IJISET - International Journal of Innovative Science, Engineering & Technology, Vol. 7 Issue 7, July 2020
ISSN (Online) 2348 – 7968 | Impact Factor (2020) – 6.72
www.ijiset.com

A Novel Biodegradable System Based On the Art Nanotechnology Combined With the Statistical Design in Saline Habitats

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ABSTRACT

The aim of this study was to investigate the biodegradability of PCL under saline conditions using the statistical methodology, including Plackett-Burman and subsequent use of the response surface methodology (RSM) combined with the art nanotechnology to elucidate the environmental factors limiting PCL biodegradation. A little information exists about the ability on the biodegradation of synthetic pollutants such as polyesters in saline environments, for this purpose, a two-level Plackett–Burman multifactorial design was used to determine the impact of 11 variables on PCL nanoparticles degradation capabilities. Among the tested variables, an increase in NH4SO4- and a decrease in NaCl and polyester concentration were found to be the most significant. Indeed, a 3.2-fold increase in PCL depolymerase enzyme activity (15.04 IU mL-1) was recorded in the preoptimized medium compared to the basal condition settings. Consequently, response surface methodology was applied to elucidate the level of the three significant factors affecting environmental PCL degradation. The Box–Behnken design identified nitrogen source (6.5-7.5 gL-1) and polyester availability (0.2-0.38 gL-1) as significant, as well as salt stress (20-40 gL-1) as a limiting factor for the production of the PCL depolymerizing enzyme. Under the optimized conditions, a 4.3 increase and hence a maximized PCL depolymerase activity of 20.2 IU mL-1 was recorded.

Keywords: Nanoparticle technology, Plackett-Burman, Box-Behnken, *Aspergillus flavus*, PCL, Biodegradation

1.INTRODUCTION

Poly(ε-caprolactone) is an aliphatic polyester that is a relatively stable synthetic polymer under usual conditions and is biodegradable under microbial attack, including river and lake waters, sewage sludge, farm soil, paddy soil, creek sediment, roadside sediment, pond sediment, and compost [1,2,3].Various simulation tests in the laboratory have been used to measure the biodegradation of plastics.

Here, the degradation might take place in compost, soil or sea water placed in a controlled reactor in a laboratory[4, 5]. Although the environmental conditions are still very close to the field-test situation, the external parameters (temperature, pH, humidity, etc.) can be controlled and adjusted and the analytical tools available are better than for field tests (e.g. for analysis of residues and intermediates, determination of CO_2 evolution or O_2 consumption). In such tests, which may be optimized for the activity of the particular microorganisms used, polymers often exhibit a much higher degradation rate than it would be observed under natural conditions [6,7]. Nevertheless, PCL biodegradation in extreme environments, especially saline and hypersaline environments has not been investigated. This is the first report on the clarification of environmental factors limiting Poly-ε-caprolactone (PCL) disintegration under salt stress. This study has been based mainly in chemical, physical, biological and statistical tools to enable mapping the best conditions for PCL depolymerization.

Therefore, the present study aimed to investigate the biodegradability of PCL under saline conditions by combining the art nanotechnology with the statistical design including Plackett-Burman and response surface methodology (RSM) [8] to optimize medium that support PCL degradation under salt stress conditions by a local *Aspergillus flavus*.

2.MATERIALS AND METHODS

2.1 Microorganism

The fungal strain used in the present work was *Aspergillus flavus* locally isolated from 'Fatnas' salt lake, Siwa, Egypt on liquid MSY-medium amended with the synthetic polyester PCL [9].

2.2 Polymer

The commercialized poly (-ε-caprolactone) (Polyscience, Inc. Warrington, USA) was provided by Dr. Dunja-Manal M. Abou-Zeid(Alexandria University, Egypt). The melting point of PCL is (Tm = 60°C) and weight-average molecular masses (Mw = 50,000 g/mol)

2.3 Medium

The modified mineral salt yeast extract medium (MSY) was used throughout this study [10]. MSY medium was used for the enrichment, screening and biodegradation tests as well as the cultivation of the isolated fungal strain.

2.4 Preparation of a stable PCL-nanoparticle suspension

100 mg of the PCL were dissolved in 10 ml acetone and suspended in double distilled water under constant mixing using an ultrasonicator (8000 U min-1) [11]. The residual acetone was removed using a rotary evaporator. To determine the polymer concentration, 5 ml of the nanoparticle suspension were dried at 40°C to constant weight to determine the concentration (w/v) of the obtained suspension (which usually did not exceed 6 mg/ml).

2.5 Crude enzyme activity

PCL depolymerizing enzyme activity in liquid cultures amended with PCL nanoparticles was determined as follows: 15 ml of the corresponding culture were centrifuged for 10 min at 14000 rpm to separate cells; sterile filtered cell free supernatant was used as the crude enzyme. The test principle is based on the decrease of the optical density (OD_{550nm}) of a stable PCL-particle suspension during enzymatic degradation. The enzyme reaction solution contained the following salts: NH_4SO_4, 1.0; KH_2PO4, 1.0; K_2HPO_4, 1.0; KCl, 0.1; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.01; FeSO4.7H2O, 0.01 and pH was adjusted to 6.5 ± 0.1. PCL suspension was added at a concentration of 0.1% (w/v). The enzyme reaction was incubated statically at 55°C for 60 min and the decrease in optical density (OD_{550nm}) of the enzyme mixture was measured. The PCL concentration was determined using a standard curve relating the optical density of the nanoparticle suspension with PCL concentration. One unit of enzyme activity (IU) is the amount of enzyme that hydrolyzed 1 mg of PCL per min. To clarify the influence of salt stress on crude enzyme activity, crude enzyme secretion and activity were tested over a test period of 7 d in MSY medium with 0.1% PCL at 30 °C. Three different conditions were tested crude enzyme activity measured in a culture (a) lacking NaCl; (b) grown on 10% NaCl; (c) lacking NaCl; however, the enzyme reaction mixture was supplemented with 10% NaCl.

2.6 Statistical analysis

A series of statistically designed studies were performed to investigate which cultivation conditions or medium components would limit PCL depolymerization. The Plackett–Burman design [12, 13, 8] was used for screening purpose and eleven independent variables were evaluated in twelve combinations organized according to the design matrix (Table 1).

Table 1. The twelfth trial Plakett-Burmann experimental design

Level	-1	0.5	0.5	0.5	0.1	0.005	0.3	0.05	0.005	6.5	static	50
	0	1	1	1	0.5	0.01	1	0.01	0.01	7	static	100
	1	5	5	5	1	0.05	3	0.5	0.02	7.5	shacked	150
Run		Independent variables:Factors under study										
		X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}
		YE	NH_4NO_3	NH_4SO_4	$MgSO_4$	$CaCl_2$	PCL	KCl	$FeSO_4$	pH	St/Sh	NaCl
1		+	-	+	-	-	-	+	+	+	-	+
2		+	+	-	+	-	-	-	+	+	+	-
3		-	+	+	-	+	-	-	-	+	+	+
4		+	-	+	+	-	+	-	-	-	+	+
5		+	+	-	+	+	-	+	-	-	-	+
6		+	+	+	-	+	+	-	+	-	-	-
7		-	+	+	+	-	+	+	-	+	-	-
8		-	-	+	+	+	-	+	+	-	+	-
9		-	-	-	+	+	+	-	+	+	-	+
10		+	-	-	-	+	+	+	-	+	+	-
11		-	+	-	-	-	+	+	+	-	+	+
12		-	-	-	-	-	-	-	-	-	-	-
13		0	0	0	0	0	0	0	0	0	0	0

The variables X1 to X8 represent the medium constituents and X9 to X11 represent environmental factors. All trials were performed in triplicates and the averages of PCL depolymerization results were treated as responses. The main effect of each variable was determined with the following equation:

$$Exi = (\sum pi+ - \sum pi-) / N$$

Where Exi is the variable main effect, pi+ and pi- are PCL depolymerase activities in trials where the independent variable (Xi) was present in high and low concentrations, respectively, and N is the number of trials divided by 2. A main effect figure with a positive sign indicates that the 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. The factor that had no effect would give a value of zero if no interactions existed. The variables whose confidence levels were greater than 90 % ($P>F\leq0.1$) were considered to have a significant influence on PCL depolymerization.

Box-Behnken: The significant independent variables influencing PCL depolymerization verified from the screening Plakett-Burman experiment were used in a second phase of medium formulation for optimum PCL degradation. The matrix of the Response Surface Methodology (RSM) [14, 15] is shown in Table 2. The most significant independent variables can be examined at three different levels, low (-), high (+) and central or basal (0). Here, the factors of NH4SO4 concentration (X1), NaCl content (X2) and PCL concentration per flask (X3) were treated as independent variables. Trials 13-16 consisted of the basal medium and represented the control.

Table 2. Box-Behnken design for three significant[1] variables that affected PCL depolymerization by *A. flavus*

Run	Factors		
	X_1 (NH_4SO_4)	X_2 (NaCl)	X_3 (PCL)
1	**0**	**-1**	**-1**
2	**0**	**+1**	**-1**
3	**0**	**-1**	**+1**
4	**0**	**+1**	**+1**
5	**-1**	**0**	**-1**
6	**+1**	**0**	**-1**
7	**-1**	**0**	**+1**
8	**+1**	**0**	**+1**
9	**-1**	**-1**	**0**
10	**+1**	**-1**	**0**
11	**-1**	**+1**	**0**
12	**+1**	**+1**	**0**
13	**0**	**0**	**0**
14	**0**	**0**	**0**
15	**0**	**0**	**0**
16	**0**	**0**	**0**

[1] based on the results of the Plackett-Burman design

Thirteen combinations and their observations in duplicates were fitted to the following second order polynomial model:

$$Y=b0+b1X1+b2X2+b3X3+b12X1X2+b13X1X3+b23X2X3+b11X12-b22X22+b33X32$$

Where, Y is PCL depolymerase activity; X1, X2 and X3 are the independent variables; b0 is the regression coefficient at center point; b1, b2 and b3 are linear coefficients; b12, b13 and b23 are second order interaction coefficients; and b11, b22, and b33 are quadratic coefficients. The value of the coefficients was calculated using Microcal Origin 6.1 software and the optimum concentrations were predicted using Microsoft Office Excel 2007. Three-dimensional graphical representations were constructed using statistical software STATSITICA 6.0 to reflect the effects as well as the interactions of independent variables on PCL degradation

Statistical analysis of data: The data of enzyme activity were subjected to multiple linear regressions using Microsoft Office Excel 2007 to estimate t-values, P-values and confidence levels which is an expression of the P-value in percent.

3. RESULTS AND DISCUSSION

3.1Screening of important medium components and environmental conditions affecting PCL depolymerisation by *A. flavus*

There are no reports available in literature regarding the environmental factors limiting PCL nanoparticle biodegradation in saline habitats. To predict the fate of such material in saline environments for pollution prevention, a two-phase optimization approach was carried out. The first step included screening for important factors that affect PCL depolymerization in shacked or static flasks. The second step included RSM to provide in-depth information about the few variables identified during screening as having the greatest impact on performances, and finally, it was aimed to validate the obtained results and elucidate the environmental factors that influence enzyme expression significantly. This approach has been widely adopted for optimizing the processes of enzymes and peptides [16], solvents [17], polysaccharides [18].

The pattern observed in the Pareto chart (Figure1) shows that there are 3 very large effects (caused by NaCl, PCL, and NH4SO4), 5 intermediate but still significant effects (pH, yeast extract, NH4NO3, FeSO4.7H2O, and MgSO4.7H2O) and then a collection of 3 smaller or insignificant effects (CaCl2.2H2O, aeration and KCL). Additionally, the statistical significance of the measured response of each independent variable was determined using the t-test (Table 3). Out of the eleven tested variables, factors having a confidence level greater than 90 % ($P>F\leq0.1$) were considered to have a significant effect on PCL depolymerisation. These results are similar to Greenland et al [19] who found that 95 % confidence intervals repeatedly in valid applications. Some investigators find confidence levels greater than 70% [20, 8], greater than 90% [21], or even greater than 95% to have a significant effect on the response [22]. Hence, variables X3 (NH4SO4), X11 (NaCl), and X6 (PCL), with confidence levels of 96%, 93%, and 92%, respectively, were selected for further studies. Consequently, the following optimized medium composition for *A. flavus* was postulated and contained in gL-1: PCL, 0.3; NH4NO3, 5; NH4SO4, 5; MgSO4.7H2O, 0.1; CaCl2.2H2O, 0.5; yeast extract, 5; KCL, 0.5; FeSO4.7H2O, 0.005; and NaCl, 50. The pH of the culture medium was adjusted to 7.5 and the inoculated culture incubated statically at 30°C. Indeed, compared to the basal condition settings the preoptimized medium showed a 3.2 fold increase (15.04 IU) in PCL depolymerase enzyme production/activity.

Table 3. Screening of critical factors for PCL depolymerisation: Main effect, *t*-value, and statistical confidence level

Component	Ranges used (g/L)	Plackett-Burman analysis		
		Main Effect	Statistical analysis	
			t-value	Statistical confidence
Yeast extract	**0.5 – 50**	**14.4**	**0.6308**	**72**
NH_4NO_3	**0.5 – 5**	**14.22**	**0.8045**	**78**
NH_4SO_4	**0.5 – 5**	**19.9**	**1.9945**	**96**
$MgSO_4.7H_2O$	**0.1 – 1**	**-10.55**	**-0.698**	**74**
$CaCl_2.2H_2O$	**0.05 - 0.5**	**7.32**	**0.4785**	**67**
PCL	**0.3 – 3**	**-21.88**	**1.5785**	**92**
KCL	**0.05 - 0.5**	**4.19**	**0.2711**	**60**
$FeSO_4.7H_2O$	**0.005 - 0.02**	**-13.75**	**-0.648**	**73**
pH	**6.5 - 7.5**	**13.62**	**-0.648**	**73**
Sh/St	**0 - 120rpm**	**-4.42**	**-0.286**	**60**
NaCl	**50 – 150**	**-22.58**	**-1.634**	**93**

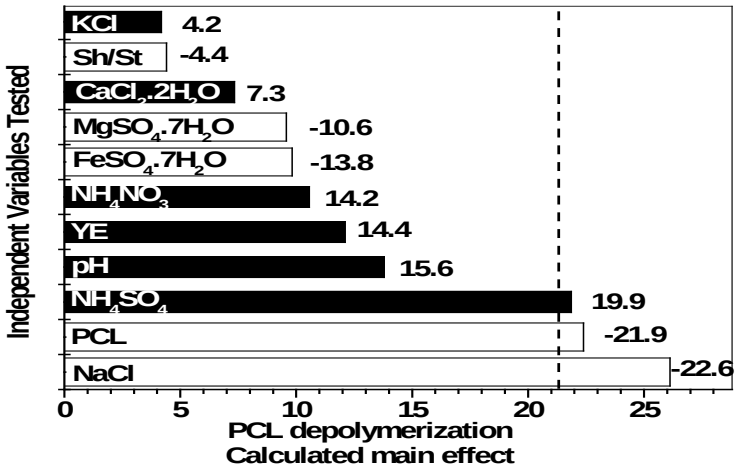

Figure 1. Pareto chart rationalizing the effect of each variable on the PCL depolymerizing activity

The obtained results clarified that depolymerase production/activity by fungus *A. flavus* was certainly enhanced by additional nitrogen sources as NH4SO4and NH4NO3but depressed by NaCl concentrations higher than 50 gL-1 (Figure 1). It is indeed known, that halotolerant fungi need nitrogenous compounds to serve as precursors for the accumulation of proline as an osmoregulator[23,24].

The fact that lower amounts of PCL (0.3 gL-1) in the culture medium are favorable for PCL depolymrerase production/activity may be referred to the fact that the applied nanoparticles have a much higher surface area reaching a 20 fold increase [25] compared to PCL powder with a particle size in the µm range. The larger surface area in turn needs a higher enzyme concentration to affect the nanoparticle size within a reasonable time span to a measurable extent.

It is worth mentioning that an elevated pH value of 7.5 was more favorable than a pH 6.5 or even 7 for best enzyme activity. Since PCL depolymerisation is a result of ester bond cleavage resulting in soluble monomers, dimers, trimers and oligomers with free carboxyl groups [26], generally culture pH decreases due to PCL depolymerization. It was previously observed, that basic pH values and high temperature favored the degradation of PCL in an aqueous environment [27]. A low pH in turn negatively affected most depolymerases [28,29]. Thus, increasing the initial pH of the fungal culture to 7.5 instead of 7 may help to partially overcome or reduce this negative effect.

3.2 Influence of salt stress on crude enzyme activity

At this phase it is still not clear whether salt stress affected only growth and consequently the amount of extracellular enzyme formed or additionally inhibited enzyme activity *per se*. Indeed, when *A. flavus* was grown in absence of salt (Figure2a) two distinct enzyme activity peaks (57% at 24 h and 43% at 5 days) were detected. However, when the fungus was grown under salt stress (Figure. 2b), the enzyme peaks appeared with delay on the third and sixth day where a maximum activity of 62% was observed.

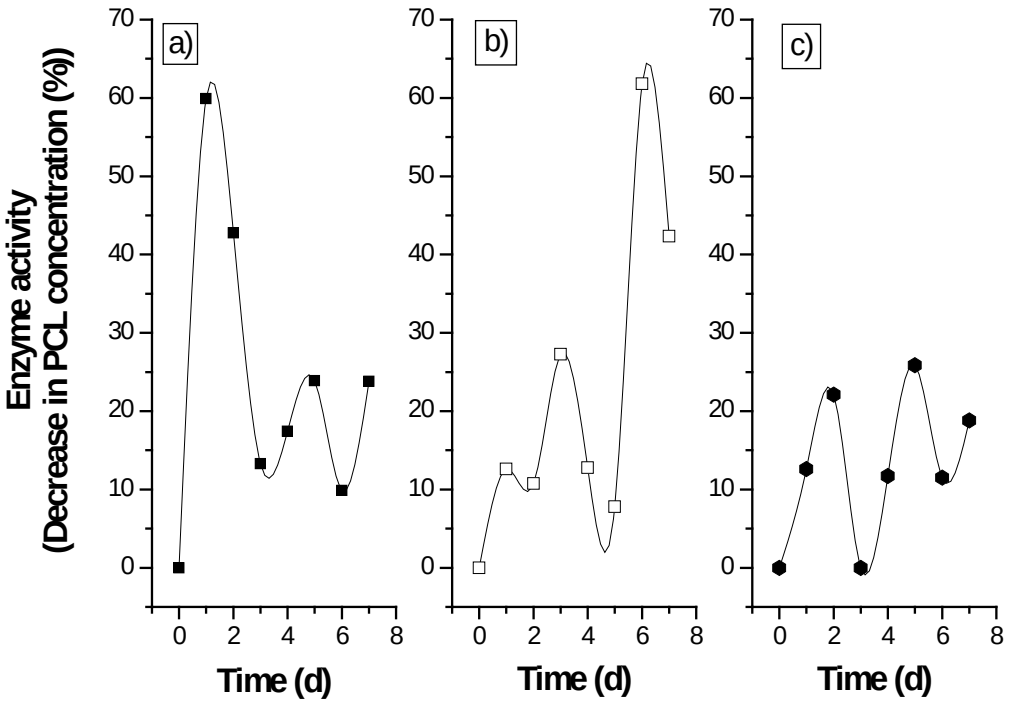

Figure2. Effect of NaCl addition (10%) on time dependent PCL depolymerizing activity at 55°C by *A. flavus*

This is explained by the fact that at the beginning of the experiment the insoluble substrate PCL was not available to support growth and reproduction and the fungus was obliged to secrete the extracellular depolymerizing enzyme. This enzyme was then available to cut the insoluble polyester chain into small, soluble and metabolizable fragments, usually monomers, dimmers, trimers and other short oligomers [25, 26]. Consequently, the fungus did not need to secrete additional amounts of depolymerizing enzyme (day 3 and 4) until the soluble fragments were catabolized. In addition, the enzyme activity apparently was negatively affected by the degradation products or the metabolites since its catalytic action rapidly decreased. However, once the growth substrate was exhausted, the fungus entered a second starvation phase which in turn resulted in a repeated secretion of the depolymerizing enzyme, which would explain the second peak on day 5. Such a degradation behavior in spurts has been previously observed by Abou-Zeid et al. [28]. When the crude enzyme of *A. flavus* grown without NaCl was exposed to 10% NaCl in the enzyme reaction mixture (Figure2c) the enzyme lost 79% of its activity and thus assures the negative impact of NaCl on the depolymerase enzyme. It is generally known that at very high salt concentrations, the abundance of interactions between the added ions and water decreases the possibilities for protein-water interactions, often resulting in the protein being precipitated from solution [30]. As shown in Fig. 2 a-c, the enzyme itself is adversely affected by the salt and not only fungal growth. This in turn implies that biodegradable plastics might persist in nutrient poor saline habitats such as desert soils or saline habitats.

3.3 Elucidation of environmental factors limiting PCL nanoparticle depolymerization by RSM using the Box-Behnken statistical design

The data in Table 4 represent the different combination levels of the most significant factors and corresponding PCL depolymerase production/activity observed after 3 days of incubation.

Table 4. Examined concentrations of the key variables and results of the Box-Behnken experiment

Trial	Concentration (gL^{-1})			Observed response
	NH_4SO_4	NaCl	PCL	Relative PCL depolymerase activity (%)
1	**5**	**25**	**0.1**	**66.05**
2	**5**	**100**	**0.1**	**45.20**
3	**5**	**25**	**0.5**	**65.60**
4	**5**	**100**	**0.5**	**46.40**
5	**3**	**50**	**0.1**	**40.34**
6	**8**	**50**	**0.1**	**75.23**
7	**3**	**50**	**0.5**	**42.41**
8	**8**	**50**	**0.5**	**77.23**
9	**3**	**25**	**0.3**	**60.20**
10	**8**	**25**	**0.3**	**79.35**
11	**3**	**100**	**0.3**	**40.54**
12	**8**	**100**	**0.3**	**49.33**
13	**5**	**50**	**0.3**	**75.20**
14	**5**	**50**	**0.3**	**76.21**
15	**5**	**50**	**0.3**	**78.20**
16	**5**	**50**	**0.3**	**80.10**

The three dimensional surface plots (Figure3 a-c) show that the PCL enzyme activity of *A. flavus* is sensitive to minor alterations of the test variables. About 6.5-7.5 gL-1 of NH4SO4 is required to compensate the effect of NaCl from 2-4% for maximum depolymerase production/activity (Figure3a). The interesting results were obtained with NaCl concentrations not exceeding 5.5%. In this case 2.2-3.8% of PCL can be optimally depolymerized within a reasonable time span (Figure3b). Further the data ascertained that the optimal depolymerization of 2-4% PCL requires sufficient concentrations of the nitrogen source NH4SO4 that should fall into the range of 6-8 gL-1 (Figure3c). Obviously the nitrogen source plays a crucial role during PCL depolymerisation. In addition, high levels of NaCl definitely negatively affected PCL depolymerization as indicated in Figure 3b. However, the salting out of the proteinaceous enzyme molecule occurs, when the salt concentration is increased, i.e. its solubility decreases and the enzyme may precipitate and hence loses activity [31].

Moreover, the shapes of the contour plots (Figure 3 a-c), circular or elliptical, indicate if the mutual interactions between the variables are significant or not [31, 8]. A circular contour plot indicates that the interactions are negligible. An elliptical nature of the contour plots indicates that interactions are significant [31]. This indicates that under salt stress conditions, the availability of nitrogen source represents a limiting factor for PCL biodegradation (Figure 3a). On the other hand, in presence of elevated salt concentrations, only a limited amount of PCL can be depolymerized, PCL depolymerase enzyme production/activity decreased rapidly above 3.5% NaCl (Figure3b). Under optimized conditions, a maximum extracellular PCL depolymerizing enzyme activity using *A. flavus* of about 20.2 IU ml-1 (4.3 fold increases) was obtained.

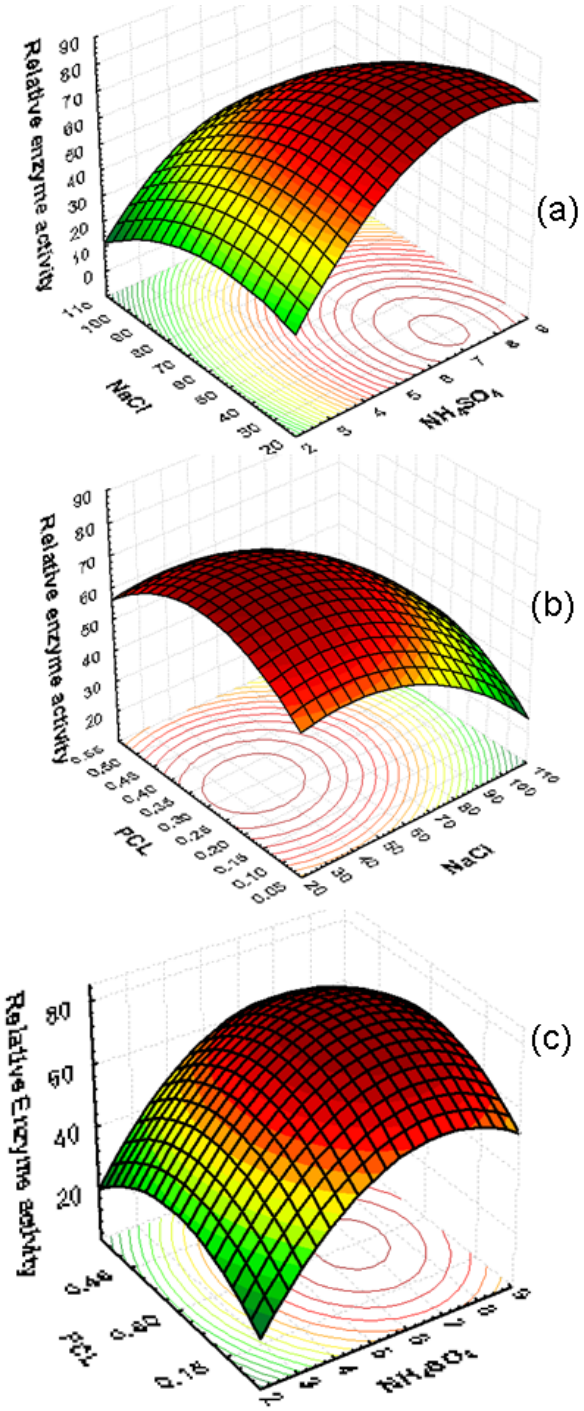

Figure3. Three and two dimensional surface plots showing PCL depolymerase production/activity by *A. flavus*

4. CONCLUCION

Conclusively it can be confirmed that the evaluation of plastic polymer biodegradability has to be comprehensive in order to minimize harmful effects of biodegradation on the environment. Not only physiological studies, such as studies of enzymatic or microbial degradation and their mechanisms, but also ecological studies are required. Studies of the distribution of degrading microorganisms and their ratios in the natural environment are necessary to gain a better understanding of plastic polymer biodegradation. Further studies with obtained isolate(s) should provide interesting insights into the mechanisms for aerobic polyester degradation under environmental stresses. No doubt, the enormous use of plastics will be the driving force for further research on their biodegradation processes.

ACKNOWLEDGEMENT

This work was supported by the Libyan Government and Alexandria University.

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