 136hr of fermentation was recorded. Therefore, the degradation percentages of 20mg/L increased from 91.83% in the basal conditions into 99.81% after optimization of Plackett-Burman and to 100% after RSM. Also, the incubation period for complete degradation was only 136hr instead of 144hr for 99.81% degradation after Plackett-Burman optimization. Three dimensional graphical representations of the regression model, called the response surface plots are presented in Fig. 3,4,5. Here, each response surface plot represented the effect of the two independent variables, holding the other variable at zero level. The calculated optimal levels of the independent variables predicted from the design were (g/L): NaCl, 0.2; $(NH_4)_2SO_4$, 1.4 and inoculums size, 0.8ml at which 100% chloroxylenol was degraded after only 134.6hr of fermentation instead of 136hr. So, as a confirmatory experiment was done, to verify the above predicted results, congruent data of the experiment and the predicted. The similarity of the predicted and the observed results confirms the validity, accuracy and applicability of RSM (Box-Behnken model) in optimization processes (Ghanem et al, 2009; Zhou et al, 2011). Therefore, response surface optimization could be successfully used to evaluate the performance in chloroxylenol degradation and to achieve higher rate of its degradation in a less fermentation period by *A.niger*. In this study, chloroxylenol concentration of 20mg/L was completely degraded after 134.6hr of incubation.

**Table 3.** Plackett-Burman experimental design of 11 variables and 13 trials.

| Trial | Independent variables ($gl^{-1}$) | | | | | | | | | | | DW (mg/50 ml) | Biodeg. (%) | Biodeg. .rate µg/day) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | G | T | N | NN | Mg | $K_2$ | K | Na | Ca | In | Ag | | | |
| 1 | 1 | -1 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | -1 | 1 | 96.1 | 80.44 | 134.06 |
| 2 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | -1 | 70.15 | 82.31 | 137.19 |
| 3 | -1 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | 65.6 | 96.06 | 160.10 |
| 4 | 1 | -1 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | 1 | 1 | 68.6 | 99.81 | 166.35 |
| 5 | 1 | 1 | -1 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | 1 | 42.7 | 74.94 | 124.90 |
| 6 | 1 | 1 | 1 | -1 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | 45.8 | 74.19 | 123.65 |
| 7 | -1 | 1 | 1 | 1 | -1 | 1 | 1 | -1 | 1 | -1 | -1 | 99.5 | 82.56 | 137.60 |
| 8 | -1 | -1 | 1 | 1 | 1 | -1 | 1 | 1 | -1 | 1 | -1 | 103.7 | 86.19 | 143.65 |
| 9 | -1 | -1 | -1 | 1 | 1 | 1 | -1 | 1 | 1 | -1 | 1 | 96.1 | 74.81 | 124.69 |
| 10 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | -1 | 1 | 1 | -1 | 91.5 | 76.81 | 128.02 |
| 11 | -1 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | -1 | 1 | 1 | 61.0 | 76.69 | 127.81 |
| 12 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 84.3 | 94.31 | 157.19 |
| 13 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 122.0 | 91.84 | 153.06 |

Glucose (G), trace elements solution (T), $(NH_4)_2SO_4$ (N), $(NH_4)NO_3$ (NN), $MgSO_4.7H_2O$ (Mg), $K_2HPO_4$ ($K_2$), $KH_2PO_4$ (K), NaCl (Na), $CaCl_2$ (Ca), inoculums size (In), and agitation (Ag). Dry weight (DW, mg/50ml), Biodegradation % (Biodeg. %), Biodegradation rate (Biodeg. rate, µg/day).

**Table 4.** Box-Behnken design for the three significant independent variables.

| Trial | Variables | | | D (%) | DR(ml/day) | DW(mg/50ml) |
|---|---|---|---|---|---|---|
| | A ($gl^{-1}$) | B ($gl^{-1}$) | C (ml) | | | |
| 1 | (-1) 0.0 | (-1) 0.4 | (-1) 0.2 | 100 | 0.194 | 121.4 |
| 2 | (-1) 0.0 | (0) 0.9 | (1) 1.4 | 100 | 0.194 | 66.6 |
| 3 | (-1) 0.0 | (1)1.4 | (0) 0.8 | 100 | 0.194 | 123.2 |
| 4 | (0) 0.1 | (-1) 0.4 | (1) 1.4 | 89.37 | 0.174 | 125.8 |
| 5 | (0) 0.1 | (0) 0.9 | (0) 0.8 | 100 | 0.194 | 69.2 |
| 6 | (0) 0.1 | (1)1.4 | (-1) 0.2 | 97.15 | 0.189 | 127.8 |
| 7 | (1) 0.2 | (-1) 0.4 | (0) 0.8 | 96.53 | 0.187 | 63.2 |
| 8 | (1) 0.2 | (0) 0.9 | (-1) 0.2 | 99.24 | 0.193 | 80.2 |
| 9 | (1) 0.2 | (1)1.4 | (1) 1.4 | 90.30 | 0.175 | 78.8 |

NaCl (A), $(NH_4)_2SO_4$ (B), Inoculum size (C), Degradation (D, %), Degradation rate (DR,ml/day)

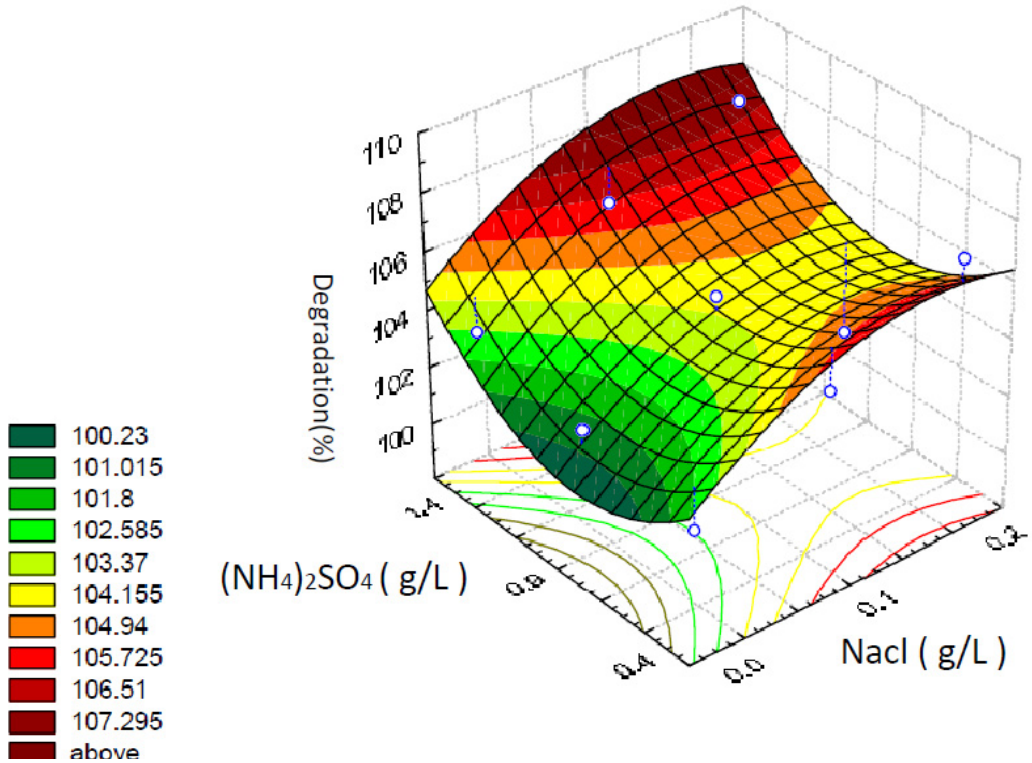


**Fig. 3.** Interaction of $(NH_4)_2SO_4$ (g/l) with NaCl (g/l) with respect to chloroxylenol degradation percentage based on RSM.

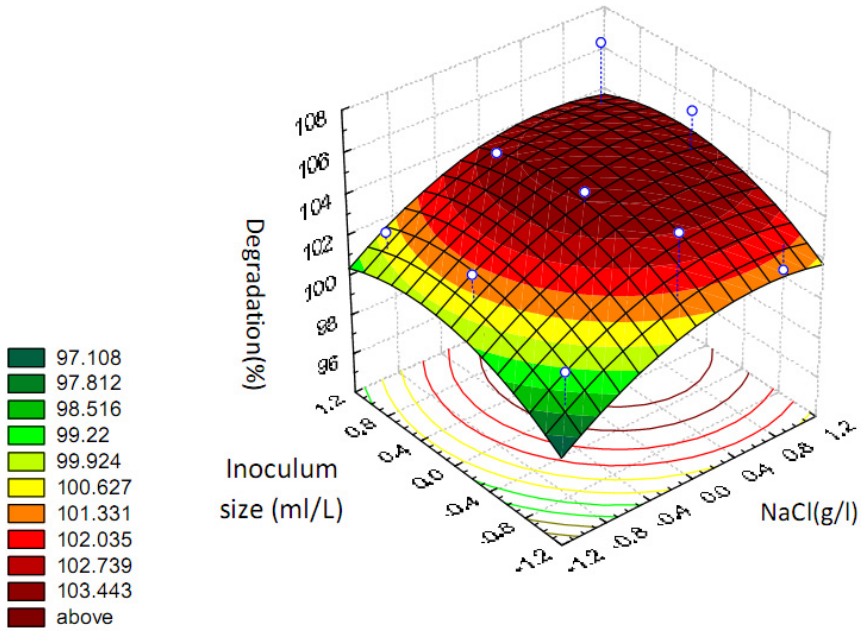


**Fig. 4.** Interaction of inoculums size (ml/l) with NaCl (g/l) with respect to chloroxylenol degradation percentage based on RSM.

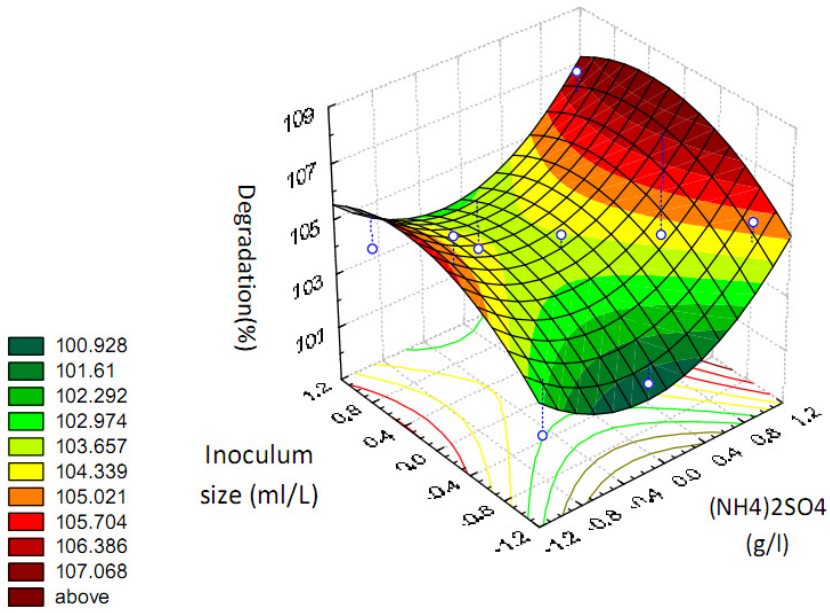


**Fig. 5.** Interaction of inoculums size (ml/l) with $(NH_4)_2SO_4$ (g/l) with respect to chloroxylenol degradation percentage based on RSM.

**Bioremediation of chloroxylenol polluted sewage**

The optimized cultural conditions were applied to bioremediate crude domestic sewage polluted with chloroxylenol at a concentration of 27.8mg/L. *A.niger* could degrade the content of the pollutant (chloroxylenol) after 8days of fermentation with a degradation rate of 7.24 mg

/day, which is less than that recorded with pure medium contaminated with 20mg/L chloroxylenol by about 2.59%. This is due to that sewage contains many contaminants and toxic materials beside chloroxylenol. These findings indicated that *A.niger* is a very efficient fungus to degrade chloroxylenol in both pure contaminated medium or in sewage polluted liquids.

## Conclusion

This was the first report applying statistical experimental designs to optimize chloroxylenol degradation by a novel *A.niger* isolate. Results suggested that statistical optimum strategy was an effective tool for optimization process parameters on chloroxylenol degradation and for advancing degradation efficiency by *A.niger*. Optimal conditions obtained in this work laid to a solid foundation for further use of this organism in the treatment of high strength chloroxylenol effluents.

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