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Full Length Research Paper

Statistical optimization of cultural conditions for chitinase production from shrimp shellfish waste by *Alternaria alternata*

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Alternaria alternata*, a local isolate from fishery polluted soil, was used successfully for the biodegradation of shrimp shellfish waste in favor of the production of highly active chitinase enzyme. Chitinase production was noticeably influenced by the culture medium and the highest enzyme production was attained through the log growth phase (96 h). Pronounced increase in chitinase production was concomitant with the finest waste size. Stagnant culture conditions were more adequate than shake cultures. Statistically based experimental designs were applied to optimize the production of chitinase by *A. alternata*. Plackett-Burman factorial design revealed that concentrations of glucose, $MnSO_4.H_2O$ and $CoCl_2$ were the most significant factors affecting the process of enzyme production. Maximum enzyme activity (8.12 U/min), which is approximately 1.8 folds the activity expressed in the basal medium, has been assayed at concentrations (g/l): glucose, 9; $MnSO_4.2H_2O$, 3.2 and $CoCl_2$, 2, after only 90 h of fermentation, when the second optimization step of Box-Behnken design was applied. The crude chitinase was characterized and maximum activity (19.53 U/min) was attained in reaction mixture of 50°C incubation temperature, 1.5 ml crude enzyme, 0.5 ml of 1% colloidal chitin, pH5 and reaction time of 10 min. The enzyme is thermostable and lost 20% of its activity when heated at 60°C for 60 min. The effect of metal ions in enzyme activity revealed that the enzyme have specific requirement of Ca and K ions for its activity. The results indicated that *A. alternata* is highly efficient fungus to produce highly active chitinase when grown in statistically optimized medium containing shrimp shellfish waste.

Key words: *Alternaria alternate*, chitinase, shellfish, statistical optimization.

INTRODUCTION

Chitin is the second most abundant renewable carbohydrate polymer in nature after cellulose and possibly the most abundant in marine environments (Bansode and Bajekal, 2006; Madigan and Martinko, 2005). It largely exists in wastes from processing of marine food products (20 to 58% of its dry weight) (Ravkumar, 2000). The waste generated from the world-wide production and processing of shellfish is a serious problem of growing magnitude. This abundant waste may pose environmental hazard due to the easy deterioration (Mejia-Saules et al., 2006). Chitinases (EC 3.2.1.14) are glycosyl hydrolases which catalyze the degradation of chitin. These enzymes have a wide range of biotechnological applications such as preparation of pharmaceutically important chitooligosaccharides and N-acetyl-D-glucosamine (Kuk et al., 2005; Pichyangkura et al., 2002; Sorbotten et al., 2005), isolation of protoplasts from fungi and yeasts (Dahiya et al., 2005), production of single-cell protein (Guevara-Gonzalez and Torres-Pacheco, 2006), control of pathogenic fungi (Chae et al., 2006) and treatment of chitinous waste (Wang and Hwang, 2001). A wide range of microorganisms have the ability to degrade chitin by producing chitinases for nutrition, antagonism and combating parasites

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(Faramarzi et al., 2009). Chitinases are produced by several fungal species (Pedraza and Lopes, 1991; Patidar et al., 2005; Yamazaki et al., 2008; Kern et al., 2009; Ghanem et al., 2010).

Studies on medium optimization for chitinases production are the worthwhile technique for multifactor experiments because it is less time consuming and capable of detecting the true optimum of the factor. In addition, medium composition greatly influence the microbial production of extracellular chitinase and their interaction play an important role in the synthesis of this enzyme. On the other hand, medium optimization is very important not only to maximize the yield productivity, but also to minimize the product cost (Al-Sarrani and El-Naggar, 2006; Youssef et al., 2006; Abdel-Fattah et al., 2007). Studies on the medium optimization for chitinase production using statistical approach have been done (Nawani and Kapandis, 2005; Al-Ahmadi et al., 2008; Faramarzi et al., 2009; Ghanem et al., 2010). The objective of the present work was to characterize the medium and growth conditions of *Alternaria alternata* for maximum biodegradation of shrimp shellfish waste in favor of the production of highly active chitinase, using statistical designs of Plackett-Burman and Box-Behnken, and to characterize the produced crude enzyme.

MATERIALS AND METHODS

Microorganism

Alternaria alternata was isolated using enrichment method (Patidar et al., 2005) from polluted soil at the market of fishery, fish sale and reparation in Jeddah, Saudi Arabia. Identification was mainly on the basis of cultural and morphological characteristics (Frey et al., 1979; Watanabe, 2002).

Shrimp shellfish waste

The waste was kindly provided by Saudi Arabian Company of Fishery in Jeddah, Saudi Arabia. The shrimp shellfish waste was washed with tap water then distilled water. Thereafter, exposed to water vapor and air dried at room temperature (Wang et al., 2006).

Inoculum and cultivation

A. alternata was maintained on potato dextrose agar (PDA), where the fungus was grown for 5 days at 30±2°C. The stocks were kept in the refrigerator and subcultured at monthly intervals (Patidar et al., 2005). Spores suspension of *A. alternata* 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 to approximately 10^6 Spores/ml. The organism was allowed to grow in 100 ml aliquots of mineral medium of the following composition (g/l): shrimp shellfish waste, 20; $(NH_4)_2SO_4$, 2; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; NaCl, 5; $CaCl_2$, 0.02; $FeSO_4.7H_2O$, traces and pH5 (Al-Nusaire, 2007) and dispensed in 250 ml Erlenmyer flasks. Standard inocula (10^6 spores/ml) were used to inoculate the flasks which were then incubated at 30±2°C under stagnant culture conditions for 7days. Thereafter, the residual fermentation products were centrifuged at 7000x g for 20 min in a cooling centrifuge. The clear supernatant was used to determine extracellular protein and considered as crude enzyme to assay chitinase activity (Rattanakit et al., 2007).

Analytical methods

Total protein assay

The extracellular protein (E.C.P) was determined colorimetrically using Biuret method (Slater, 1986).

Chitinase activity assay

It was measured using colloidal chitin as substrate (Bindo et al., 2005). Enzyme solution (0.5 ml) was added to 0.5 ml of substrate solution, which contained 1% colloidal chitin in phosphate buffer (0.05 M, pH 5.2) and 1 ml distilled water. The mixture was then incubated in shaking water bath at 50°C for 10 min, thereafter 3 ml of 3,5-dinitrosalicyclic acid reagent was added. The mixture was then placed in a boiling water bath for 5 min, after cooling, the developed color, as indication to the quantity of released N-acetylglucosamine (NAGA), was measured spectrophotometrically at 575 nm. The amount of NAGA was calculated from standard curve of NAGA.

Chitinase activity (C.A) (U/min) = the amount of enzyme releasing 1 µmol NAGA /min from colloidal chitin, under assay conditions (Bindo et al., 2005).

$$\text{Specific activity (S.A)} = \frac{\text{Chitinase activity (U/min)}}{\text{Extracellular protein}}$$

$$\text{Relative activity (R.A)} = \frac{\text{Highest chitinase activity (A)} - \text{Chitinase activity of the treatment (B)}}{\text{Highest chitinase activity (A)}} \times 100$$

Then the value is subtracted from 100.

Effect of cultivation medium

To test the quantity and quality of ingredients of the cultivation medium on shrimp shellfish waste biodegradation in favor of production of active chitinase, different five media were tested, as follows (g/100 ml): 1) K_2HPO_4, 0.1; $MgSO_4.7H_2O$, 0.05; $NaNO_3$, 0.1 (Wang et al., 2006); 2) Peptone, 0.03; yeast extract, 0.03; K_2HPO_4,0.07; KH_2PO4, 0.03; $MgSO_4.7H_2O$, 0.05; Rose Bengal, 0.05 (Rattanakit et al., 2003); 3) $(NH_4)_2SO_4$, 0.2; K_2HPO_4,1; $MgSO_4.7H_2O$, 0.05; KCl, 0.05; NaCl, 0.5; $CaCl_2$, 0.02; $FeSO_4.7H_2O$, traces (Al-Nusaire, 2007); 4) Glucose, 0.3; peptone, 0.1; $(NH_4)_2SO_4$, 0.14; urea, 0.03; $MgSO_4.7H_2O$, 0.03; $FeSO_4.7H_2O$, 0.5; $MnSO_4.2H_2O$, 0.16; $ZnSO_4.7H_2O$, 0.14; $CoCl_2$, 0.2 (Rattanakit et al., 2002); 5) KH_2PO4, 0.3; K_2HPO_4, 0.1; $MgSO_4.7H_2O$, 0.07; $(NH_4)_2SO_4$, 0.14; NaCl, 0.05; yeast extract, 0.05; peptone, 0.05 (Kim et al., 1988). Each medium contain 2 g shrimp shellfish waste. Initial pH was adjusted at 5. Standard inoculum (10^6 spores/ml) of *A. alternata* was used and the flasks were stagnantly incubated at 30±2°C for 7 days. Thereafter, the necessary analyses were carried out.

Effect of different sizes of shrimp shellfish waste

The best formulated medium (4) that favored production of highly

Table 1. Plackett-Burman experimental design for 11 variables and 14 trials.

Trial	Independent variables (g/100ml)											Response C.A (U/min)
	SH	GL	PE	NH	UR	MG	FE	MN	ZN	CO	SP	
1	3.0(+)	0.0(-)	0.2(+)	0.0(-)	0.0(-)	0.0(-)	1.0(+)	0.32(+)	0.3(+)	0.0(-)	2×10^6(+)	1.59
2	3.0(+)	0.6(+)	0.0(-)	0.3(+)	0.0(-)	0.0(-)	0.0(-)	0.32(+)	0.3(+)	0.4(+)	5×10^5(-)	2.17
3	1.5(-)	0.6(+)	0.2(+)	0.0(-)	0.1(+)	0.0(-)	0.0(-)	0.0(-)	0.3(+)	0.4(+)	2×10^6(+)	1.74
4	3.0(+)	0.0(-)	0.2(+)	0.3(+)	0.0(-)	0.06(+)	0.0(-)	0.0(-)	0.0(-)	0.4(+)	2×10^6(+)	1.00
5	3.0(+)	0.6(+)	0.0(-)	0.3(+)	0.1(+)	0.0(-)	1.0(+)	0.0(-)	0.0(-)	0.0(-)	2×10^6(+)	2.12
6	3.0(+)	0.6(+)	0.2(+)	0.0(-)	0.1(+)	0.06(+)	0.0(-)	0.32(+)	0.0(-)	0.0(-)	5×10^5(-)	3.44
7	1.5(-)	0.6(+)	0.2(+)	0.3(+)	0.0(-)	0.06(+)	1.0(+)	0.0(-)	0.3(+)	0.0(-)	5×10^5(-)	1.66
8	1.5(-)	0.0(-)	0.2(+)	0.3(+)	0.1(+)	0.0(-)	1.0(+)	0.32(+)	0.0(-)	0.4(+)	5×10^5(-)	1.30
9	1.5(-)	0.0(-)	0.0(-)	0.3(+)	0.1(+)	0.06(+)	0.0(-)	0.32(+)	0.3(+)	0.0(-)	2×10^6(+)	1.13
10	3.0(+)	0.0(-)	0.0(-)	0.0(-)	0.1(+)	0.06(+)	1.0(+)	0.0(-)	0.3(+)	0.4(+)	5×10^5(-)	1.34
11	1.5(-)	0.6(+)	0.0(-)	0.0(-)	0.0(-)	0.06(+)	1.0(+)	0.32(+)	0.0(-)	0.4(+)	2×10^6(+)	5.79
12	1.5(-)	0.0(-)	0.0(-)	0.0(-)	0.0(-)	0.0(-)	0.0(-)	0.0(-)	0.0(-)	0.0(-)	5×10^5(-)	1.07
13(basal)	2.0(0)	0.3(0)	0.1(0)	0.14(0)	0.03(0)	0.03(0)	0.5(0)	0.16(0)	0.14(0)	0.2(0)	1×10^6(0)	4.46
14	3.0(+)	0.6(+)	0.2(+)	0.3(+)	0.1(+)	0.06(+)	1.0(+)	0.32(+)	0.3(+)	0.4(+)	2×10^6(+)	5.58

SH, shrimp shellfish; GL, glucose; PE, peptone; NH, $(NH_4)_2SO_4$; UR, urea; MG, $MgSO_4.7H_2O$; FE, $FeSO_4.7H_2O$; MN, $MnSO_4.2H_2O$; ZN, $ZnSO_4.7H_2O$; CO, $CoCl_2$; SP, spore number. The (-) indicates the low level, (+) indicates the high level and (0) indicates the basal level.

active chitinase was fortified by shrimp shellfish waste (2%) of different sizes of 0.15 (basal), 0.30, 12.5, 25 and 50 (almost original size) mm^2. Incubation period was 7 days (168 h).

Time course study of chitinase production

A. alternata was allowed to grow in 100 ml aliquots of the best medium (4) for 8 days under stagnant culture conditions at 30±2°C. Thereafter, the necessary analyses were carried out after 48 h of growth every 24 h.

Influence of aeration in production of highly active chitinase

The test fungus was allowed to grow under the best gained cultural conditions of cultivation medium (4), incubation period (96 h) and size of shrimp waste (0.15 mm^2). A group of the inoculated flasks were incubated for (96 h) stagnantly (lower aeration) in an incubator at 30±2°C. The other was incubated under shaking (higher aeration) at 150 rpm for (96 h) at 30±2°C.

Statistical optimization

Plackett-Burman design

As a preliminary optimization experiment, various medium components and environmental factors, eleven independent variables were screened in 14 combinations (Table 1), have been evaluated, based on the Plackett-Burman factorial design (Plackett and Burman, 1946). All trials were performed in triplicate and the average of production of highly active chitinase observations were treated as responses. The main effect of each variable was simply calculated as the difference between the average of measurements made at the high setting (+) and the average of measurements observed at the low setting (-) of the factor. Plackett-Burman experimental design is based on the first order model:

$$Z = b_0 + \sum b_i X_i$$

Where Z is the response (chitinase activity), b_0 is the model intercepts, b_i is the linear coefficient and X_i is the level of the independent variable.

This model does not describe interaction among factors and it is used to screen and evaluate the important factors that influence the response.

Box-Behnken design

In order to describe the nature of the response surface in the experimental region and elucidate the optimal concentrations of the most significant independent variables, a Box-Behnken design (Box and Behnken, 1960) was applied which is a response surface methodology (RSM).

Table 2. Box-Behnken factorial design for three independent variables and at 84, 90 and 96 h of incubation.

Trial	Variables (g/l)			Chitinase activity (U/min) at different incubation periods (h)		
	Glucose(X1)	$MnSO_4.2H_2O$(X2)	$CoCl_2$(X3)	84	90	96
1	0.3 (-)	0.16 (-)	0.2 (-)	2.04	2.28	1.97
2	0.3 (-)	0.32 (0)	0.6 (+)	1.04	1.28	1.47
3	0.3 (-)	0.48 (+)	0.4 (0)	2.08	2.22	2.39
4	0.6 (0)	0.16 (-)	0.6 (+)	5.12	5.39	5.20
5	0.6 (0)	0.32 (0)	0.4 (0)	5.70	5.76	5.80
6	0.6 (0)	0.48 (+)	0.2 (-)	6.16	6.40	6.03
7	0.9 (+)	0.16 (-)	0.4 (0)	5.77	7.32	7.00
8	0.9 (+)	0.32 (0)	0.2 (-)	7.44	7.63	7.34
9	0.9 (+)	0.48 (+)	0.6 (+)	7.32	7.32	7.16

As presented in Table 2, factors of highest confidence levels namely; glucose (X1), $MnSO_4.2H_2O$ (X2) and $CoCl_2$ (X3) were divided into three levels (low, basal and high) coded (-, 0, +). According to the applied design nine combinations were executed and their observations were fitted to the following polynomial model:

$$Z = b_0 + b_1X1 + b_2X2 + b_3X3 + b_{11}X1^2 + b_{22}X2^2 + b_{33}X3^2$$

Where Z is the dependent variable (chitinase activity), X1, X2, X3 are the independent variables as mentioned earlier; b_0 is the regression coefficient at the center point; b_1, b_2 and b_3 are linear coefficients and b_{11}, b_{22}, and b_{33} are quadratic coefficients.

The values of the coefficients as well as the optimum concentrations were calculated using statistical software. The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2.

Characterization of crude chitinase

The effect of some factors that influence chitinase activity in the reaction mixture as substrate level, enzyme concentration, temperature and pH was studied. The thermal stability of the crude chitinase at temperatures 40 to 80°C at different time intervals of 15, 30 and 60 min, was also elucidated. The response of chitinase activity to some metals as K, Na, Mg, Mn, Ca, Cu, Zn, Cd, Hg, Pb was studied at 1 mM concentration of each metal salt. Each experiment was carried out in triplicate and the obtained results were the arithmetic mean.

RESULTS AND DISCUSSION

Effect of fermentation medium

The formulation of medium (4) fortified *A. alternata* by nutrients, quantitatively and qualitatively, in favor of highly active chitinase formation (3.86 U/min). The ingredients of media 5, 1, and 2 have suppressive influences in chitinase production (0.49, 0.57, and 0.73, respectively). Chitinase production, under the experimental conditions of medium (4), showed about 1.6 fold increase of the basal medium (3) (Figure 1). This may due to the richness of medium (4) in Co, Zn, Mn, Fe and urea as well as glucose, as compared to the tested media. It was reported that medium composition is one of the main factors that enhance chitinase production by micro-organisms (Al-Ahmadi et al., 2008; Akhir et al., 2009; Faramarzi et al., 2009). In accordance with these findings, it was indicated that medium (4) was the most favorable for chitinase production by *Aspergillus* spp. from fish scales waste (Rattanakit et al., 2002; Ghanem et al., 2010).

Effect of shrimp shellfish waste size

To study the effect of grinding of shrimp waste, as a physical treatment, on the availability of chitin in shellfish to the attack of chitinolytic enzymes and hence production of chitinases, different shrimp shellfish sizes (0.15, 0.30, 12.5, 25 and 50 mm^2) were prepared and added at 20 g/l of medium (4). The results (Figure 2) indicate that the finest shellfish sizes comparable to the highest chitinase production and vice versa. Under these conditions the enzyme showed about 2.6 fold increase as the shellfish size reduced by grinding from 50 to 0.15 mm^2. This may be attributed to increase of surface/ volume ratio, as a result to grinding, and hence increased surfaces for enzyme catalysis and other metabolic activities. Grinding also may attain chitin particles in shellfish more exposed and accessible for the enzymes than that in larger sizes. It was indicated by Rattanakit et al. (2003) and Ghanem et al. (2010) that larger sizes of fish scales were more convenient for higher chitinase production by *Aspergillus* spp. than finest scales. This due to the differences between constituents and architecture of fish scales and shrimp shellfish wastes and also test organisms.

Time course study of chitinase production

The results (Figure 3) indicated that the highest chitinase activity (4.47 U/min) and extracellular protein (1 g/100 ml medium) was recorded after 96 h (4 days) of growth.

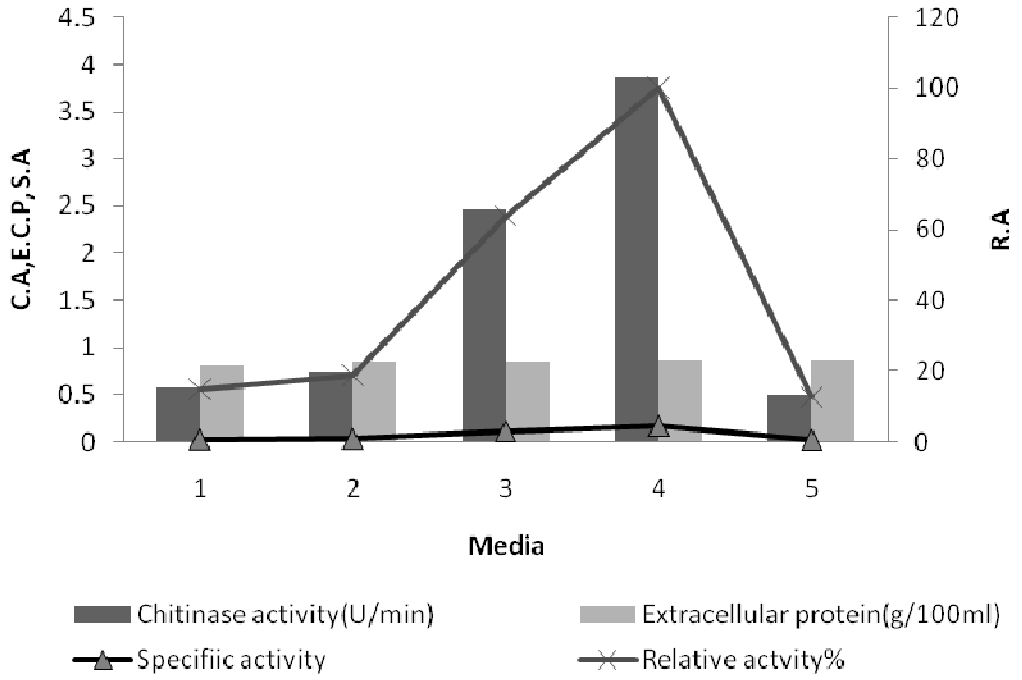

Figure 1. Effect of different cultivation media on extracellular protein and chitinase activity of *Alternaria alternate*.

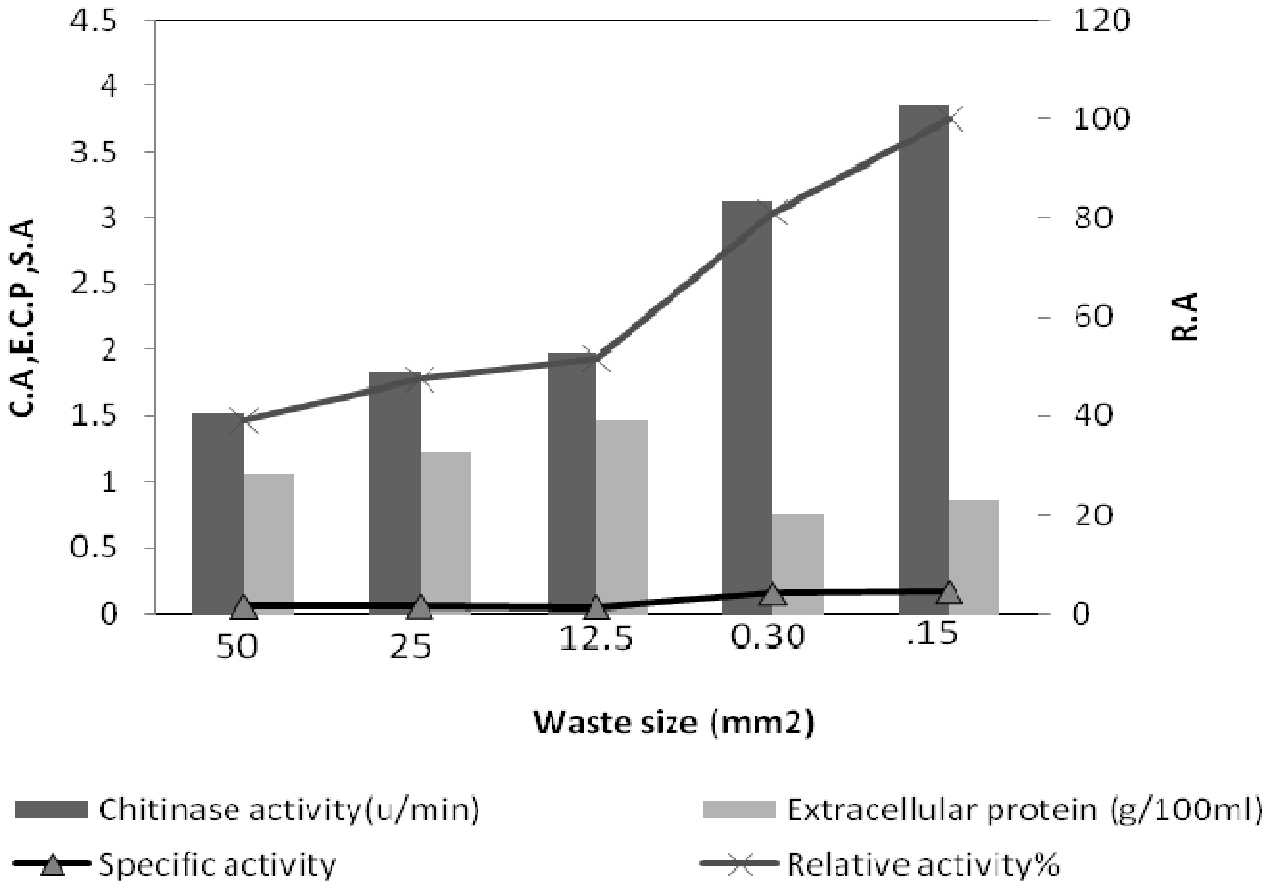

Figure 2. Effect of shrimp shellfish size (mm^2) on extracellular protein and chitinase activity of *A. alternata*.

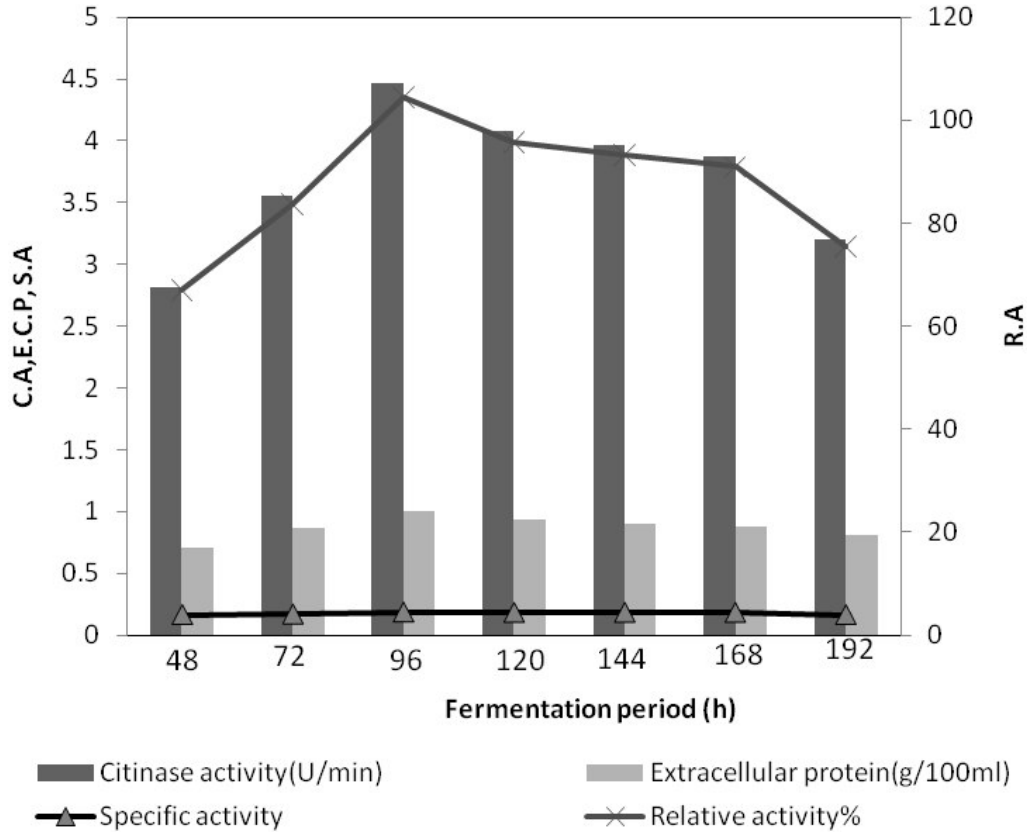

Figure 3. Effect of fermentation period on extracellular protein and chitinase activity of *A. alternata*.

While, lower or higher growth periods of *A. alternata* were concomitant with lower enzyme and extracellular protein production. It was reported by Sandhya et al. (2004) and Ghanem et al. (2010) that 4 days of growth of *Tichoderma harziaum* on shrimp shellfish and *A. terreus* on fish-scales was optimal for chitinase production. While, Patidar et al. (2005) and Sharaf (2005) indicated that from 6 to 7 days of growth were accompanied by the highest chitinase production by *Penicillium chrysogenum* and *A. alternata*, respectively. All these findings indicated that highest chitinase production takes place at the logarithmic growth phases of different fungi. It is well known that the highest production of enzymes including hydrolytic enzymes (chitinases) takes place at the accelerated growth phase of microorganisms.

Influence of aeration

The biodegradation processes are mostly more efficient under aerobic conditions. So, to monitor how much bio-degradation of shrimp shellfish waste in favor of chitinase formation by *A. alternata*, stagnant and shake cultures were compared. The results (Figure 4) indicated that stagnant culture conditions fortified the fungus with optimal aeration responsible for chitinase production by about 2.06% than shake one. In accordance with these findings, Rattanakit et al. (2007) and Patidar et al. (2005) found that chitinase production by *Aspergillus* spp. and *P. chrysogenum*, respectively, using shrimp shellfish waste was more efficient under stagnant cultures. Also, Ghanem et al. (2010) supported the same finding using fish scales waste. However, Rattanakit et al. (2003) indicated that 200 rpm was more convenient for chitinase production than stagnant one. This discrepancy is related to differences in physiology of producing organism, medium components, chitinaceous waste, and volume of medium/flask…..etc.

Evaluation of the most significant medium constituents affecting chitinase production using Plackett-Burman design

The best tested medium (4) of Rattanakit et al. (2002), which stimulate chitinase production, of pH 5, inoculated by 1 ml (10^6 spores), dispensed in 250 ml Erlenmeyer flasks, and incubated stagnantly at 30±2°C for 96 h (4 days) was used as the basal conditions of cultivation. The design was applied with 14 different fermentation con-ditions (trials) as shown in Table 1. The results indicated that levels of factors at trial 11 were the best. The main

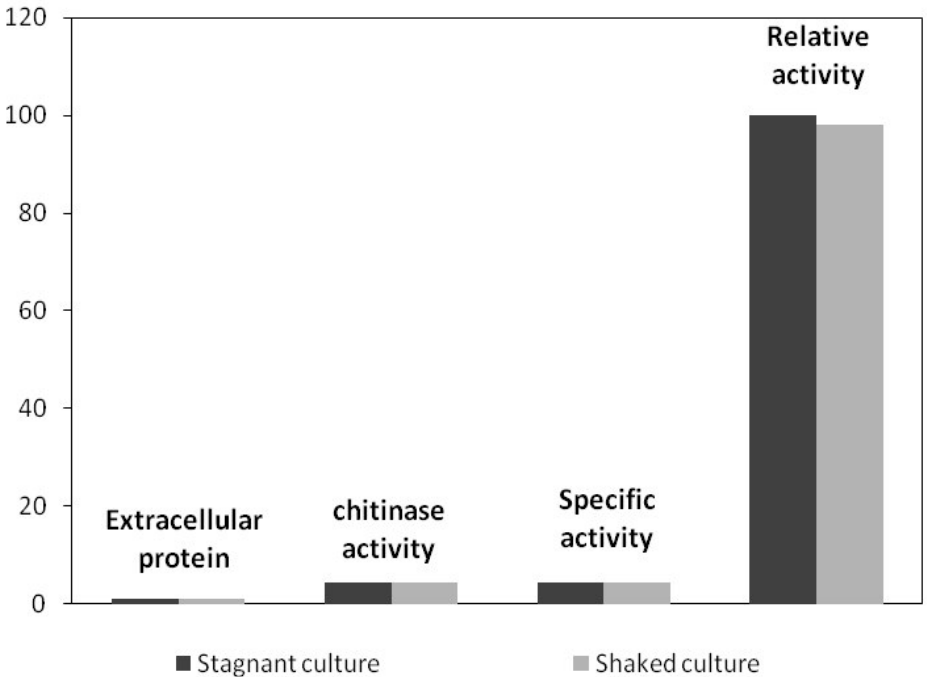

Figure 4. Effect of aeration on extracellular protein and chitinase activity of *A. alternata*.

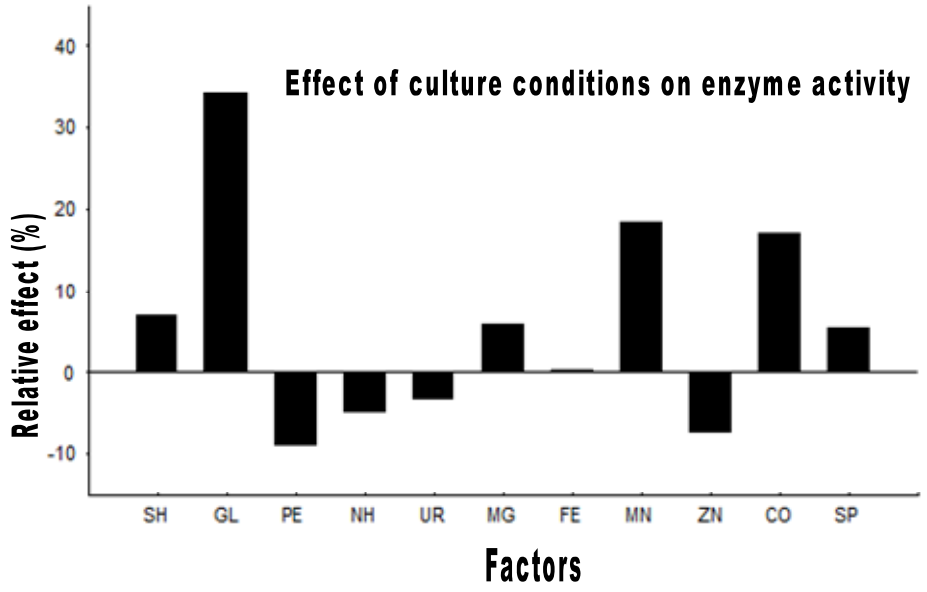

Figure 5. Proportional effect of applied factors based on results of Plackett-Burman design.

effect for each variable was estimated and the results (Figure 5) revealed that the most significant three factors which were more effective in chitinase production were glucose, $MnSO_4.2H_2O$ and $CoCl_2$. While, shrimp shellfish waste, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$ and spores number (inoculum size) showed positive non-significant effect on chitinase production. However, the main effect of peptone, $(NH_4)_2SO_4$, urea and $ZnSO_4.7H_2O$ was negative. It is well known that Mn and Co ions act as activators and cofactors in many enzyme systems responsible for hydrolysis of carbohydrates, fats and proteins (Bowman and Russell, 2001), while glucose is the most assessable simple carbon source to micro-organisms. So, *A. alternata* is in needing it to initiate

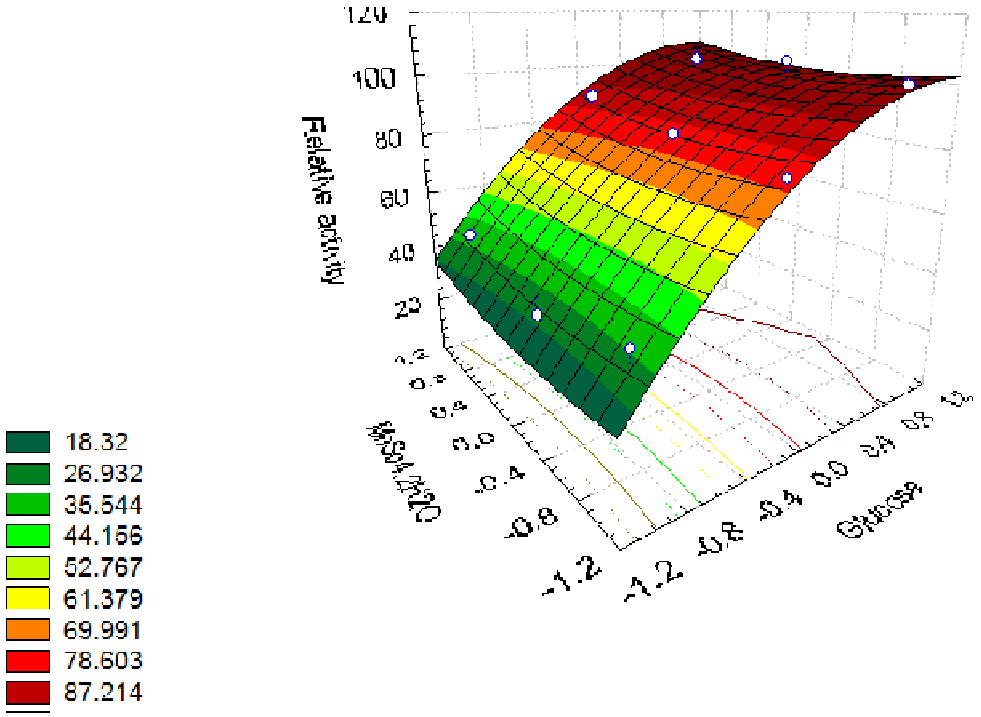

Figure 6. Effect of glucose and $MnSO_4.2H_2O$ and their interactive effect of chitinase production.

growth that enables it to hydrolyze the complex chitin polymer. According to recorded results, medium formula which was predicted to be near optimum was (g/l): Shrimp shellfish waste,15; glucose, 6; $MgSO_4.7H_2O$, 0.6; $FeSO_4.7H_2O$, 10; $MnSO_4.2H_2O$, 3.2; $CoCl_2$, 4 and 2×10^6 spores/100ml (inoculum).

A verification experiment demonstrated that the enzyme activity expressed in this medium was increased by 29.5% (5.79 U/min) when compared to the basal conditions (4.46 U/min).

Optimization of the factors affecting chitinase production using Box-Behnken design

In order to approach the optimum response region of chitinase production, significant independent variables (glucose, $MnSO_4.2H_2O$ and $CoCl_2$) were further explored by applying response surface methodology, each at three levels according to Box and Behnken (1960). Table 2 represents the design matrix of the coded variables together with the experimental results of chitinase activity. All cultures were performed in 100 ml aliquots in triplicates and the average of observations was used. The optimal levels of the three examined independent variables as predicted from the model (trial 8) are (g/l): glucose, 9; $MnSO_4.2H_2O$, 3.2 and $CoCl_2$, 2. It was reported by Ghanem et al. (2010) that 0.65 and 0.47% of glucose and $MnSO_4.2H_2O$, respectively, highly induced chitinase production by *A. terreus* from fish scales waste. In order to verify the optimization results, an experiment was carried out under the predicted optimal conditions, where the extracellular protein and chitinase activity were monitored at different time intervals of 84, 90 and 96 h. The basal culture medium of Plackett-Burman design was used as control. As shown in Table 2, the optimized medium formula recorded an actual maximum chitinase activity of 8.15 U/min, which is of more than 40% as the pre-optimized control medium and at 90 h of fermentation instead of 96 h. Figures 6, 7 and 8 show graphically, the relationship and interaction between the independent variables (glucose, $MnSO_4.2H_2O$ and $CoCl_2$) and response (chitinase activity). A verification experiment was accomplished and revealed approximately 99.68% MODEL validity. The great similarity between predicted and observed results in this application confirm the accuracy and applicability of Box-Behnken model in optimization process (Al-Ahmadi et al., 2008; Lopes et al., 2008; Akhir et al., 2009; Ghanem et al., 2009, 2010).

It is also likely that the optimized culture conditions accelerated the rate of chitinase expression in *A. alternata*, as it showed maximum enzyme activity within

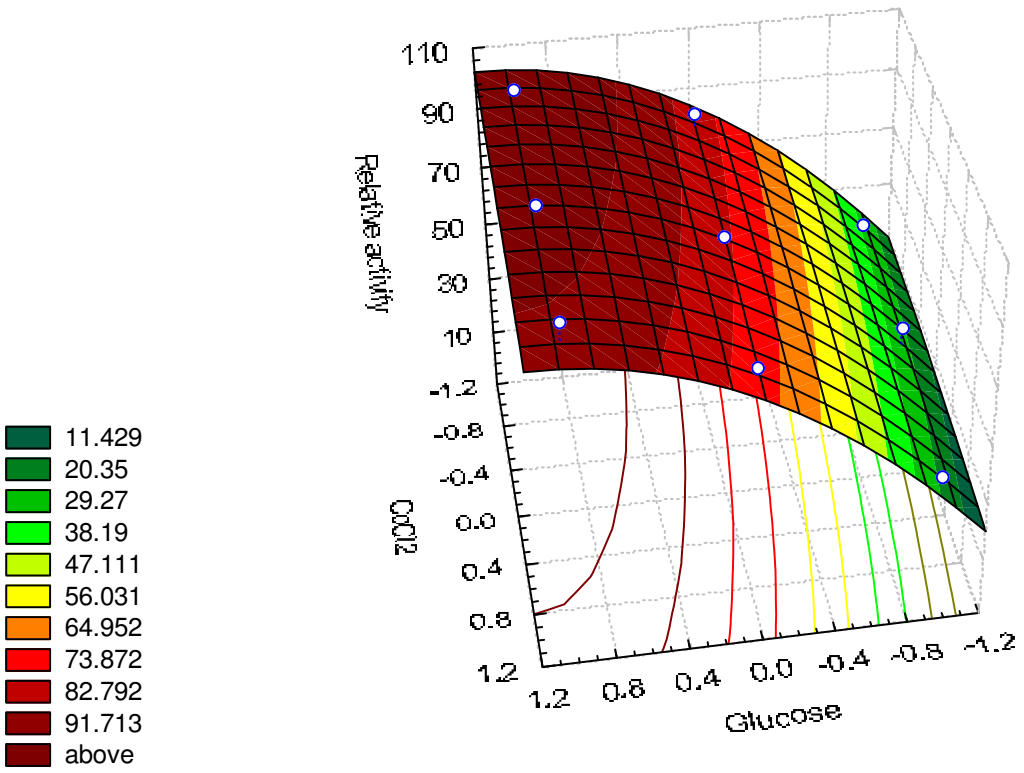

Figure 7. Effect of glucose and $CoCl_2$ and their interactive effect of chitinase production.

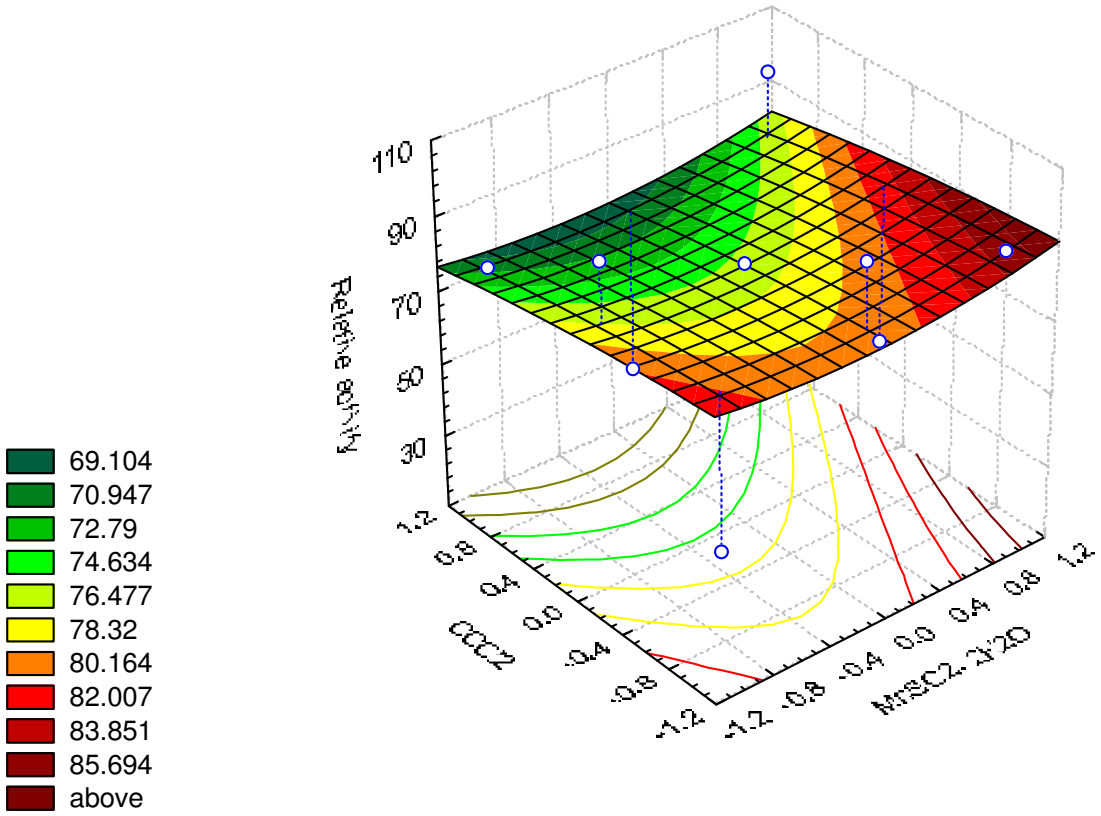

Figure 8. Effect of $MnSO_4.2H_2O$ and $CoCl_2$ and their interactive effect of chitinase production.

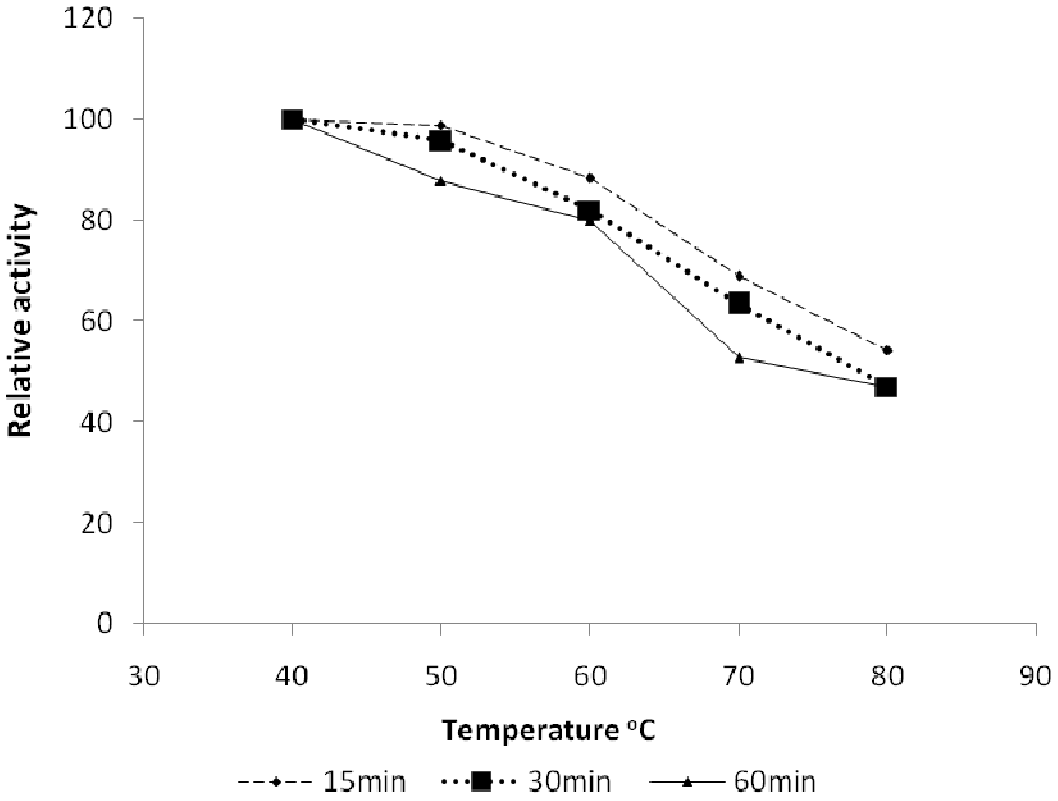

Figure 9. Thermal stability of crude chitinase produced by *A. alternata* from shrimp shellfish waste.

90 h instead of 96 h in the case of control culture.

Characterization of crude enzyme

The crude chitinase produced by *A. alternata* cultivated for 90 h under the optimized factors for maximum chitinase production using Box-Behnken design, was obtained by centrifugation at 7000x g for 20 min in cooling centrifuge. The supernatant culture filtrate is used as a crude chitinase. The effect of temperature of the reaction mixture, crude enzyme concentration, substrate level of colloidal chitin, pH of the reaction mixture using phosphate buffer and the reaction time, revealed that the optimal factors for maximum crude chitinase activity were incubation temperature of 50°C, 1.5 ml of crude enzyme, 0.5 ml of 1% colloidal chitin, and pH 5 for 10 min of reaction time. Under these conditions the enzyme activity (15.61 U/min) showed more than 92% as that of basal medium (8.12 U/min). These results are comparable to those obtained with *A. terreus*, *A. alternata*, *P. aculeatum*, *P. janthinellum* and *T. harzianum* (Fenice et al., 1998; Saad and Nawar, 2001; Binod et al., 2005; Sharaf, 2005; Lee et al., 2009; Alaa-El-Dein et al., 2010; Garg and Gupta, 2010; Ghanem et al., 2010) for the tested characteristics. Crude chitinase is thermostable (Figure 9) and lost only 20% of its activity when heated in the absence of its substrate at 60°C for 60 min. This finding is comparable to that of *A. terreus* (Ghanem et al., 2010) and is superior to that of *A. alternata* (Sharaf, 2005), *A. terreus* (Al-Nusaire, 2007) and *Bacillus* sp. S22 (Al-Mahboob, 2010). The effect of $CuSO_4.5H_2O$, NaCl, KCl, $MgSO_4.7H_2O$, $CaCl_2$, $ZnSO_4.7H_2O$, $CdCO_3$, $HgCl_2$ and $PbCl_2$ at 1mM on chitinase activity revealed that *A. alternata* crude chitinase appears to have specific requirement of Ca and K ions for higher enzyme activity (19.53 and 17.041 U/min, respectively). While, Zn, Cu, Na and Mn were of inhibitory action not more than 10% and to a noticeable extent Mg ions resulted in 30% inhibition, as compared to their absence. However, addition of Pb, Hg and Cd ions resulted in drastic enzyme inhibition, ranged between 95 to 81%.

In harmony with these findings it was reported that chitinase of *T. harzianum* and *A. alternata* was activated by Ca ions. Meanwhile Mn and Zn ions gave little enzyme inhibition. The addition of Pb and Hg ions resulted in higher enzyme inhibition (Saad and Nawar, 2001; Sharaf, 2005).

REFERENCES

Abdel-Fattah YR, El-Helow ER, Ghanem KM, Lotfy WA (2007). Application of factorial designs for optimization of avicelase production by a thermophilic *Geobacillus* Isolate. J. Microbiol., 2: 13-23.

Akhir SM, Abd-Aziz S, Salleh MM, Raman Ra, Lllia RM, Hassan MA (2009). Medium optimization of chitinase enzyme production

from shrimp waste using *Bacillus licheniformis* TH-1 by response surface methods. Biotechnology, 8(1): 120-125.
Alaa-EL-Dein MS, Hosny NA, El-Shayeb A, Abood AM, Abdel-Fattah (2010). A potent chitinolytic activity of marine actinomycete sp. and enzymatic production of chitooligosaccharides, Aust. J. Basic Appl. Sci., 4: 615-623.
Al-Ahmadi KJ, Yazdi MT, Najafi MF, Shahverdi AR (2008). Optimization of medium and cultivation conditions for chitinase production by newly isolated *Aeromonas* sp. Biotechnology, 7(2): 266-272.
Al-Mahboob SA (2010). Isolation, purification and charechterization of chitinase from a chitinolytic bacterium isolated from Jeddah Region and its potential for the biocontrol of some pathogenic fungi . M.Sc. Thesis, Faculty of Science, King Abul-Aziz University, Jeddah, Saudia Arabia.
Al-Nusaire TS (2007). Degradation of some marine polymer by microorganism isolated from Al-Jouf Region. M.Sc. Thesis, Faculty of Education for Girls, King Abul-Aziz University, Jeddah, Saudia Arabia.
Al-Sarrani AQ, El-Naggar MY (2006). Application of Plackett-Burman factorial design to improve citrinin production in *Monascus ruber* batch cultures. Bot. Sci., 47 (2):167-174.
Bansode VB, Bajekal SS (2006). Characterization of chitinases from microorganisms isolated from Lonar Lake. Indian J. Biotechnol., 5: 364-367.
Binod P, Pusztahelyi T, Nagy V, Sandhya C, Szakács G, Pócsi I, Pandey A (2005). Production and purification of extracellular chitinases from *Penicillium aculeatum* NRRL 2129 under solid-state fermentation. Enzy. Microbial Technol., 36: 880-887.
Bowman BA, Russell RM (2001). Present knowledge in nutrition. 8th ed. Washington, Dc. ILSI Press, pp. 426-438.
Box G, Behnken D (1960). Some new three level designs for the study of quantitative variables, Technometrics, 2: 455–475.
Chae D, De Jin R, Hwangbo H, Kim Y, Park R, Krishnan H, Kim K (2006). Control of late blight (*Phytophthora capsici*) in pepper plant with a compost containing multitude of chitinase-producing bacteria, Biocontrol, 51: 339-351.
Dahiya N, Tewari R, Hoondal G (2006). Biotechnological aspects of chitinolytic enzymes: A review. Appl. Microbiol. Biotechnol., 71: 773-782.
Faramarzi MA, Fazeli M, Yazdi MT, Adrangi S, Al-Ahmadi KJ, Tasharrofi N, Mohseni FA (2009). Optimization of cultural conditions for production of chitinase by a soil isolate *Massilia timonae*. Biotechnol Bioseparation, 8(1): 93-99.
Fenice M, Leuba JL, Federici F (1998). Chitinolytic enzyme activity of *Penicillium janthinellum* P9 in bench-top bioreactor. J. Fermentation Bioeng., 86: 620-623.
Frey D, Oldfield RJ, Bridger RC (1979). Color atlas of pathogenic fungi, printed by Smeets, Holland, pp. 92-93.
Garg N, Gupta H (2010). Isolation and purification of fungal pathogen (*Macrophomina phaseolina*) induced chitinase from moth beans (*Phaseolus aconitifolius*). J. Pharm. Bioallied Sci., 2: 38-43.
Ghanem KM, Al-Garni SM, Al-Makishah NH (2010). Statistical optimization of cultural conditions for chitinase production from fish scales waste by *Aspergillus terreus*. Afr. J. Biotechnol., 9(32): 5135-5146.
Ghanem KM, Al-Garni SM, Al-Shehri AN (2009). Statistical optimization of cultural conditions by response surface methodology for phenol degradation by a novel *Aspergillus flavus* isolate. Afr. J. Biotechnol., 8: 3576-3583.
Guevara-Gonzalez RG, Torres-Pacheco I (2006). Advances in Agricultural and Food Biotechnology. Kerala, India, Research Signpost.
Kern M, Maraschin S, Vom Endt D, Schrank A, Vainstein M, Pasquali G (2009). Expression of a chitinase gene from *Metarhizium anisopliae* in tobacco plants confers resistance against *Rhizoctonia solani*. Appl. Biochem. Biotechnol., 160: 1933-1946.
Kim YY, Kim YW, Kim KS (1988). Studies on the enzymatical properties of *Streptomyces* sp. S-45 isolated from soil. Res. Reports Rural Dev. Admin., (Korea R.) 21: 129-134.
Kuk JH, Jung WJ, Jo GH, Kim YC, Park RD (2005). Production of N-acetyl-β-D-glucoseamine from chitin by *Aeromonas* sp. GJ-18 crude enzyme. Appl. Microb. Biotechnol., 68: 384-389.
Lee YG, Chung KC, Wi SG, Lee JC, Bae HJ (2009). Purification and properties of a chitinase from *Penicillium* sp. LYG 0704. Protein Expression Purif., 65: 244-250.
Lopes MA, Gomes DS, Bello-Koblitz MG, Pirovani CP, Cascardo J,Goes-Neto A, Michell F (2008). Use of response surface methodology to examine chitinase regulation in the basidiomycete *Moniliophthora perniciosa*. Mycol. Res., 112: 399-406.
Madigan M, Martinko J (2005). Brock Biology of Microorganisms, Pearson Prentice Hall , New Jersey, USA.
Mejia-Saules JM, Waliszewski KN, Garcia MA, Cruz-Camarillo R (2006). The use of crude shrimp shell powder for chitinase production by *Serratia marcescens* WF. Food Technol. Biotechnol., 44(7): 646-651.
Nawani NN, Kapadnis BP (2005). Optimization of chitinase production using statistics based experimental design. Process Biochem., 40: 651-660.
Patidar P, Agrawal D, Banerjee T, Patil S (2005). Optimisation of process parameters for chitinase production by soil isolates of *Penicillium chrysogenum* under solid substrate fermentation. Process Biochem., 40: 2962-2967.
Pedraza RM, Lopez RE (1991). Chitinase activity in germinating cells of *Mucor rouxii*. Antonie van Leeuwenhoek, 59: 183-189.
Pichyangkura R, Kudan S, Kutiyawong K, Sukwattanasinitt M (2002). Quatitative production of 2-acetamido-2-deoxy-D-glucose from crystalline chitin by bacterial chitinase. Carbohydr. Res., 337(6): 557-559.
Plackett RL, Burman JP (1946). The design of optimum multifactorial experiments. Biometrika 33: 305-325.
Rattanakit N, Plikomol A, Yano S, Wakayama M, Tachiki T (2002). Utilization of shrimp shellfish waste as a substrate for solid-state cultivation of *Aspergillus* sp. S1-13: evaluation of a culture based on chitinase formation which is necessary for chitin-assimilation. J. Biosci. Bioeng., 93: 550-556.
Rattanakit N, Yano S, Plikomol A, Wakayama M, Tachiki T (2007). Purification of *Aspergillus* sp. S1-13 chitinases and their role in saccharification of chitin in mash of solid-state culture with shellfish waste. J. Biosci. Bioeng., 103: 535-541.
Rattanakit N, Yang S, Wakayama M, Plikomol A, Tachiki T (2003). Saccharification of chitin using solid-state culture of *Aspergillus* sp. S1-13 with shellfish waste as a substrate. J. Biosci. Bioeng., 95: 391-396.
Ravi Kumar MNV (2000). A review of chitin and chitosan applications. Reactive and Functional Polymers., 46: 1-27.
Saad MMS, Nawar LS (2001). Chitinase formation by *Trichoderma harzianum* isolates. The J. Agric. Sci., 26: 537-549.
Sandhya C, Adapa LK, Nampoothiri KM, Binod P, Szakacs G, Pandey A (2004). Extracellular chitinase production by *Trichoderma harzianum* in submerged fermentation. J. Basic Microbiol., 44: 49-58.
Sharaf EF (2005). A potent chitinolytic activity of *Alternaria alternata* isolated from Egyptian black sand. Polis J. Microbiol., 54: 145-151.
Slater RJ (ed.) (1986). Experiments in Molecular Biology. Clifton, New Jersey, USA: Humana Press. p. 269.
Sorbotten A, Horn SJ, Eijsink VG, Varum KM (2005). Degradation of chitosans with chitinase B from *Serratia marcescens*. Production of chitooligosaccharides and insight into enzyme processivity. Febs. J., 272(2): 538-549.
Wang SL, Hwang JR (2001). Microbial reclamation of shellfish wastes for the production of chitinases. Enzyme Microbial. Technol., 28: 376-382.
Wang SL, Lin TY, Yen YH, Liao HF, Chen YJ (2006). Bioconversion of shellfish chitin wastes for the production of *Bacillus subtilis* W-118 chitinase. Carbohydr. Res., 341: 2507-2515.
Yamazaki H, Tanaka A, Kaneko J, Ohta A, Horiuchi H (2008). *Aspergillus nidulans* ChiA is a glycosylphosphatidylinositol (GPI)-anchored chitinase specifically localized at polarized growth sites. Fungal Gene. Biol., 45: 963-972.
Youssef AS, El-Naggar MY, El-Aassar SA, Beltagy IA (2006). Optimization of culture conditions for β-Mannanase production by a local *Aspergillus niger* isolate. Int. J. Agric. Biol., 8(4): 539-545.