Journal of Applied Pharmaceutical Science Vol. 7 (02), pp. 067-075, February, 2017
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2017.70208
ISSN 2231-3354

Sequential optimizations of *Aspergillus awamori* EM66 exochitinase and its application as biopesticide

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ARTICLE INFO

Article history:
Received on: 27/06/2016
Accepted on: 10/09/2016
Available online: 27/02/2017

Key words:
Aspergillus awamori; Bio pesticides; Central composite design; Exochitinase.

ABSTRACT

Sequential optimization strategy was achieved based on statistical experimental designs for enhancement of *Aspergillus awamori* EM66 exochitinase production. Firstly, 2-level Plackett-Burman design was accomplished for distinguishing between the bioprocess parameters, which improve the exochitinase production. Second optimization step was implemented through central composite design (CCD), for optimization variables amounts which have the highest positive effect on exochitinase production. Maximum activity (5998mU/ml) for exochitinase reported approximately 22 fold increase compared to the basal medium activity. Mortality 92%, 86.67% and 65.67% was recorded when the partial pure fungal exochitinase was applied to the diet of the greater wax moth larvae, *Galleria mellonella*, the cotton leaf worm, *Spodoptera littoralis*, and the black cutworm, *Agrotis ipsilon*, respectively. The results indicated to the importance of *Aspergillus awamori* EM66 exochitinase as an effective bio- pesticide.

INTRODUCTION

Hard environments are permanently predictable to accord unrivalled microorganisms have new feature. Honey osmophilic property commended it to be good medium for dominant spores worthy to produce bio-products with unique properties (Esawy *et al*., 2011; Esawy *et al*., 2012; Esawy *et al.*, 2013). Chitin, is a β-1,4-linkedN-acetylglucosaminehomopolymer. It is consider the second most copious polysaccharide in nature. Chitin is considered as one of the main component of exoskeletons of some insect, fungus cell walls and crustacean shells (Gooday, 1990; Crosby and Alfred, 2004). It is also exists in several body ingredients of invertebrates and as a bacterial product (Tanaka *et al*., 1999; Nawani and Kapadnis, 2005). Chitinases (E.C.3.2.1.14) is enzymes have the ability to hydrolyze in soluble chitin to its oligo and monomeric components. Chitinases could be divided into two main enzymes, exochitinases and endochitinases (Graham and Sticklen, 1994). Endochitinases (EC 3.2.1.14) can split chitin at random internal sites yielded a soluble, low-molecular mass multimers of N-Acetylglucosamine (GlcNAc) like chitotetrose, chitotriose, and diacetylchitobiose. Exochitinase consisted of two subcategories: β-1, 4 N-acetyl glucosaminidases and exochitinases (EC 3.2.1.29), the aim of this work which can stimulate the release of diacetylchitobiose starting at the non-reducing end of chitin chains **(**Chuan, 2006). Many researchers pay attention to chitinases because of their wide range of biotechnological applications, especially in chitooligosaccharides production, glucosamines, and GlcNAc have an massive pharmaceutical potential (Singh *et al*., 2009) and, Chitinases can be also used as bio-control agents against fungal phytopathogens in agriculture field due to their ability to hydrolyze the chitinous fungal cell wall (Maisuria *et al*., 2008). Crop pests control by the use of chitinase holds a great promise as an alternative to the use of chemicals (Kramer, 1997). Several years ago, statistical design experimental techniques have been employed for reaching to the most favorable conditions. It was achieved by evaluating the parameters effects and conquer the abuse of factor interactions (Ren *et al*., 2006, Liu *et al*., 2013).

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Response surface method (RSM) is one of the popular techniques that are used widely in the biotechnology industry (Wejse *et al*., 2003, Rui *et al*., 2009). RSM is a combination of mathematic and statistical techniques for experiments design, building models, estimating factors effects, and looking for the optimum conditions. RSM is a convenient road for developing optimum processes with precise conditions that has also minimized production cost of many processes by efficient screening process parameters. The optimal conditions or the region that fits the operation specification can be determined by the RSM via a curvature approach (Elibol and Dursun, 2002).

Egyptian cotton leaf worm, *Spodoptera littoralis* (Lepidoptera: Noctuidae), an important pest that led to vast loss in vegetables varieties, fodder, and fiber crops (Güz *et al*., 2013). It is damages wide types of crops, such as cotton, tobacco, and corn in countries located around the Mediterranean Basin and in Southeast Asia (Balachowsky, 1972, Sneh *et al*., 1981). Number of moth species offensive honey bees, honey polen and wax. *Galleria mellonella*, causes serious losses to commercial beekeepers every year. Black cutworm causes an economic threat to many agricultural crops. In Pennsylvania field crops, it is most often a pest of corn. It is also cause trouble in wheat and tobacco (Robinson *et al*., 2001).

The present report is an attempt to improve exochitinase production by honey isolates *Aspergillus awamori* EM66 through optimization of medium components. The enzyme was partially purified by 30% acetone. Finally, the study focused in the insecticidal activity against three serious insects which destroy valuable and economic crops, the greater wax moth larvae, *Galleria mellonella*, the Egyptian cotton leaf worm, *Spodoptera littoralis* and the black cutworms, *Agrotis ipsilon.*

MATERIALS AND METHODS

Microorganisms and maintenance

The fungal used throughout this work, was previously identified as *Aspergillus awamori* EM66 (Kansoh *et al*., 2015) based on morphological characterization and 18S rRNA sequence analysis. *A. awamori*EM66 was routinely grown on Potato dextrose agar (PDA) medium at 30°C and preserved at − 80ºC in 50% (v/v) glycerol.

Exochitinase assay

Exochitinase activity was determined according to the method of (Matsumoto *et al*., 2004) using the chromogenic substrate p- nitro phenyl-β-D-N-acetylglucoseaminide (PNP-β-GlcNAc) as a substrate. One unit of the enzyme activity was acquainting as the enzyme amount releasing 1µmol of P-nitrophenol per minute under the specified assay conditions.

Enzyme production conditions

Initially, the basal medium (BM) for exochitinase production contained (g%): (Chitin,0.5; peptone, 0.5; yeast extract, 0.3; $NaNO_3$, 0.2; K_2HPO_4, 0.1; KCl, 0.05; $MgSO_4.7H_2O$, 0.001 pH was adjusted to 6.0.Conical flasks (250 ml) had 50 ml of (BM) were inoculated with one ml of spore suspension include approximately (1×10^6CFU/ml).

Inoculated flasks were incubated at 30 ℃ for 6 days in a rotary shaker adjusted at 200 rpm. At the end of incubation period cultures were centrifuged for 15 min using the cooling centrifuge. Culture supernatant was used as the crude enzyme. Results reported are the average values with standard deviations.

Experimental designs

Plackett–Burman design

For multivariable processes such as biochemical systems, in which numerous potentially influential factors were involved, it is needful to analyze the process with an initial screening design prior to optimization (Box *et al*., 1978). Plackett–Burman experimental design (Plackett and Burman,1946) was used to evaluate the relative importance of various nutrients for exochitinase production by *A. Awamori* EM66 in submerged fermentation.

Fifteen components were selected for the study, each variable represented at two levels, high value (+1) and low value (−1) in 16 trials as shown in (Table 1).

Incubation time, pH, glucose, insect, soya bean, chitin, wheat flour, $NaNo_3$, $CuSO_4$, $MgSO_4$, K_2HPO_4, $ZnSO_4$, $FeSO_4$, $MnSO_4$, $CaCl_2$, each row represented a trial run and each column represented an independent variable concentrations. Plackett–Burman experimental design was based on the first order linear model:

$$Y = B_0 + \Sigma B_i X_i \quad \text{Eq. 1}$$

Where, Y was the response (exochitinase production), B_0 was the model intercept and B_i was the variables estimates. The effect of each variable was estimated by following equation,

$$E(X_i) = 2(\Sigma M_i^{+} - M_i^{-})/N \quad \text{Eq. 2}$$

Where, E (X_i) was the effect of the tested variable. M_i^{+} *and* M_i^{-} represented exochitinase production from the trials where the variable (X_i) measured was present at high and low concentrations, respectively and N was the number of trials in Eq. 2. The standard error (SE) of the concentration effect was the square root of the variance of an effect, and the significance level (*p*-value) of each concentration effect was determined using student's *t*-test

$$t(X_i) = E(X_i)/\text{SE} \quad \text{Eq. 3}$$

Where, E (X_i) was the effect of variable X_i.

Table1. Coded levels and real values for Plackett-Berman experiment

Trial No	X_1 Time	X_2 pH	X_3 Glucose	X_4 Insect	X_5 Soy	X_6 Chitin	X_7 Flour	X_8 $NaNO_3$	X_9 $CuSO_4$	X_{10} $MgSO_4$	X_{11} K_2HPO_4	X_{12} $ZnSO_4$	X_{13} $FeSO_4$	X_{14} $MnSO_4$	X_{15} $CaCl_2$	Exochitinase mU/ml
1	+1(6)	+1(8)	-1(0.2)	+1(0.1)	-1(0.2)	+1(0.5)	-1(0.2)	+1(0.02)	+1(0.001)	+1(0.1)	-1(0.1)	-1(0)	-1(0)	+1(0.001)	-1(0.01)	996
2	-1(4)	+1(8)	+1(0.5)	-1(0)	+1(0.6)	-1(0.1)	+1(1)	-1(0.01)	+1(0.001)	+1(0.1)	+1(0.2)	-1(0)	-1(0)	-1(0)	+1(0.05)	1009
3	+1(6)	-1 (6)	+1(0.5)	+1(0.1)	-1(0.2)	+1(0.5)	-1(0.2)	+1(0.02)	-1(0)	+1(0.1)	+1(0.2)	+1(0.001)	-1(0)	-1(0)	-1(0.01)	1034
4	-1(4)	+1 (8)	-1(0.2)	-1(0)	+1(0.6)	-1(0.1)	+1(1)	-1(0.01	+1(0.001)	-1(0.05)	+1(0.2)	+1(0.001)	+1(0.001)	-1(0)	-1(0.01)	1126
5	-1(4)	-1(6)	+1(0.5)	-1(0)	+1(0.6)	+1(0.5)	-1(0.2)	+1(0.02)	-1(0)	+1(0.1)	-1(0.1)	+1(0.001)	+1(0.001)	+1(0.001)	-1(0.01)	1131
6	-1(4)	-1(6)	-1(0.2)	-1(0)	-1(0.2)	+1(0.5)	+1(1)	-1(0.01	+1(0.001)	-1(0.05)	+1(0.2)	-1(0)	+1(0.001)	+1(0.001)	+1(0.05)	1236
7	+1(6)	-1(6)	-1(0.2)	+1(0.1)	+1(0.6)	-1(0.1)	+1(1)	+1(0.02)	-1(0)	+1(0.1)	-1(0.1)	+1(0.001)	-1(0)	+1(0.001)	+1(0.05)	1176
8	+1(6)	+1 (8)	-1(0.2)	+1(0.1)	-1(0.2)	+1(0.5)	-1(0.2)	+1(0.02)	+1(0.001)	-1(0.05)	+1(0.2)	-1(0)	+1(0.001)	-1(0)	+1(0.05)	2045
9	+1(6)	+1(8)	+1(0.5)	+1(0.1)	-1(0.2)	-1(0.1)	+1(1)	-1(0.01	+1(0.001)	+1(0.1)	-1(0.1)	+1(0.001)	-1(0)	+1(0.001)	-1(0.01)	1031
10	-1(4)	+1(8)	+1(0.5)	-1(0)	-1(0.2)	-1(0.1)	-1(0.2)	+1(0.02)	-1(0)	+1(0.1)	+1(0.2)	-1(0)	+1(0.001)	-1(0)	+1(0.05)	1266
11	+1(6)	-1(6)	+1(0.5)	+1(0.1)	+1(0.6)	-1(0.1)	-1(0.2)	-1(0.01)	+1(0.001)	-1(0.05)	+1(0.2)	+1(0.001)	-1(0)	+1(0.001)	-1(0.01)	876
12	-1(4)	+1(8)	-1(0.2)	-1(0)	+1(0.6)	+1(0.5)	-1(0.2)	-1(0.01)	-1(0)	+1(0.1)	-1(0.1)	+1(0.001)	+1(0.001)	-1(0)	+1(0.05)	1351
13	+1(6)	-1(6)	+1(0.5)	+1(0.1)	+1(0.6)	+1(0.5)	+1(1)	-1(0.01)	-1(0)	-1(0.05)	+1(0.2)	-1(0)	+1(0.001)	+1(0.001)	-1(0.01)	974
14	-1(4)	+1(8)	-1(0.2)	-1(0)	-1(0.2)	+1(0.5)	+1(1)	+1(0.02)	-1(0)	-1(0.05)	-1(0.1)	+1(0.001)	-1(0)	+1(0.001)	+1(0.05)	1049
15	+1(6)	-1(6)	+1(0.5)	+1(0.1)	+1(0.6)	-1(0.1)	+1(1)	+1(0.02)	+1(0.001)	-1(0.05)	-1(0.1)	-1(0)	+1(0.001)	-1(0)	+1(0.05)	926
16	+1(6)	-1(6)	-1(0.2)	+1(0.1)	-1(0.2)	-1(0.1)	1(0.2)	-1(0.01)	-1(0)	1(0.05)	-1(0.1)	-1(0)	-1(0)	-1(0)	-1(0.01)	1341

Real values (given in parentheses) are in (%)

Central composite design

After the components identification which affecting the production by Plackett–Burman design three variables (Chitin, $MnSO_4$, and $CaCl_2$ concentrations) for exochitinase were chosen for response surface methodology of central composite design (CCD). CCD proposed by (Adinarayana *et al*., 2003; Awad *et al*., 2013) was selected for this study. A 2^3 factorial design with six star points and six replicates at the central points were used to fit the second-order polynomial model. The experimental design consisted of 20 runs and the independent variables were studied at five different levels. The experimental design used for the study was represented in Table 3. All the experiments were done in triplicate and the average of exochitinase production obtained was taken as the dependent variable or response (*Y*). The second-order polynomial coefficients were calculated and analyzed using the 'SPSS' software (Version 16.0). Second degree polynomials, Eq.(4), which includes all interaction terms, were used to calculate the predicted response:

$$Y\ activity = ß_0 + ß_1 X_1 + ß_2 X_2 + ß_3 X_3 + ß_{11} X_1^2 + ß_{22} X_2^2 + ß_{33} C_3^2 + ß_{12} X_1 X_2 + ß_{13} X_1 X_3 + ß_{23} X_2 X_3 \quad \text{Eq. (4)}$$

Where, Y activity was the predicted production of exochitinase (mU/ml), X1, X2 and X3 were the independent variables corresponding to the concentration of Chitin, $MnSO_4$, and $CaCl_2$ respectively. ß0 was the intercept, $ß_1$, $ß_2$, $ß_3$ were linear coefficients, $ß_{11}$, $ß_{22}$,$ß_{33}$ were quadratic coefficients, $ß_{12}$, $ß_{13}$, $ß_{23}$ were cross product coefficients. Statistical analysis of the model was performed to evaluate the variance analysis (ANOVA). Statistical significance of the model equation was determined by Fisher's test value, and the proportion of variance explained by the model was given by the multiple coefficient of determination for each variable. The quadratic models were represented as contour plots (3D) and response surface curves were generated by using STATISTICA (0.6).

Partial purification

The optimized culture supernatant (crude exochitinase) of *Asperigillus awamori* EM66 produced on the previous medium was fractionated using ethanol concentrations (30-80%). The precipitate was obtained by centrifugation (10000 xg, 15 min. at 4°C) and suspended in an appropriate volume of 0.05 M acetate buffer (pH 5.0). The enzyme activity was determined for each fraction as described before. Also, protein content was evaluated for each fraction as described by Lowry *et al*., (1951).

Insect bioassay

Insects

Fourth instars larvae of three lepiopteran insect pests were used to test the exochitinase efficiency. These tested insects were the great wax moth larvae, *Galleria mellonella*, the Egyptian cotton leaf worm, *Spodoptera littoralis* and the black cutworms, *Agrotis ipsilon*. Greater wax moth culture *G. Mellonella* was preserve, on a modified artificial diet according to (Metwally *et al*., 2012). Cultures of the black cut worm, *A. ipsilon* (Huf.) and the cotton leaf worm, *S. littoralis* (Boisd.) larvae were reared on castor leaves *Ricinus communis* L. according to (Hussein, 2004).

Bioassay

Exochitinase application occurred through mixing with insect media. Two ml of the partial pure enzyme suspension was mixed with 50 g of the artificial diet for the greater wax moth in a 150 CC cups (Metwally *et al*., 2012). For the other two pests, the leaves of castor were sprayed with 2 ml enzyme suspension and left to dry in a 9 cm Petri dish lined with no.1 Whatman-filter paper. Five insect were added for each cup and Petri dish and 15 replicates for each tested insect. The treatments were kept in 25 ± 1°C and larval mortalities were recorded after 48h.

RESULTS AND DISCUSSION

At the preliminary stage of our study, the honey isolate *A. awamori* EM66 was tested for exochitinase production. The result indicated that it was exochitinase producer with activity equal (270 mU/ml) and 176.36 mg protein. Consequently, the specific activity was evaluated to be 1.531 U/mg by using the basal medium under submerged fermentation condition. Production of chitinase by *Aspergillus* spp. was studied in many reports as a potential producer for both endochitinase and exochitinase (Vionis *et al*., 1996, Xia *et al*., 2001, Nawani *et al*., 2002, Rattanakit *et al*., 2007, Brzezinska and Jankiewicz, 2012).

Exochitinase production optimization by multi-factorial experiments

In this work a sequential optimization approaches were used. The first trail aimed to screen the nutritional factors influencing growth of *A. awamori* EM66 with respect to exochitinase production. The second step aimed to optimize the influencing factors which controlling enzyme production process.

Estimation of the factors affecting exochitinase productivity

Firstly, the Plackett–Burman design was done to explain the relations between different medium components. Fifteen factors (X_1-X_{15}) included culture conditions and medium components were selected for optimization process. Exochitinase average activity for the different attempts were formulated as mU /ml and represented in Table 1. Main effect was estimated as the difference between both the averages of measurements made at the high level (+1) and at the low level (-1) of that factor. Table 1 showed a wide divergence from 876 to 2045 mU /ml on exochitinase activity. Factors effects on the enzyme activity were estimated and showed graphically in Fig. 1. It was introduced the view for factor ranking estimated by Plackett–Burman design. This variation reflected medium optimization significant to reach high productivity. Data analysis from Plackett–Burman tested contained a first order (main effects) model. Regression coefficients analysis for examined variables for the exochitinase were :incubation time, pH, glucose, dried insects, soya bean, chitin, wheat flour, $NaNO_3$, $CuSO_4$, $MgSO_4$, K_2HPO_4, $ZnSO_4$, $FeSO_4$, $MnSO_4$, $CaCl_2$. Chitin, $MnSO_4$, $CaCl_2$, $FeSO_4$, $NaNO_3$, K_2HPO_4, $CuSO_4$ and incubation period showed positive effect on exochitinase activity. Wheat flour, soya bean, glucose, pH, $MgSO_4$, $ZnSO_4$ and dried insect, were contributed negatively. First order model explained the link between the fifteen factors and the exochitinase activity which presented as follows:

$$Yactivity=853.0953+4.532X_1+38.26265X_2-987.144X_3+4152.63X_4-528.983X_5-9.469752X_6 -373.737X_7-1768.74X_8+26445.02X_9-529.596X_{10}+2311.588X_{11}+193353.7X_{12}+181015.2X_{13}-10997.6X_{14}+4499.06X_{15}$$ Eq.(5)

Table 2 revealed the t-test, p effect and confidence level. The variables reported confidence level above 98% in the Plackett–Burman design which were selected for further optimization; they were (Chitin, $MnSO_4$ and $CaCl_2$). Many reports showed the importance of chitin as a significant factor affecting chitinase production. It was reported that chitin is an essential factor influencing chitinase production by *Streptomyces lividans* (Vionis *et al*., 1996). In similar, colloidal chitin was proved to be the best substrate for production of chitinase in Microbispora sp. (Nawani *et al*., 2002).

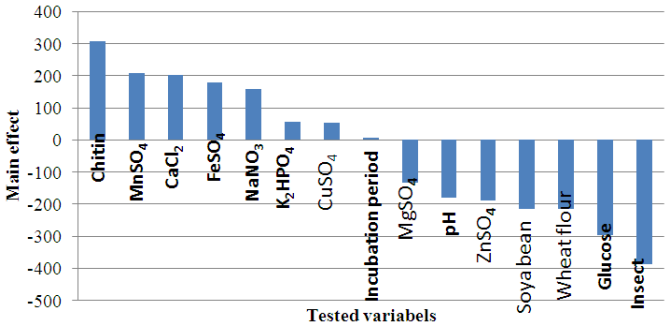

Fig. 1: Effect of culture conditions and medium composition on exochitinase (mU/ml) produced by *Aspergillus awamori* EM66

Table 2. Statistical analysis of Plackett- Burman design showing coefficient values, effect, t-and P- values for each variable on exochitinase analysis

Exochitinase analysis	Variables				
	Coefficient	Effect	*t*-test	*P*-value	Confidence level (%)
Intercept	853.0953				
Incubation period	4.532	6.75	0.086573	0.4661	53
pH	-38.26265	-190	-2.436866	0.0139	98
Glucose	-987.144	-297	-3.809206	0.0009	99
Insect	-4152.63	-386	-4.950686	0.0001	99
Soya bean	-528.983	-216	-2.770332	0.0071	99
Chitin	9.469752	206	2.642076	0.0092	99
White Flour	-373.737	-216	-2.770332	0.0071	99
$NaNO_3$	1768.74	158	2.026446	0.0304	96
$CuSO_4$	26445.02	53	0.679757	0.2535	74
$MgSO_4$	-529.596	-134	-1.718632	0.0531	94
K_2HPO_4	2311.588	57	0.73106	0.238	76
$ZnSO_4$	-193353.7	-180	-2.30861	0.0178	98
$FeSO_4$	181015.2	180	2.30861	0.0178	98
$MnSO_4$	10997.6	307	3.937462	0.0007	99
$CaCl_2$	4499.06	203	2.603599	0.01	99

Chitin also was an important factor for chitinase production by Streptomyces sp. Da11 (Han *et al*., 2008). Similar to

our result, $MnSO_4$ was found to enhance chitinase production by *Aspergillus terreus* (Ghanem *et al*., 2010). It was also mentioned as one of the most important factors affecting chitinase production by *Bacillus thuringiensis* (Sarrafzadeh and Hussein, 2012). On contrary, (Sharaf 2005) reported that $MnSO_4$was an inhibitor for chitinase production by *Alternaria alternate*. Our results also showed that $CaCl_2$ activated the production of exochitinase. Similarly, it was found that $CaCl_2$ activated *Pantoea dispersa* chitinase production (Gohel *et al*., 2004). Building on previous results, a medium including (g %), (glucose, 0.2; insect, 0.1; soyabean, 0.2; wheat flour, 0.2; $NaNO_3$, 0.02; $CuSO_4$, 0.001; $MgSO_4$, 0.05; K_2HPO_4, 0.2; $FeCl_3$, 0.001; $CaCl_2$, 0.05 at pH; 8). It was used as a plan medium for further investigations and the cultures were incubated for 6 days.

Optimization of the culture conditions by central composite design

For searching to the proper concentration for the most effected medium components (chitin, $MnSO_4$ and $CaCl_2$) showed confidence level with about 98% supported the highest chitinase productivity in the Plackett–Burman design. CCD experimental was employed coded and un-coded level of the three independent variables was recorded in Table 3. Also, the results in table.3. referred to the CCD experimental plan, the observed and predicted exochitinase production. Multiple regression analysis of the experimental data reported the following second order polynomial Eq. (6):

$$Y_{activity} = -4462.211 + 4591.092\ X_1 + 1.160E6\ X_2 + 62355.551X_3 - 604.012X_1^2 - 5.749E7X_2^2 - 87493.312X_3^2 - 74853.051X_1X_2 - 16412.830X_1X_3 - 3.522E6X_2X_3 \quad Eq.6$$

Where Y activity was the response (exochitinase production) and X_1, X_2 and X_3 were the coded values of the test variables (chitin, $MnSO_4$ and $CaCl_2$) respectively. The graphical representations of the regression equation was represented in the three-dimensional response surface and the two-dimensional contour plots graphs. They were fundamentally important for showing the relations between the interaction effects of the estimated factors and the response value. Fig. 2. A–C showed the response surface and contour plots of chitin and $MnSO_4$, $MnSO_4$ and $CaCl_2$, also chitin and $CaCl_2$ on exochitinase production respectively, the other component was fixed at zero level. Regression analysis results are shown in Table 4. analyzing the results of central composite designed experiments data. It is recognized the increase in t-value magnitude, the decrease in p-value, resulting more consideration of the corresponding coefficient (Aravindan and Viruthagiri, 2007). This implies that the variable with the largest effect represented in the linear effect of the $CaCl_2$ concentration and the squared term of the $CaCl_2$ concentration. In this finding, it was reported that $CaCl_2$, played a role to predict optimum chitinase production (Gohel *et al*., 2004). In this finding, it was reported that $CaCl_2$, played a role to predict optimum chitinase production (Gohel *et al*., 2004). Table 5. showed that F-value obtained by (ANOVA) analysis was (5.622) confirming the model significant. Moreover Prob> F (0.006) was less than 0.05 showed highly significant. (R^2) was evaluated as 0.914 for exochitinase activity (a value of R^2> 0.75 insuring the model aptness). This result indicated that the statistical model recorded 91.4% of variability in the response. Model goodness could be tested by the determination of coefficient (R^2) and correlation coefficient (R). The R^2 value is always between 0 and 1. The closer the R^2 to 1, referred to model powerful and better predicted response (Munk *et al*., 1963). R value (0.8340) concerning (Eq. 6) was near 1. This pointed to a close coincide among the experimental results and the theoretical values determined by equation model.

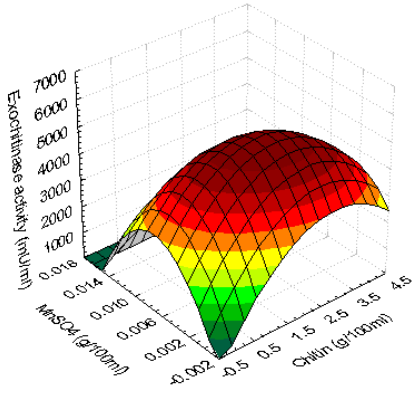

Fig 2. A. Response surface plot of exochitinase production by *Aspergillus awamori* EM66 showing the interactive effects of different concentrations of chitin and $MnSO_4$ at X_3= 0

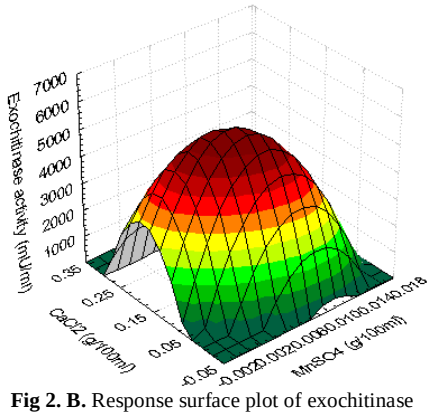

Fig 2. B. Response surface plot of exochitinase production by *Aspergillus awamori* EM66 showing the interactive effects of different concentrations of $CaCl_2$ and $MnSO_4$ at X_1= 0

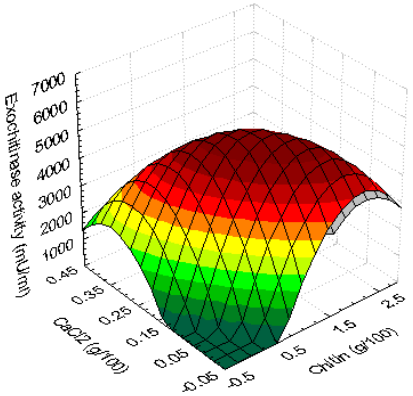

Fig 2. C. Response surface plot of exochitinase production by *Aspergillus awamori* EM66 showing the interactive effects of different concentrations of chitin and $CaCl_2$at X_2= 0

Table 3: Central composite design (CCD) consisting of 20 experiments for three experimental factors in coded and actual values for the production of exochitinase by *Aspergillus awamori* EM66.

Trial No.	Factors levels						Exochitinase (mU/ml)	
	Chitin (X_1)		$MnSO_4$ (X_2)		$CaCl_2$ (X_3)			
	Coded	Actual (g%)	coded	Actual (g%)	Coded	Actual (g%)	Experimental	Predicted
1[a]	-1	0.5	-1	0.0020	-1	0.05	1072.00 ±79	1834
2[a]	+1	2.0	-1	0.0020	-1	0.05	4445.80 ± 22	5000
3[a]	-1	0.5	+1	0.0080	-1	0.05	4112.36 ± 56	4063
4[a]	+1	2.0	+1	0.0080	-1	0.05	5574.40 ±16	6555
5[a]	-1	0.5	-1	0.0020	+1	0.2	5227.39 ± 136	5618
6[a]	+1	2.0	-1	0.0020	+1	0.2	3890.07 ± 67	4094
7[a]	-1	0.5	+1	0.0080	+1	0.2	3778.93 ± 14	4678
8[a]	+1	2.0	+1	0.0080	+1	0.2	3445.49 ± 124	3477
9[b]	-2	0.25	0	0.0040	0	0.1	3112.06 ± 17	3834
10[b]	+2	4.0	0	0.0040	0	0.1	4556.94 ± 51	4147
11[b]	0	1.0	-2	0.0010	0	0.1	4401.55 ± 78	3919
12[b]	0	1.0	+2	0.016	0	0.1	514.56 ± 5	523
13[b]	0	1.0	0	0.0040	-2	0.025	3290.10 ± 73	3687
14[b]	0	1.0	0	0.0040	+2	0.4	2058.24 ± 102	1688
15[c]	0	1.0	0	0.0040	0	0.1	5998.91 ± 374	5256
16[c]	0	1.0	0	0.0040	0	0.1	5968.90 ± 124	5256
17[c]	0	1.0	0	0.0040	0	0.1	5986.05 ± 16	5256
18[c]	0	1.0	0	0.0040	0	0.1	5994.62 ±282	5256
19[c]	0	1.0	0	0.0040	0	0.1	5977.47±181.11	5256
20[c]	0	1.0	0	0.0040	0	0.1	5998.33 ±249	5256

[a] Fractional 2^3 factorial design, [b] star points, [c] central points.

Table 4: Model coefficients and Analysis of variance (ANOVA) test estimated by multiples linear regression for exochitinase.

Term	Regression coefficient	Standard error	*t- test*	*P-value*
Intercept	-4462.211	1958.690	-2.278	0.046
X_1	4591.092	1423.223	3.226	0.009
X_2	1.160E6	355805.862	3.261	0.009
X_3	62355.551	14232.234	4.381	0.001
X_1^2	-604.012	214.858	-2.811	0.018
X_2^2	-5.749E7	1.343E7	-4.281	0.002
X_3^2	-87493.312	21485.780	-4.072	0.002
X_1X_2	-74853.051	144157.809	-.519	0.615
X_1X_3	-16412.830	5766.312	-2.846	0.017
X_2X_3	-3.522E6	1.442E6	-2.443	0.035

F value =5.622; P value = 0.006; R^2= 0.914; R=.8340

Table 5: Summary of the mortality percentages of the three lepidopteran pests after treatment with crude exochitinase.

Pest	% Larval Mortality	% Pupal Mortality	% Total Mortality
Galleria mellonella	84	8	92
Spodopteralittoralis	76	10.67	86.67
Agrotisipsilon	58.67	6.67	65.67

Model Validation

Validation was carried out under optimized medium conditions. It was predicted by the polynomial model. The experimental exochitinase production of 5886mU/ml was recorded. This result was close to its predicted value (5200mU/ml) after 6 days of fermentation validating the proposed model. An overall 22.2fold increased in exochitinase production was being achieved after RSM application. This reflected the success and value of optimization process. Many reports were mentioned in optimization of exochitinase productivity by response surface methodology. Exochitinase produced by marine isolate *Pantoea dispersa increased* about 3.9% after optimization by RSM (Gohel *et al*., 2004). *Aeromonass chubertii* chitinase increased about 1.6 % more than primary medium (Liu *et al*., 2013). On the other hand, chitinase produced by *Streptomyces* sp. Da11 associated with the South China Sea sponge *Craniella australiensis* increased about 39% with (RSM) optimization (Han *et al*., 2008). The optimization strategy led to an increased in chitinase production in the strains *Streptomyces sp.* NK1057, NK528 and NK951 by 29, 9.3 and 28%, respectively (Sarrafzadeh and Hussein, 2012).

Exochitinase productivity by *Aspergillusa wamori EM66* under submerged fermentation was effective and worthy optimized by using Plackett–Burman design and central composite design for selecting the statistically important factors and evaluation their optimal concentrations and illustrated graphically by second order polynomial model prepared by central composite design. For determination the relationship between the three factors and the exochitinase yield. Final components concentrations optimized with RSM medium were g%: (Glucose, 0.2; insect,0.1; Soyabean,0.2; Chitin,1.0; Wheat flour,0.2; $NaNO_3$,0.02; $CuSO_4$, 0.001; $MgSO_4$, 0.05; K_2HPO_4,0.2; $FeCl_3$,0.001; $MnSO_4$, 0.004; $CaCl_2$, 0.1).The initial pH was limited to 8 and the cultures were incubated for 6 days.

Enzyme partial purification

The crude enzyme was fractionated by using absolute ethanol (30-80%) W/V. The most promising fraction was obtained at 30% this result suggested that the exochitinase had low molecular weight. Its total activity and protein content recorded 23.34 U and 0.837 mg, respectively. This result referred to about 18 times purification fold compared to the preliminary crude culture filtrate. Accordingly, the specific activity was calculated to be 27.89 U/mg. The high specific activity of this enzyme gave it tremendous interest; since it referred that it approaches to purity.

Efficiency of the exochitinase against some lepidopteran pests

The partial pure enzyme was tested against three serious lepidopteran pests, larvae of the black cutworm, *A. ipsilon,* larvae of the Egyptian cotton leaf worm, *S. littoralis* and larvae of the greater wax moth, *G. mellonella.* The results in table 5.showed that exochitinase had a great effect on the larvae of the three tested pests. The maximum effect of the enzyme was noticed with exochitinase addition to the artificial diet of *G. mellonella* with a mortality percentage of 84% for the larvae and 8% for pupae and the total percentage reached to 92%. The treated larvae stopped feeding and suffered from recognizable blacking and severe rot which leads to their death (Fig.3, 4).

Fig 3: Effect of partial purified exichitinase on larvae of the great wax moth, *Galleria mellonella*,A. larva after treatment, B. larva without treatment.

Fig 4: Effect of partial purified exichitinase on different stages of larvae of the great wax moth, *Galleria mellonella*

When the exochitinase sprayed on castor leaves and fed to the cotton leaf worms, 86.67% total mortality of *S. littoralis* was recorded. Meanwhile, less effect of the enzyme (the total mortality reach to 65.67%) was noticed with the treatment of larvae *of A. ipsilon* with the enzyme. Great attention to the chitinolytic enzymes has been progress due to their possible involvement as defensive agents against chitin-including pestiferous and pathogenic organisms, like insects, nematodes, and fungi (Munk *et al*., 1963, Carr and Klessig, 1989, Linthorst, 1991, Sahai and Manocha, 1993). The peritrophic membrane and exoskeleton of insects act as physicochemical barriers to environmental hazards and predators. Both are composite materials made up primarily of chitin and protein, Chitinase of family 18 and 19 have a catalytic reaction and can be described as being similar to lysozyme and chitosanase in its mode of action (Sahai and Manocha, 1993). Another effect of chitinase could be revealed to that the microbial chitinases could be partially digesting the peritrophic membrane. This helping the microbes and their toxins in peritrophic membrane penetration (Smirnoff and Valero, 1983, Sneh *et al*., 1983, Shahabuddin and Kaslow, 1993, Wiwat *et al*.1996, Chandrasekaran *et al*., 2012).

CONCLUSIONS

This study is aiming to improve the exochitinase productivity for honey isolate *Aspergillus awamori EM66.* Statistical experimental design was used for optimization process. Estimation the constituents optimal concentrations have clear influencing on enzyme productivity can be obtained by a highly significant quadratic polynomial equation obtained by the central composite design. A high similarity between the predicted and experimental results was noticed. This reflected RSM accuracy and applicability to optimize the exochitinase production process. Mortality (92%) was recorded when partial pure enzyme was applied to the diet of both larva and pupal of *Galleria mellonella* wax, followed 86.67% and 65.67% mortality when fungal exochitinase was applied for larva of *Spodoptera littoralisn and Agrotis ipsilon respectively*. *Aspergillus awamori* EM66 exochitinase could be serving as an effective biopesticide to control harmful pests which destroy importantly economic crops instead of hazardous chemical pesticide.

Financial support and sponsorship: Nil.

Conflict of Interests: There are no conflicts of interest.

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How to cite this article:

Awad GEA, Abdel Wahab WA, Hussein M, El-diwany A, Esawy MA. Sequential optimizations of *Aspergillus awamori* EM66 exochitinase and its application asbio- pesticide. J App Pharm Sci, 2017; 7 (02): 067-075.