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Optimization of Process Parameters for Chitinase Production by a Marine Aspergillus Flavus MK20

Mohammed Rawway[1,*] *, Ehab Aly Beltagy*[2], *Usama Mohamed Abdul-Raouf*[1], *Mohamed Ahmed Elshenawy*[2] *and Mahmud Saber Kelany*[3] .

[1]Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Assiut, Egypt.
[2]Microbiology Department, National Institute of Oceanography and Fisheries, Alexandria, Egypt.
[3]Microbiology Department, National Institute of Oceanography and Fisheries, Suez, Egypt.

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Abstract: Chitinases assume a critical part in the disintegration of chitin and result in the usage of chitin as a renewable source. A sum of 27 chitinolytic microorganisms was segregated from the sediment samples. Taking after essential and auxiliary screening in colloidal chitin medium, the most potential strains showed the most elevated chitinolytic activity was genetically identified as *Aspergillusflavus* MK20. Colloidal chitin at concentration 0.3 gm/100 ml as well as the concentration of yeast extract and the nitrogen source at the same concentration gave 620.5 U/ml.Expansion in chitinase yield was watched when Placket Burman and box behnken were applied on the selected isolate. The optimum ingredients consisting of yeast extract, peptone, and dipotassium hydrogen phosphate for chitinase generation (1520.41 U/ml) by the isolate were observed to be 3.5 g/l, 2 g/l and 3 g/l separately. The outcomes got in the present work demonstrated that isolated fungi had an intense maker of chitinase and the catalyst can be capitalized for the biodegradation of chitinous end products and may discover applications as biocontrol operators against organisms.
Keywords: Aspergillus flavus, Colloidal chitin, Chitinase.

1 Introduction

Chitin is considered one of the most abundant polymer in marine waste that contributes to the mechanical strength of chitin containing organisms which in turn represents as the agent for chitin containing waste management [1, 2].

Marine chitin wastes represented in Crab shell, Snail shell, Shrimp shell and fish scales can used be as substrate for chitinase enzymes [2]. Shrimp byproducts were used as carbon source for production of chitinase enzyme from *Purpureocillum lilacinum* CFRNT 12[3]. Also, Krishnaveni and Ragunathanproduced chitinase enzymes from shrimp wastes (4.7 U/min), Snail shell (4.3 U/min), Crab shell (4.2 U/min) and fish scales (3.7 U/min) which can degrade (0.24g – 0.11g – 0.46g – 1.49g) of Crab shell, Snail shell, Shrimp shell and fish scales respectively by *Aspergillus terreus* CBNRKR KF 529976.

The optimization studies of chitinase production was enhanced by response surface methodology (RSM) based on statistical experimental design. **Patil and Jadhav (2014)** were improving the productivity of chitinase by using three substrates (wheat, rice and red gram) in experimental design for *penicillium ochrochloron* in the presence of four variables [4]. Optimization experiments can take advantages to get economical chitinase production using chitin residues.

The proteins representing the chitinase enzymes have significant role in biological control as antibacterial and antifungal activities [1, 5]. In this investigation, factorial design and Box-Behnken design were used towards the economic production of chitinase from ***Aspergillus flavus* MK20.**

2 Experimental Section

2.1 Materials

Potato dextrose agar [6], Sabouraud dextrose broth [7], chitin from shrimp shells (poly-(1→4)-β-N-acetyl-D-glucosamine) Sigma-Aldrich Co., Muller-Hinton agar [8]. All other reagents and chemicals were of analytical

2.2 Method

*Corresponding author e-mail: m_rawway@azhar.edu.eg

2.2.1 Chitinolytic Microorganism's Isolation

Marine sediment samples were collected from Gulf of Suez (Egypt) and were immediately transferred to the laboratory condition [9]. Approximately one gram of sediment samples was aseptically transferred into 9 ml of filtrated seawater. Samples were inoculated on Potato dextrose agar medium. After that, all isolates were purified and tested for chitinase production using sucrose yeast extract peptone medium which containing g/l (colloidal chitin 3.0g, yeast extract 3.0g, peptone 5.0g, di potassium hydrogen phosphate 1.0g, potassium chloride 2.0g, sodium nitrate 2.0g, magnesium sulphate 0.5g, ferrous sulphate 0.01g and pH 5.6 ±0.2), incubated at 28 ºC for 7days in shaker incubator 150rpm [10].

2.2.2 Preparation of Colloidal Chitin

The method was anticipated according to [10, 11]. Five grams of chitin was added to 50 ml of conc. HCl and kept aside for 30 min. with occasional shaking. Cold water (250 ml) was then added and colloidal chitin precipitate was washed 2 to 3 times with water. The pH was adjusted to 7.0 with 8N NaOH. Finally the precipitate was collected. Freeze dried and stored at 4 ºC for further use.

2.2.3 Estimation of Protein Contents

The Lawry method was described to estimate the protein content in the samples using bovine serum albumin as standard protein according toLowry method[9].

2.2.4 Enzyme Assay

Chitinase activity was estimated by calculating the amount of reducing sugar secreted using Di-nitrosalicylic acid (DNS) method at 575 nm [12, 13]. A reaction mixture containing 0.3 % chitin powder (100 micron) and supernatant (100 micron) was maintained at pH 7.0 and 40 ºC for 20 min. After incubation, 400 micron of DNS reagent was added and the reaction mixture was heated in water bath at 100 ºC for 5-10 min. Two ml of sodium potassium tartrate (40 %) was added to the mixture, cooled and the absorbance was read at 575 nm. Chitinase activitydefined as the amount of chitinase that catalysis the release of mg/ml of reducing sugar per min under assay condition [14].

2.2.5 Optimization of Process Parameters

Chitin as a sole carbon source was studied while the nitrogen sources for each were studied including yeast extract, peptone and $NaNO_3$ **(Table 1)**. The design was modified for the optimization of successive parameters influencing chitinase production. The Placket-Burman design was studied on the medium components by different concentrations with seven variables at three equidistant levels (-1, 0 and +1) as shown in **Table 2**[10].

For the experimental result, the effect of factors is defined using a first order polynomial equation model [Equation (1)] for the PB design;

$$Y = \beta_0 + \Sigma\, \beta_t X_t \qquad (1)$$

$t=1$

Where Y is the response, β_0 is a constant, β_t is a linear coefficient and X_t is the coded factor level.

Placket-Burman results have been used in the second experimental design (Box-Behnken design). Three variables were examined in fifteen trials at three equidistant levels (-1, 0 and +1). The Box-Behnken design is depicted in **table 3** [15, 16].

Predicting chitinase activity was calculated using the second order polynomial equation:

$$Y = \boldsymbol{\beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1 X_1 + \beta_{22} X_2 X_2 + \beta_{33} X_3 X_3} \qquad (2)$$

Where Y is predicted response, $\boldsymbol{\beta_0}$ is intercept $\boldsymbol{\beta_1}$, $\boldsymbol{\beta_2}$ and $\boldsymbol{\beta_3}$ are linear coefficients, $\boldsymbol{\beta_{11}}$,$\boldsymbol{\beta_{22}}$, and $\boldsymbol{\beta_{33}}$ are squared coefficients, $\boldsymbol{\beta_{12}}$,$\boldsymbol{\beta_{13}}$, and $\boldsymbol{\beta_{23}}$ are interaction coefficients, and $\boldsymbol{X_1}$, $\boldsymbol{X_2}$, and $\boldsymbol{X_3}$ are independent variables. By using this equation, it is possible to evaluate the linear, quadratic and interactive effects of the independent variables on the response appropriately.

2.2.6 Statistical Analysis

The results were statistically analyzed by using analysis of variance (ANOVA) techniques, where a P-value of ≤ 0.05 was regarded as significant [17].

3 Results and Discussion

3.1 Microorganisms and Screening of Chitinase Production

The increase of enzyme products often depends on screening a large number of microorganisms for an enzyme activity [19].In this study, Chitinolytic strains were isolated from wastewater beaches located in Gulf of Suez. The total of 27 isolated fungi were purified by repeated sub-culturing on the Sabouraud dextrose agar medium and incubated at 28 °C. The isolates were identified based on the colony morphology basis of colloidal chitin degradation and zone of clearance.Four isolates were selected for secondary screening using minerals synthetic broth medium and for the chitinase activity(**figure 1 and 2)**. The most active isolate (4 M) was identified based on the molecular

identificationand confirmed to be *Aspergillus flavus* MK20 (MF538738).Eighteen S rRNA sequence analysisis used for the identification of fungi [20].

This paper is a study to optimize the production of chitinase enzyme by *Aspergillus flavus*, where the placket-purman and Box-Behnken designs are used as significant modules for achieving this goal.Based on maximum chitinase production, the highest activity recorded was (620.5 mg/l enzyme activity) after 7 days of incubation. Sample (4M) which identified as *Aspergillus flavus* was selected for further and more investigations. The identification was done to 4 M isolate basing on 18S rRNA gene sequencing. Sequences were further analyzed using BLAST from the National Center of Biotechnology Information (NCBI) website. The percentages of sequence matching were also analyzed and the sequence was submitted to NCB1-Gene Bank which showing 97% homology of 4 M isolate with *Aspergillus flavus* accession number JQ269824 [18] and according to the Gen Bank data the isolate 4 M was identified as*Aspergillus flavus* MK20with accession number (MF538738)

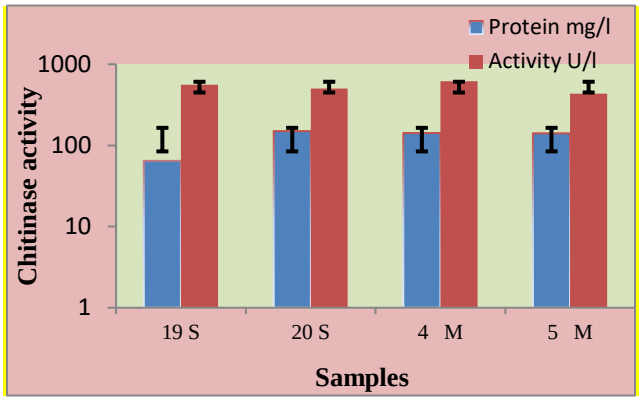


Figure 1. Detection of most active isolate producing chitinase enzyme

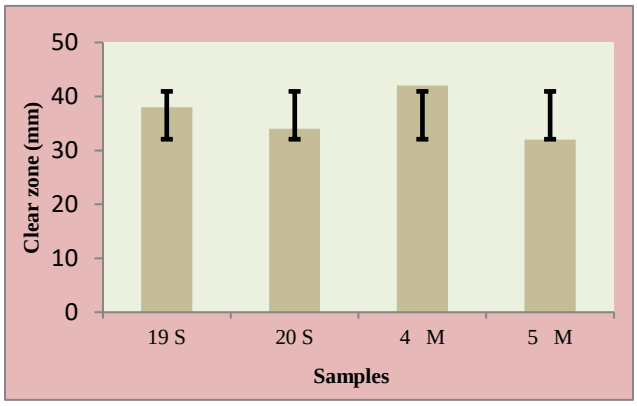


Figure 2. Detection of most active isolate producing chitinase enzyme by well activity (*mm*)

3.2 Optimization Process

As shown in **table 1**, seven culture variables were examined and the design was applied with 9 different incubation conditions. The culture filtrate of each trial was taken where the protein content, chitinase activity, specific activity and clear zone were determined.All experiments were performed in duplicates and the averages of results of chitinase activity (U/L) and protein content (mg/l) are calculated. The principal statistical analysis of this experiment is shown in **Table 1 and 3**. The main effect of each variable upon enzyme production as well as protein content was studied **(Figure 3).** The data indicated that, the presence of high levels of chitin, dipotassium hydrogen phosphate, yeast extract, potassium chloride and pH in the growth medium affects chitinase production by *Aspergillus flavus* MK20(MF538738) positively. On the other hand, the same figure suggests that the presence of peptone and sodium nitrate at their lowest levels would result in high chitinase activity.

The main effect results pointed out those high levels of dipotassium hydrogen phosphate, yeast extract and peptone in the medium with low levels of chitin, potassium chloride, sodium nitrate and pH induced the existence of high protein content in the culture filtrate of the immobilized fungal cells.

According to the result, it can be predicted that, the optimum medium for producing an extracellular chitinase from the culture of *A. flavus* with a relatively high chitinase activity is (g/ l): Chitin, 3.5 ; K_2HPO_4, 1.4; yeast extract, 3.5; peptone, 4.0; KCL, 3.0; $NaNo_3$, 1.0; pH, 6.2. Different fungal isolates have been reported for chitinase production such as *Purpureocillium lilacinum* CFRNT12 [3], *Penicilliumochrochloron* MTCC 517 [4], *Aspergillus flavus* CFR 10 &*Fusarium oxysporum* CFR 8 [21] and *Penicillium oxalicum* SAEM-51 [22]. By *Aspergillus flavus* in the present study, a maximum chitinase activity was detected after seven days of incubation. Similar result was reported by **Nidheesh *et al.*, 2015** while maximum chitinase activity was detected after seven days [3]. The primary reasons for the reduction in chitinase production might be due to the lack of nutrients in the culture medium or the production of toxic chemicals in the medium, which in turn disabling the enzyme action. This result corresponds to **Aliabadi *et al.*, 2016**in terms of interpretation the reduction of the chitinase activity by *Cohnella sp.* A01 after 48 hours [23].

Regarding the placket Burman design, the medium ingredients optimization; the optimum concentration of carbon, nitrogen, and phosphorus sourcesfor maximum enzyme production by *Aspergillus flavus* were determined where chitin was applied as a sole carbon source for enhancing chitinase enzyme production with *Aspergillus flavus* [24], and yeast extract, peptone with sodium nitrate as nitrogen sources to synthesis protein and nucleic acid

[25, 26], while dipotassium hydrogen phosphate was recognized as the best phosphorus source for the enzyme production by *Aspergillus flavus*, in concordance with chitinase biosynthesis by *Vibrio alginolyticus* JN863235 [27], *Aspergillus terreus* 7452-09 [10] and *Paenibacillus sp*. D1 [28].

Box-Behnken is complete three-level factorial design. In this design, two levels factorial design is repeated over different sets of variables. According to placket-Burman design the most effective variables were yeast extract, peptone and dipotassium hydrogen phosphate. These variables were introduced in Box-behnken design for enhancing chitinase production**(Table 3)**.

By applying multiple regression analyses on the experimental data, the following second order polynomial equation was found to explain the enzymatic production as a function of the three variables studied (Eq. 2):

Y = 1121.735 + (-361.575X1) + (28.328X2) + (-100.318X3) + (-96.1715X1X2) + (-22.113X1X3) + (106.3936X2X3) + (-181.055X1X1) + (-122.742X2X2) + 395.9806X3X3

Where Y represents chitinase production (U/l) and X1, X2 and X3 are coded values of dipotassium hydrogen phosphate, yeast extract and peptone, respectively.

As can be seen from **Table 3**, yeast extract, peptone and dipotassium hydrogen phosphate were significant (*P-value*< 0.05). The squared correlation coefficient (R2) and adjusted R2 were calculated to be 0.86 and 0.89, respectively. Through solving Equation and analyzing response surface plots, the optimum levels of peptone, yeast extract, and K_2HPO_4were deduced theoreticallyto be2.0, 3.5, and 3.0g/L, respectively. At these concentrations, predicted chitinase activity was calculated to be 1121.735 U/l.

The **figure (4)** represents the combined effect of di potassium hydrogen phosphate, yeast extract and peptone on chitinase production using colloidal chitin as a substrate. **Figure (4-A)** shows that, (2.0g/l) of peptone combined with (3.5g/l) of yeast extract gave the highest response, while **figure (4-B)** shows that, (3.5g/l) of yeast extract combined with (3.0g/l) of K_2HPO_4 gave the highest response. Also, the **figure (4-C)**, (3.0g/l) of K_2HPO_4 combined with (2.0g/l) of peptone gave the highest response.

Three dimensional plot charts of box-behnken design and solver equation were demonstrated that, the (2.0g/l) of peptone and (3.5g/l) of yeast extract combined with (3.0g/l) of K_2HPO_4 gave the highest response. This results indicated that, nitrogen and phosphate source are positively effect on the production of chitinase enzyme. This result concomitant with **Meruvu and Donthireddy (2014)** in terms of using nitrogen sources in their optimization process [29].

In order to evaluate the accuracy of the applied optimization designs, a verification experiment was carried out. The predicted optimum levels of independent variables were examined and compared to the basal condition through measuring the protein content and chitinase activity. It was found that the chitinase activity reached about 1520.41U/l, which is approximately 2.45 times higher than that obtained from the basal medium (620.5 U/l).As a result of verification test for optimized medium, a maximal chitinase production (1314.1 U/l) was achieved after 7 days of submerged cultivation of *Aspergillus flavus* with colloidal chitin as carbon source, and higher than those obtainde by **Nidheesh *et al.*, 2015, Patil & Jadhav, 2014 Narayanan *et al.*, 2013and Jenifer *et al.*, 2014** where the chitinase production recorded (41.78 U/l – 142.7 U/l – 1216.659 U/l – 42.86 U/l) respectively [3, 4, 10, 30].

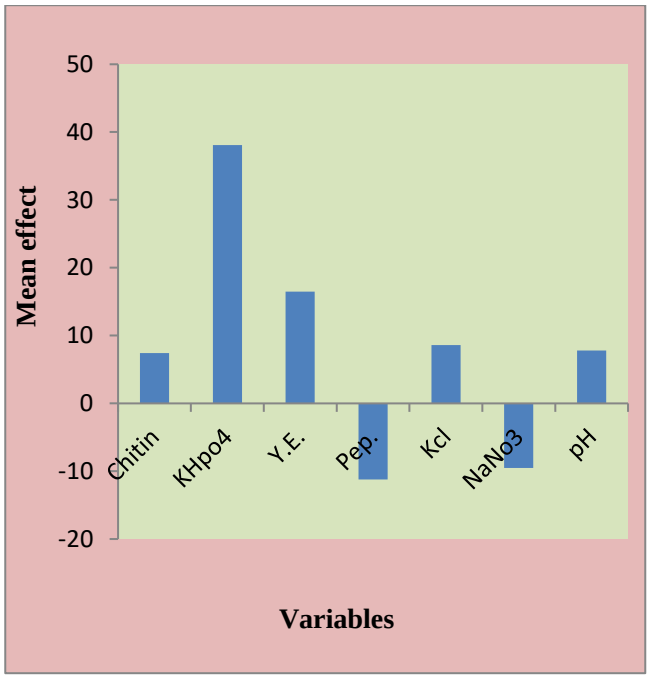


Figure 3. Main effect of Placket-Burman variables on chitinase production by *Aspergillus flavus*

Table1. Placket-Burman Design. Effect of various components on chitinase production per 100ml.

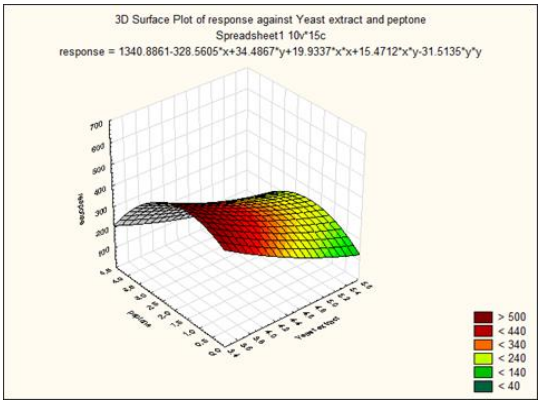
	Factors symbols per 100ml (g)							Result			
Trials	**Chitin**	**K_2HPO_4**	**Y.E.**	**Pep.**	**KCl**	**$NaNO_3$**	**pH**	**Activity (U/l)**	**Protein (mg/l)**	**Sp. Activity (U/l)**	**Clear Zone mm**
1	-(0.25)	-(0.06)	-(0.25)	(0.6)	(0.3)	(0.3)	-(5)	428.73	121.42	3.53	22
2	(0.35)	-(0.06)	-(0.25)	-(0.4)	-(0.1)	(0.3)	6.2	473.94	116.9	4.1	31
3	-(0.25)	(0.14)	-(0.25)	-(0.4)	(0.3)	-(0.1)	6.2	597.89	99.02	6.04	29
4	(0.35)	(0.14)	-(0.25)	(0.6)	-(0.1)	-(0.1)	-(5)	546.58	103.02	5.31	28
5	-(0.25)	-(0.06)	(0.35)	(0.6)	-(0.1)	-(0.1)	6.2	492.74	114.86	4.29	32
6	(0.35)	-(0.06)	(0.35)	-(0.4)	(0.3)	-(0.1)	-(5)	542.01	94.32	5.75	31
7	-(0.25)	(0.14)	(0.35)	-(0.4)	-(0.1)	(0.3)	-(5)	574.01	81.89	7.01	32
8	(0.35)	(0.14)	(0.35)	(0.6)	(0.3)	(0.3)	6.2	606.01	101.9	5.95	35
9	(0.3)	(0.1)	(0.3)	(0.5)	(0.2)	(0.2)	5.6	586.71	93.63	6.27	26
Effect	7.4	38.1	16.5	-11.2	8.6	-9.5	7.8	-	-	-	-
SS (v)	0.83	0.11	0.83	2.33	0.4	0.44	285	-	-	-	-
F-value	3.98	4.28	3.98	1^{-4}	1.1	.009	1.0	-	-	-	-

Table 2 . Main effect and *t*-value results of multi-factorial independent variables affecting the production of chitinase.

	symbols	**Levels per 100 ml**			**Main effect**	***t-value***
		(-1)	(0)	(+1)		
Chitin	**Chitin**	(0.25)g	(0.30)g	(0.35)g	7.4	.344
Di potassium hydrogen phosphate	**K_2HPO_4**	(0.06)g	(0.1)g	(0.14)g	38.1	.206
Yeast Extract	**Y.E.**	(0.25)g	(0.30)g	(0.35)g	16.5	1.24
Peptone	**Pep.**	(0.40)g	(0.50)g	(0.60)g	-11.2	-.026
Potassium chloride	**KCl**	(0.10)g	(0.20)g	(0.30)g	8.6	.249
Sodium nitrate	**$NaNO_3$**	(0.10)g	(0.20)g	(0.30)g	-9.5	-.009
pH	**pH**	5.0	5.6	6.2	7.8	.739

Table 3. Box-Behnken design matrix for *Aspergillus flavus* to optimize the chitinase production

	Factors symbols per 100ml (g)										Result
Trials	**Y.E.**	**Pep.**	**K_2HPO_4**	**Chitin**	**KCL**	**$NaNO_3$**	**pH**	**$MgSO_4$**	**$FeSO_4$ (10^{-3})**	**Temp.**	**Activity (mg/l)**
1	(0.45)	+(0.4)	-(0.1)	0.35	0.3	0.2	6.2	0.05	1	28	118.6
2	+(0.55)	+(0.4)	(0.2)	0.35	0.3	0.2	6.2	0.05	1	28	277.2
3	(0.45)	(0.2)	(0.2)	0.35	0.3	0.2	6.2	0.05	1	28	769.9
4	+(0.55)	-(0.05)	(0.2)	0.35	0.3	0.2	6.2	0.05	1	28	474.7
5	- (0.35)	-(0.05)	(0.2)	0.35	0.3	0.2	6.2	0.05	1	28	753.5
6	+(0.55)	(0.2)	+(0.3)	0.35	0.3	0.2	6.2	0.05	1	28	312.4
7	(0.45)	-(0.05)	+(0.3)	0.35	0.3	0.2	6.2	0.05	1	28	747.1
8	(0.45)	(0.2)	(0.2)	0.35	0.3	0.2	6.2	0.05	1	28	935.1
9	+(0.55)	(0.2)	-(0.1)	0.35	0.3	0.2	6.2	0.05	1	28	560.1
10	-(0.35)	(0.2)	+(0.3)	0.35	0.3	0.2	6.2	0.05	1	28	1036.9
11	-(0.35)	(0.2)	-(0.1)	0.35	0.3	0.2	6.2	0.05	1	28	1314.1
12	-(0.35)	+(0.4)	(0.2)	0.35	0.3	0.2	6.2	0.05	1	28	409.9
13	(0.45)	- (0.05)	-(0.1)	0.35	0.3	0.2	6.2	0.05	1	28	626.9
14	(0.45)	(0.2)	(0.2)	0.35	0.3	0.2	6.2	0.05	1	28	185.8
15	(0.45)	+(0.4)	+(0.3)	0.35	0.3	0.2	6.2	0.05	1	28	724.5
Variable	**X1**	**X2**	**X3**	**X1X2**	**X1X3**	**X2X3**	**X1X1**	**X2X2**	**X3X3**		**Response**
Effect	-361.6	28.33	-100.32	-96.17	-22.11	106.4	-191.1	-122.7	395.98		
P-value	-0.03	-0.012	-0.034	-0.9	-0.84	0.53	0.79	-0.33	0.53		
T-value	-2.5	-0.18	-0.68	-0.51	-0.13	0.42	0.83	-0.64	1.69		
F-value	0.868890										



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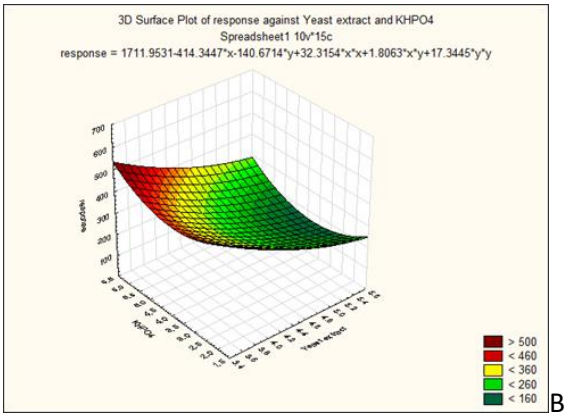

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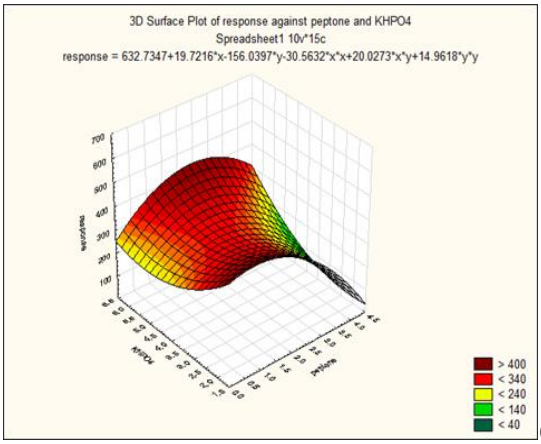

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Figure 4. Three-dimensional response surface curve showing the effect of interactions of (A) yeast extract and peptone, (B) yeast extract and KHPO4, (C) peptone and KHPO4 in incubation period of Aspergillus sp. to producing chitinase enzyme.

4 Conclusions

Chitinase plays an important role in the decomposition of chitin and potentially in the utilization of chitin as a renewable resource. The result concluded that, ***Aspergillus flavus*** have theability to produce a valuable production of chitinase
with optimized medium containing yeast extract, peptone and dipotassium hydrogen phosphate. This
enzyme may also be useful in the management of sea food waste industries.

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