Full Length Research Paper

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

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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 2 mg/L of chloroxylenol after 7 days of fermentation. It also has a high capacity to degrade 91.83% of higher chloroxylenol concentration of 20 mg/L after 6 days of incubation on mineral medium amended with 2 g/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* was able to degrade completely (100%) chloroxylenol (20 mg/L) after 134.6 h 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 27.8 mg/L of chloroxylenol by *A. niger*. The fungus efficiently degraded chloroxylenol after 8 days of fermentation.

Key words: Chloroxylenol degradation, *Aspergillus niger*, 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 a 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 bacteria to produce ATP, whose deficiency leads to 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

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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 METHODS

Microorganism

A. 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 5 days at 30°C. The stocks were kept in the refrigerator and subcultures at monthly intervals were done. Spores suspension of *A. niger* was prepared by washing 5 days old culture slants with sterilized saline solution (0.9% NaCl) and shaking vigorously for 1 min. Spores were counted by a haemocytometer to adjust the count approximately to 5×10^6 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, 2 ml 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 250 ml Erlenmeyer flasks containing 50 ml of the basal medium. Flasks were inoculated with standard inoculums (5.0×10^6 spores/ml) and incubated in shaking incubator (180 rpm) at 30°C. After degradation for 168 h (7 days), the fermentation media were centrifuged at 7000xg for 20 min 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 510 nm, according to standard methods (APHA, 1998).

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

Effect of chloroxylenol (CDP) concentration

The effect of chloroxylenol concentration (2 to 20 mg/L) on degradation activity of *A. niger* was carried out using 250 ml Erlenmeyer flasks containing 50 ml of basal medium and incubated at 30°C for 168 h.

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, 5 ml (modified) (Santos and Linardi, 2004); V) K_2HPO_4, 1.0; yeast extract, 2; sucrose, 0.2; $NaNO_3$, 3.0; KCl, 3.5; $MgSO_4.7H_2O$, 5.0; $ZnSO_4.7H_2O$, 0.1, $CuSO_4.7H_4O$, 0.05; 1 ml 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 168 h the chloroxylenol concentrations were determined. The following equations were used to calculate the biodegradation percentage, rate and efficiency.

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

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

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

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

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

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.

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:

$$Y = b_0 + b_1 A + b_2 B + b_3 C + b_{12} AB + b_{13} AC + b_{23} BC + b_{11} A^2 + b_{22} B^2 + b_{33} C^2$$

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 46.1 software and the optimum concentrations were predicted using Microsoft Excel 2007. 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 7 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 27.8 mg/L. Where, the optimized medium components were dissolved in sewage water having 27.8 mg/L of chloroxylenol, and after sterilization the medium inoculated and incubated under the optimized culture conditions. Thereafter, residual chloroxylenol was estimated after 6, 7, and 8 days.

RESULTS AND DISCUSSION

Degradation of different concentrations of chloroxylenol

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

The efficiency of *A. niger* to degrade different concentrations of chloroxylenol (Figure 1) showed that as the concentration of chloroxylenol increased ten times (2 to 20mg/L) the degradation efficiency showed only less than 9% decrease (99.72 to 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 to that recorded with phenol degradation. So, as *P.chrysogenum* could degrade 100 mg phenol/L (Leitao et al., 2007) two

species of the same genus (*P.corylophilum* and *P.chrysogenum*) failed to degrade 2 mg/L of chloroxylenol in our work. It was found that *A. terreus* degraded 1200 mg phenol/L (Garcia et al., 2000) and this species degraded only 55.62% of 2 mg/L of chloroxylenol. It was reported that increasing the concentration of a phenolic compound may lead 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.

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 (252 mg/100 ml medium). The data indicated that no correlation between fungal growth outputs and its degradation efficiency. Thus, the same growth yields were recorded from media (II, III, 160 mg/100 ml) and also from media (IV, VII, 120 mg/100 ml) 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 such as glucose activates cell viability and degradation process (Topp and Hanson, 1988). In addition, 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 (Figure 2) indicated that the highest chloroxylenol degradation (91.83%) and the high growth yields (240 mg/100 ml) were achieved after 144 h (6 days) 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).

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. 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 (0.1 g/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 (0.9 g/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 (0.8 ml= 4×10^6 spores). The importance of inoculums size for degradation of phenol compound was reported (Zhou et al., 2011). The preoptimized 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, 4 ml; pH 6; inoculums size, 0.8 ml; chloroxylenol, 20 mg and agitation, 250 rpm at 30°C for 144 h.

A confirmatory experiment was conducted to verify the pre-optimized medium resulting from Plackett-Burman design. The results of confirmatory test are congruent (99.81% degradation after 144 h) indicating the 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

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

Medium	Final pH	Dry weight (mg/50ml)	Biodegraded CDP (µg/50 ml)	Biodegradation (%)	Biodegradation rate (µg/day)	Biodegradation 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

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% significance)
		-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
KH_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

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

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

G, Glucose; T, trace elements solution; N, $(NH_4)_2SO_4$; NN, $(NH_4)NO_3$; Mg, $MgSO_4.7H_2O$; K_2, K_2HPO_4; K, KH_2PO_4; Na, NaCl;, Ca, $CaCl_2$; In, inoculums size; Ag, agitation. DW, dry weight.

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

Trial	Variables			D (%)	DR(μg/day)	DW(mg/50 ml)
	A (gl^{-1})	B (gl^{-1})	C (ml)			
	(-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

A, NaCl; B, $(NH_4)_2SO_4$; C, inoculum size; D, degradation; DR, degradation rate; DW, dry weight.
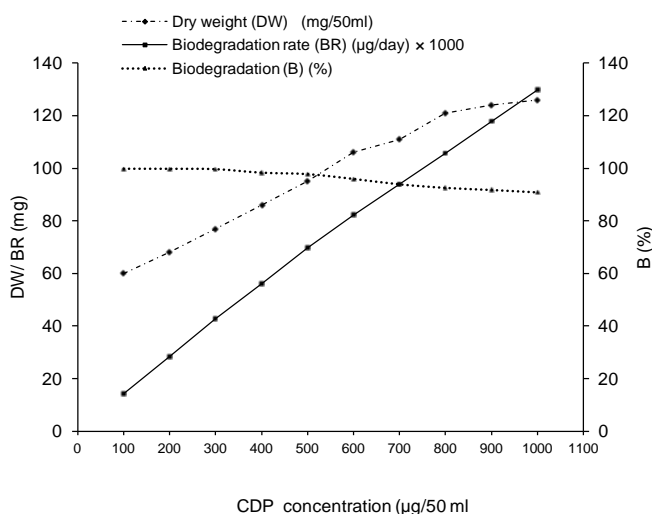
Figure 1. Efficiency of *A. niger* to degrade different concentrations of chloroxylenol (CDP).

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 is 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, 0.2 ml. Under these conditions, 100% of chloroxylenol degradation after only 136 h of fermentation was recorded. Therefore, the degradation percentages of 20 mg/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 136 h instead of 144 h for 99.81%

degradation after Plackett-Burman optimization. Three dimensional graphical representations of the regression model, called the response surface plots are presented in Figures 3, 4 and 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, 0.8 ml at which 100% chloroxylenol was degraded after only 134.6 h of fermentation instead of 136 h.

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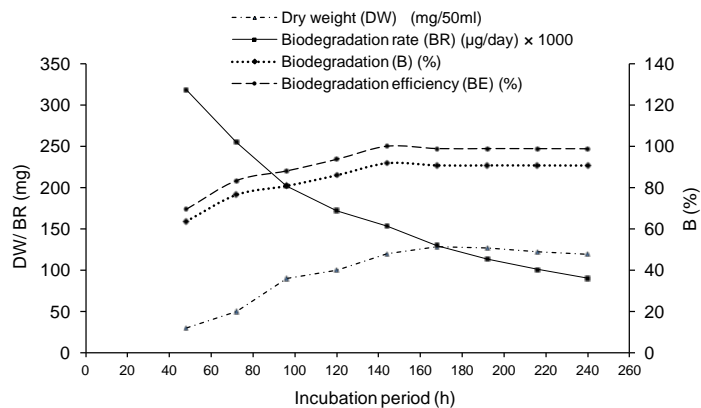
Figure 2. Effect of incubation period on the efficiency of *A. niger* to degrade chloroxylenol (CDP).
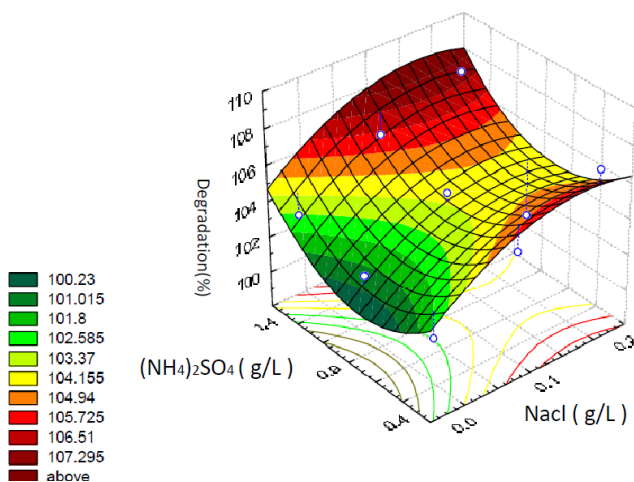

Figure 3. Interaction of $(NH_4)_2SO_4$ (g/l) with NaCl (g/l) with respect to chloroxylenol degradation percentage based on RSM.

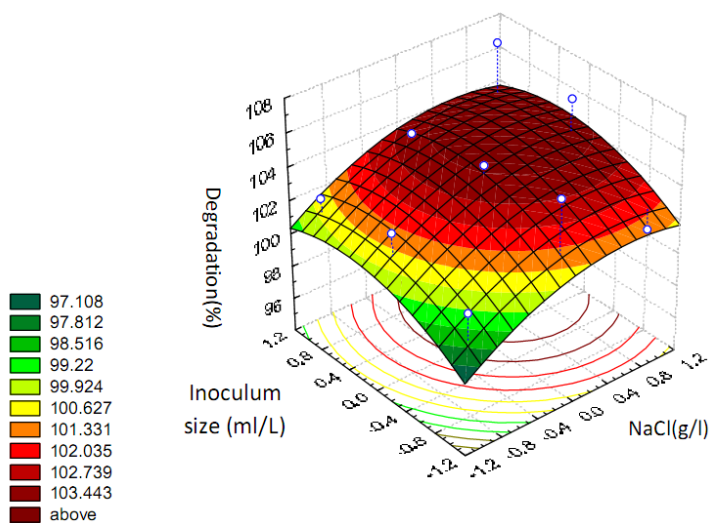**Figure 4.** Interaction of inoculums size (ml/l) with NaCl (g/l) with respect to chloroxylenol degradation percentage based on RSM.
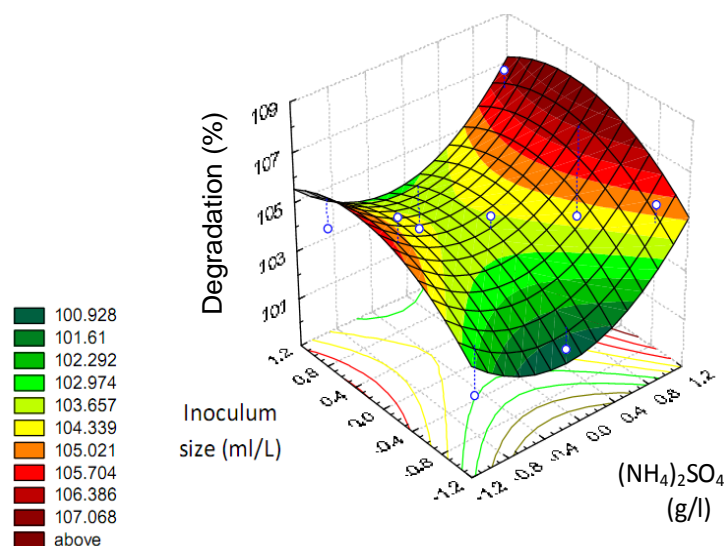
■	100.928
■	101.61
■	102.292
■	102.974
■	103.657
■	104.339
■	105.021
■	105.704
■	106.386
■	107.068
■	above

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

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 20 mg/L was completely degraded after 134.6 h of incubation.

Bioremediation of chloroxylenol polluted sewage

The optimized cultural conditions were applied to bioremediate crude domestic sewage polluted with chloroxylenol at a concentration of 27.8 mg/L. *A. niger* could degrade the content of the pollutant (chloroxylenol) after 8 days of fermentation with a degradation rate of 7.24 mg /day, which is less than that recorded with pure medium contaminated with 20 mg/L chloroxylenol by about 2.59%. This is due to the fact 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

To the best of our knowledge, this is the first report applying statistical experimental designs to optimize chloroxylenol degradation by *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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