African Journal of Biotechnology Vol. 9(32), pp. 5135-5146, 9 August, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

*Full Length Research Paper*

# Statistical optimization of cultural conditions for chitinase production from fish scales waste by *Aspergillus terreus*

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Accepted 3 June, 2010

***Aspergillus terreus*, a local isolate from fishery polluted soil, was used successfully for the biodegradation of parrot fish-scales 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 acceleration growth phase (96 h). Pronounced decrease in chitinase production was concomitant with the sizes of fish-scales; larger sizes (normal, non-grinded) were the best for chitinase production than the finest grinded scales. Stagnant culture conditions were more favorite for chitinase production than shaked culture. Statistically based experimental designs were applied to optimize the production of chitinase by *A. terreus*. Eleven culture parameters were examined for their significance as effectors of chitinase expression using the Plackett-Burman factorial design. Concentrations of $FeSO_4.7H_2O$, glucose and $MnSO_4.2H_2O$ were the most significant factors affecting the process of enzyme production. The second optimization step was to figure out the levels of these three independent variables that generate maximum chitinase activity, using the Box-Behnken design. Maximum enzyme activity (4.309 u/min), which is approximately 1.81 folds the activity expressed in the basal medium, has been assayed at concentrations (g/l): $FeSO_4.7H_2O$ (9.5), glucose (6.5) and $MnSO_4.2H_2O$ (4.7), after 90 h of fermentation. A verification experiment was accomplished and revealed approximately 99% model validity. The crude chitinase was characterized and maximum activity was obtained in reaction mixture of 50°C incubation temperature, 2 ml crude enzyme, 0.5 ml of 10% colloidal chitin, pH 6 and reaction time of 10 min. The enzyme is thermostable and lost only less than 10% 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 Cu, Ca, Zn and Mn ions for its activity.**

**Key words:** Chitinases, *Aspergillus terreus*, fish-scales, statistical optimization.

## INTRODUCTION

Chitinase is the second most abundant polysaccharide in nature after cellulose, and it largely exists in wastes from processing of marine food products (crab, shrimp and krill shells as well as fish scales). About $10^{11}$ ton of chitin is produced annually in the aquatic biosphere alone (Wang and Chio, 1998). The waste generated from the world-wide production and processing of shell-fish and fish scales 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 chitooligo-saccarides 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), preparation of single-cell protein (Vyas and Deshpande, 1991), control of pathogenic fungi (Mathivanan et al., 1998) and treatment of chitinous waste (Wang and Hwang, 2001).

Chitinases are present in a wide rang of organisms including bacteria, fungi, insects, higher plants and animals, and play important physiological and ecological roles

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(Cody et al., 1990; Duo-Chuan, 2006). Chitinases are produced by several bacterial species (Gohel et al., 2004; Wang et al., 2005; 2006; Al-Ahmadi et al., 2008), and fungal species (Humphreys and Goody, 1984; Charnley, 1999; Patidar et al., 2005; Rattanakit et al., 2007). 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 and productivity, but also to minimize the product cost (Abdel-Fattah et al., 2005). Studies on the medium optimization for chitinase production using the statistical approach have been done by Nawani and Kapandis (2005), Al-Ahmadi et al. (2008), Akhir et al. (2009) and Faramarzi et al. (2009).

The objective of the present study was to characterize the medium and growth conditions of *Aspergillus terreus* for maximum biodegradation of fish-scales waste in favor of the production of highly active chitinase, using statistical design of Plackett-Burman and Box-Behnken, and to characterize the produced crude chitinase.

## MATERIALS AND METHODS

### Microorganism

*A. terreus* was isolated from polluted soil at the market of fishery, fish sale and reparation in Jeddah, Saudi Arabia. Identification was done mainly on the basis of cultural and morphological characteristics (Frey et al., 1979; Watanabe, 2002; CBS, 2006).

### Fish-scales waste

The waste was kindly provided by Saudi Arabian Company of Fishery in Jeddah, Saudi Arabia. Fish scales of parrot fish were especially collected for their large size (average 20 $mm^2$) and more availability than the other fish scales. The scales waste was washed with tap water then distilled water. Thereafter, exposed to water vapor and air dried at room temperature (Wang et al., 2005, 2006).

### Inoculum and cultivation

*A. terreus* was maintained on Zcapek's Dox agar slants, where the fungus was grown for 5 days at 30 ± 2°C. The stocks were kept in the refrigerator and subcultured at monthly intervals. Spores suspension of *A. terreus* 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 14.6 x $10^6$spores/ml. The organism was allowed to grow in 100 ml aliquots of mineral salt medium of the following composition(g/l): fish scales 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 pH 6 (Al-Nusaire, 2007) and dispensed in 250 ml Erlenmyer flasks. Standard inocula (14.6 x $10^6$spores/ml) were used to inoculate the flasks which were then incubated at 30 ± 2°C under shaking conditions (otherwise stated) at 150 rpm for 5 days. Thereafter, biomass was picked up, washed thoroughly to determine the dry weight at 80°C for constant weight. The residual fermentation products were centrifuged at 5000 rpm for 15 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 was determined calorimetrically using Biuret methods.

### Chitinase activity assay

Chitinase activity 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 10% 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-dinitrosalicylic acid reagent was added. The mixture was 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 (u/min) = the amount of enzyme releasing 1 µmol NAGA per min from colloidal chitin, under the assay conditions (Bindo et al., 2005).

$$\text{Specific activity} = \frac{\text{Chitinase activity (u/min)}}{\text{Extracellular protein}}$$

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

Then the value is subtracted from 100.

### Effect of cultivation medium

To test the quantity and quality of ingredients of the cultivation medium on fish scales biodegradation in favor of production of active chitinase, different six 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., 2005); 2) Peptone, 0.03; yeast extract, 0.03; $K_2HPO_4$, 0.07; $KH_2PO_4$, 0.03; $MgSO_4.7H_2O$, 0.05; Rose Bengal, 0.05 (Rattanakit et al., 2003); 3) $(NH_4)_2SO_4$, 0.2; $K_2HPO_4$, 0.1; $MgSO_4.7H_2O$, 0.05; KCl, 0.05; NaCl, 0.05; traces of $CaCl_2$ and $FeSO_4.7H_2O$ (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) Pancreatic digest of casein, 0.5; peptic digest of fresh meat, 0.5; glucose, 2.0 (Coutino et al., 2006); 6) $KH_2PO_4$, 0.3; $K_2HPO_4$, 0.1; $MgSO_4.7H_2O$, 0.07; $(NH_4)_2SO_4$, 0.14; NaCl, 0.05; $CaCl_2$, 0.05; yeast extract, 0.05; peptone, 0.05 (Kim et al., 2005). Each medium contain 2 g of fish scales waste. Initial pH was adjusted at 6. Standard inocula (14.6 x 106spores/ml) of *A. terreus* were used and the flasks were incubated under shacked conditions for five days at 30 ± 2°C. Thereafter, the necessary analyses were carried out.

**Table 1.** Plackett-Burman experimental design for 11 variables and 13 trails.

| Trail | Independent variables(g/100ml) | | | | | | | | | | | Response chitinase activity (µ/min) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SH | GL | PE | NH | UR | MG | FE | MN | ZN | CO | SP | |
| 1 | 4.0(+) | 0.1(-) | 0.2(+) | 0.0(-) | 0.0(-) | 0.01(-) | 0.8(+) | 0.32(+) | 0.30(+) | 0.05(-) | $79x10^7$(+) | 1.650 |
| 2 | 4.0(+) | 0.5(+) | 0.0(-) | 0.3(+) | 0.0(-) | 0.01(-) | 0.2(-) | 0.32(+) | 0.30(+) | 0.40(+) | $9.8x10^6$(-) | 2.664 |
| 3 | 1.5(-) | 0.5(+) | 0.2(=) | 0.0(-) | 0.1(+) | 0.01(-) | 0.2(-) | 0.08(-) | 0.30(+) | 0.40(+) | $79x10^7$(+) | 0.697 |
| 4 | 4.0(+) | 0.1(-) | 0.2(+) | 0.3(+) | 0.0(-) | 0.05(+) | 0.2(-) | 0.08(-) | 0.07(-) | 0.40(+) | $79x10^7$(+) | 0.730 |
| 5 | 4.0(+) | 0.5(+) | 0.0(-) | 0.3(+) | 0.1(+) | 0.01(-) | 0.8(+) | 0.08(-) | 0.07(-) | 0.05(-) | $79x10^7$(+) | 1.829 |
| 6 | 4.0(+) | 0.5(+) | 0.2(+) | 0.0(-) | 0.1(+) | 0.05(+) | 0.2(-) | 0.32(+) | 0.07(-) | 0.05(-) | $9.8x10^6$(-) | 1.137 |
| 7 | 1.5(-) | 0.5(+) | 0.2(+) | 0.3(+) | 0.0(-) | 0.05(+) | 0.8(+) | 0.08(-) | 0.30(+) | 0.05(-) | $9.8x10^6$(-) | 2.363 |
| 8 | 1.5(-) | 0.1(-) | 0.2(+) | 0.3(+) | 0.1(+) | 0.01(-) | 0.8(+) | 0.32(+) | 0.07(-) | 0.40(+) | $9.8x10^6$(-) | 2.029 |
| 9 | 1.5(-) | 0.1(-) | 0.0(-) | 0.3(+) | 0.1(+) | 0.05(+) | 0.2(-) | 0.32(+) | 0.30(+) | 0.05(-) | $79x10^7$(+) | 0.984 |
| 10 | 4.0(+) | 0.1(-) | 0.0(-) | 0.0(-) | 0.1(+) | 0.05(+) | 0.8(+) | 0.08(-) | 0.30(+) | 0.40(+) | $9.8x10^6$(-) | 0.889 |
| 11 | 1.5(-) | 0.5(+) | 0.0(-) | 0.0(-) | 0.0(-) | 0.05(+) | 0.8(+) | 0.32(+) | 0.07(-) | 0.40(+) | $79x10^7$(+) | 2.736 |
| 12 | 1.5(-) | 0.1(-) | 0.0(-) | 0.0(-) | 0.0(-) | 0.01(-) | 0.2(-) | 0.08(-) | 0.07(-) | 0.05(-) | $9.8x10^6$(-) | 0.634 |
| 13 (basal) | 2.0(0) | 0.3(0) | 0.1(0) | 0.14(0) | 0.3(0) | 0.03(0) | 0.5(0) | 0.16(0) | 0.14(0) | 0.2(0) | $14.6x10^6$(0) | 1.392 |

SH, fish scales waste; 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.

### Time course study of chitinase production

*A. terreus* was allowed to grow in 100 ml aliquots of the best previous medium (4) and favored production of highly active chitinase for 8 days under shacked conditions at 30 ± 2°C. Thereafter, the necessary analyses were carried out.

### Effect of different sizes of fish scales waste

The best formulated medium (4) that favored production of highly active chitinase was fortified by fish scales (2%) of different sizes of 20 (basal- original size), 10, 8, 4, 0.3 and 1.5 $mm^2$. Incubation period was 4 days (96 h).

### Influence of aeration in production of highly active chitinase

*A. terreus* was allowed to grow under the best gained cultural conditions of cultivation medium (4), incubation period (4 days) and size of fish scales waste (20 $mm^2$). A group of the inoculated flasks were incubated for 4 days stagnantly (lower aeration) in an incubator at 30 ± 2°C, while the other was incubated under shaking (higher aeration) at 150 rpm for 4 days 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 13 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 that factor. Plackett-Burman experimental design is based on the first order model:

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

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 (He et al., 2004; Box and Behnken, 1960) was applied which is a response surface methodology (RSM). As presented in Table 2, factors of highest confidence levels namely; $FeSO_4.7H_2O$ (X1), glucose (X2) and $MnSO_4.2H_2O$ (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 second order

**Table 2.** Box-Behnken factorial experimental design for three independent variables.

| Trial | Variables ($gl^{-1}$) | | | Chitinase activity (µ/min) | | |
|---|---|---|---|---|---|---|
| | $FeSO_4.7H_2O$ ($x_1$) | Glucose ($x_2$) | $MnSO_4.2H_2O$ ($x_3$) | 1st experiment and 5 h incubation | 2nd experiment and 90 h incubation | 3rd experiment and 95 h incubation |
| 1 | 6.5(-1) | 3.5(-1) | 1.7(-1) | 0.486 | 0.559 | 0.753 |
| 2 | 6.5(-1) | 5.0(0) | 4.7(+1) | 1.297 | 1.514 | 1.448 |
| 3 | 6.5(-1) | 6.5(+1) | 3.2(0) | 1.524 | 1.931 | 1.960 |
| 4 | 8.0(0) | 3.5(-1) | 4.7(+1) | 1.567 | 2.127 | 2.025 |
| 5 | 8.0(0) | 5.0(0) | 3.2(0) | 2.191 | 2.730 | 2.575 |
| 6 | 8.0(0) | 6.5(+1) | 1.7(-1) | 3.204 | 3.819 | 3.655 |
| 7 | 9.5(+1) | 3.5(-1) | 3.2(0) | 1.897 | 2.482 | 2.381 |
| 8 | 9.5(+1) | 5.0(0) | 1.7(-1) | 2.486 | 3.348 | 3.243 |
| 9 | 9.5(+1) | 6.5(+1) | 4.7(+1) | 3.501 | 4.309 | 4.230 |

polynomial model:

$$Z = b_0 + b_1 X1 + b_2 X2 + b_3 X3 + b_{11} X1^2 + b22\ X\ 2^2 + b_{33} X3^2$$

Where Z is the dependent variable (chitinase activity), X1, X2 and X3 are independent variables as mentioned above; $b_0$ 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 between 40 - 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 and EDTA was studied at 1 and 10 mM concentrations of each metal salt. Each experiment was carried out in triplicate and the obtained results were the arithermatic mean.

## RESULTS AND DISCUSSION

### Effect of cultivation medium

In order to verify the most adequate nutrient, ingredients of cultivation medium favor chitinase production through fish scales degradation by *A. terreus* and six different media were tested. The results (Figure 1) indicated that formulation of medium 4 (g/l): fish scales waste, 20; glucose, 3; peptone, 1; $(NH_4)_2SO_4$, 1.4; urea, 0.3; $MgSO_4.7H_2O$, 0.3; $FeSO_4.7H_2O$, 5.0; $MnSO_4.2H_2O$, 1.6; $ZnSO_4.7H_2O$, 1.4; $CoCl_2$, 2.0 (Rattanakit et al., 2002), fortified the test organism by nutrients, qualitatively and quantitatively, in favor of chitinase formulation and fungal growth. This may be due to the richness of this medium in Co, Zn, Mn and Fe, as well as urea beside glucose and peptone, as compared to the other tested media. It was reported that medium composition is one of the main factors that enhance chitinase production by microorganisms (Al-Ahmadi et al., 2008; Akhir et al., 2009; Faramarzi et al., 2009). Rattanakit et al. (2002) indicated that the previous fermentation medium was the most favorable for chitinase production by *Aspergillus* sp.

### Time course study of chitinase production

Time course of growth and active chitinase production by *A. terreus* were studied through 192 h of fermentation.

The fungus was allowed to grow under shaked conditions (150 rpm) for 8 days (192 h) on aliquots of 100 ml medium (medium 4) dispensed in 250 ml Erlenmeyer flasks. After 48 h of growth, the necessary analyses were carried out every 24 h. As shown in Figure 2, the first 5 days (120 h) represent the logarithmic growth phase of *A. terreus*. Thus, maximum fungal biomass (380 mg/ 100 ml) was estimated at 120 h of growth followed by growth yield decrease as the cultivation period extended to 192 h (8 days). However, the highest chitinase activity was recorded after 96 h (4 days) of growth and it was concomitant with low extracellular protein (about 90 mg/100 ml) produced by the fungus. These indicated that chitinase with high activity is produced at the beginning of the log phase of fungal growth. In

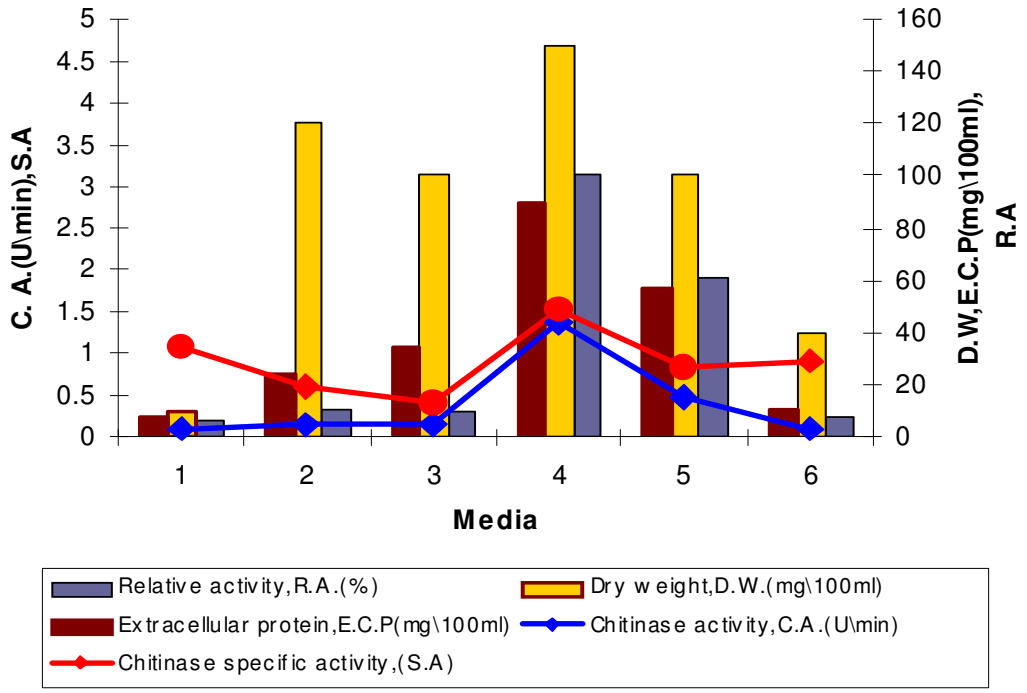


**Figure 1.** Effect of different cultivation media on dry weight, extracellular protein and chitinase activity of *A. terreus*.

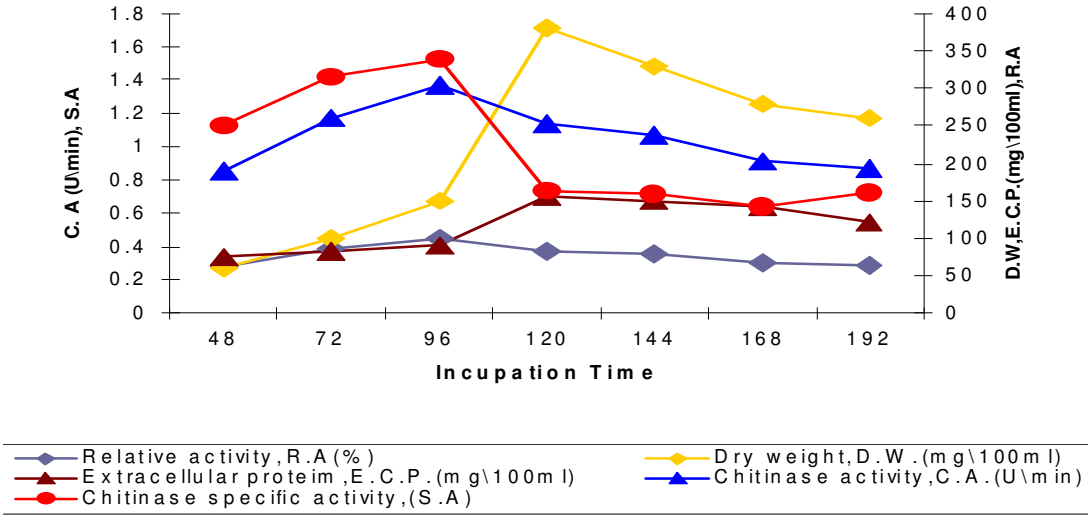


**Figure 2.** Growth, extracellular protein and chitinase activity of *A. terreus* as grown on basal medium for 192 h (8 days).

accordance with these findings, it was reported by Sandhya et al. (2004) that 96 h of growth was optimum for chitinase production by *Trichoderma harzianum* and also for *Metarhizium verrucaria* when grown in medium containing fish scales waste as the sole carbon source (Vyas and Deshpande, 1998). *Penicillium chrysogenium* needs from 6 - 7 days incubation for maximum chitinase production (Patidar et al., 2005).

## Effect of parrot fish scales size

To study the effect of grinding of fish scales, as a physical treatment on the availability of chitin to the attack of chitinolytic enzymes and hence production of chitinases, different sizes [20 (original basal), 10, 8, 4, 0.3 and 1.5 $mm^2$] were prepared and added at 20 g/l of medium 4. The results in Figure 3 indicate that as the finest fish

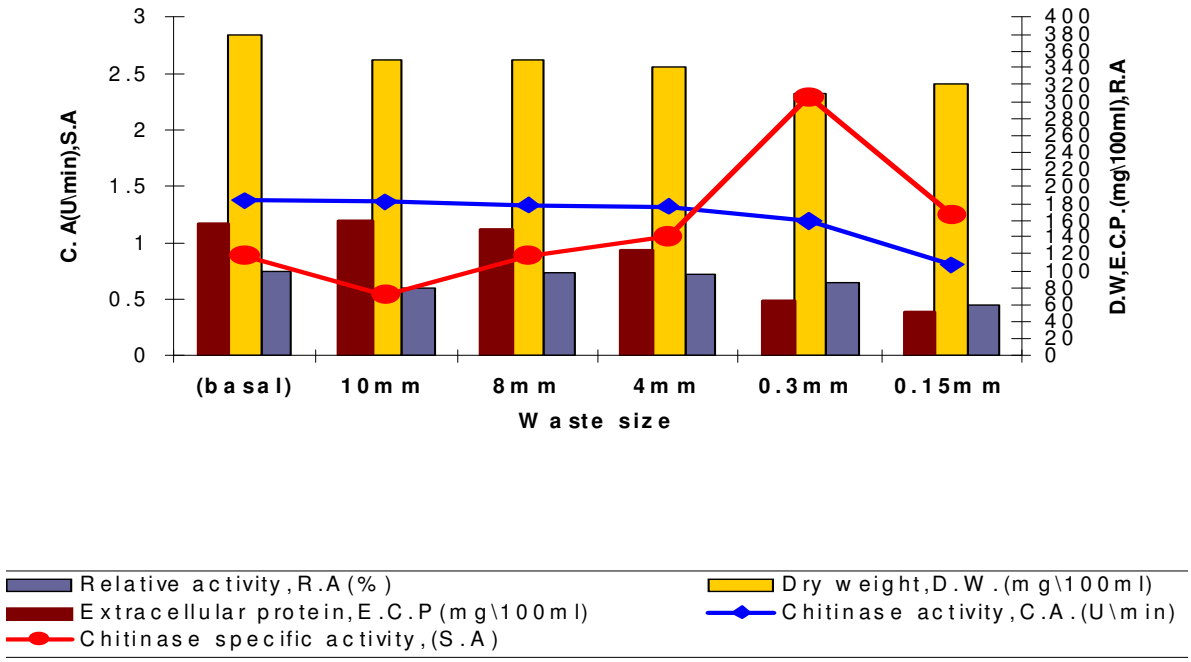


**Figure 3.** Effect of parrot fish scales size ($mm^2$) on growth, extracellular protein and chitinase activity of *A. terreus* – basal (20 $mm^2$).

scales sizes, the lowest chitinase activities were recorded and vice versa. While the mould growth yields exhibited lower values not exceeding about 18%, under the same conditions. Thus, the highest chitinase production, extracellular protein and dry weight production were obtained with untreated (normal) fish scales. These may be explained that grinding process exposes some toxic contents and increase their availability and cause inhibitory effect in chitinase production by the fungus. But in complete scales, these inhibitory constituents are more hidden and less available than finest scales. Also, grinding increase surface/volume ratio than none grinding, thus increasing the effect of the substances of inhibitory action to enzyme production. Grinding may deform the architecture of the chitin that resulted in lower chitinolytic activity of the fungus. In accordance with our findings, Rattanakit et al. (2003) reported that as the size of fish scales waste increase, chitinase production by *Aspergillus* sp. decreases.

## Culture condition

The biodegradation processes are mostly more efficient under aerobic conditions. So, to monitor how much biodegradation of fish scales waste is in favor of chitinase formulation by *A. terreus*, stagnant and shaked cultures were compared. The results in Figure 4 indicated that stagnant culture conditions fortified *A. terreus* with optimal aeration in favor of chitinase production, dry weight yields and extracellular protein more than shaked cultures. In accordance with these findings, Rattanakit et al. (2007) found that chitinase production by *Aspergillus* sp. was more efficient under stagnant culture conditions using fish scales waste. The same finding was also indicated by Patidar et al. (2005) by *P. chrysogenum*.

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

A medium of the composition (g/l): fish scales waste, 20; glucose, 3; peptone, 1; $(NH_4)_2SO_4$, 1.4; urea, 0.3; $MgSO_4.7H_2O$, 0.3; $FeSO_4.7H_2O$, 5.0; $MnSO_4.2H_2O$, 1.6; $ZnSO_4.7H_2O$, 1.4; $CoCl_2$, 2.0; pH 6 (Rattanakit et al., 2002), inoculum 1 ml (14.6 x $10^6$ spores)/100 ml medium dispensed in 250 ml Erlenmeyer flasks and incubated stagnantly at 30 ± 2°C for 4 days (96 h) was used as the basal conditions of cultivation. The design was applied with 13 different fermentation conditions (trials) as shown in Table 1. All experiments were performed in triplicates and the results (averages of the observations) were presented. Table 1 represents the design matrix together with response observations. The results indicated that levels of factors at trial 11 were the best. The main effect for each variable was estimated and the results presented graphically in Figure 5 revealed that the most significant three factors which were more effective were $FeSO_4.7H_2O$, glucose and $MnSO_4.2H_2O$. While $(NH_4)_2SO_4$, $CoCl_2$ and $ZnSO_4.7H_2O$ showed positive non significant effect on chitinase production. However, the

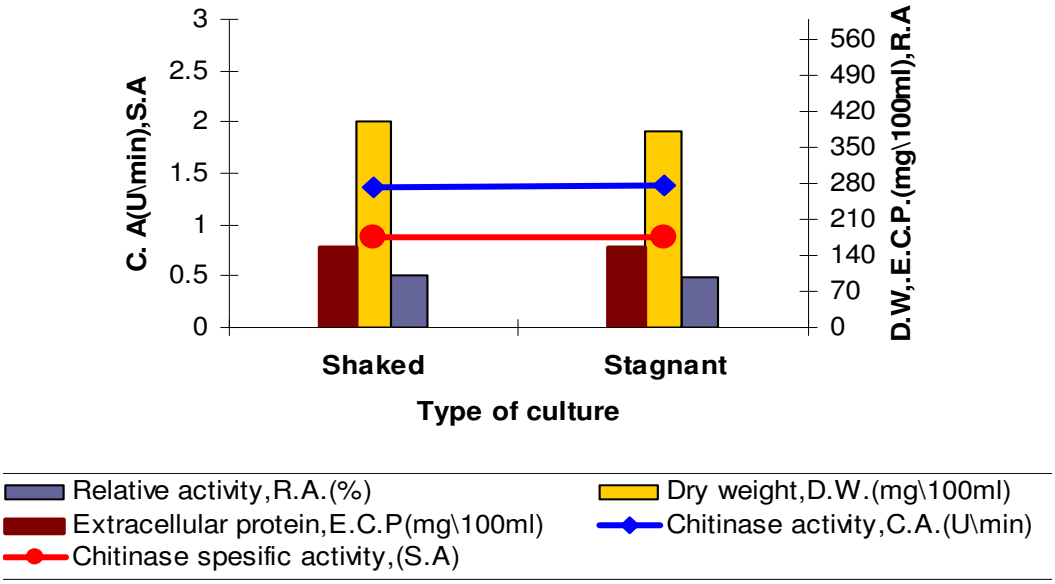


**Figure 4.** Effect of culture condition on growth, extracellular protein and chitinase activity of *A. erreus.*

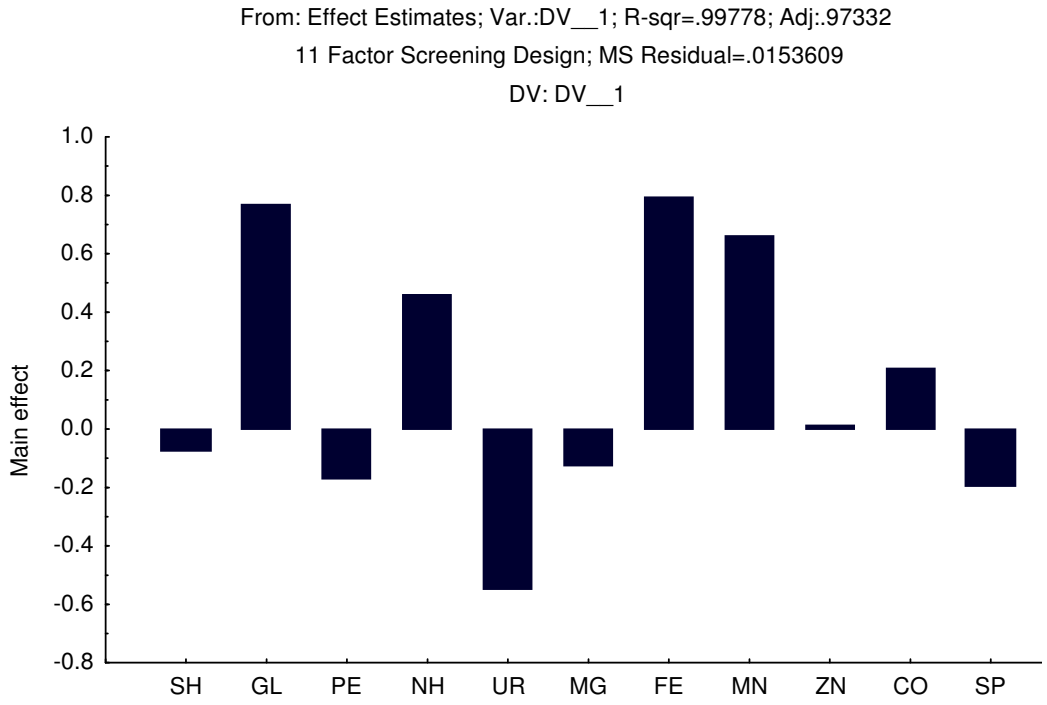


**Figure 5.** Proportional effect of applied factors based on results of Placket- Burman design.

main effect of urea, size of inoculum, peptone, $MgSO_4.7H_2O$ and fish scales waste level was negative. It is well known that Mn and $Fe^{2+}$ act as activators and cofactors in enzymes responsible for hydrolysis of carbohydrates, fats and proteins (Bowman and Russell, 2001), while glucose is the most assessable carbon source to microorganisms and *A. terreus* needs it to initiate growth that enable it to hydrolyse the complex polymer of chitin.

According to the results obtained, medium formula which was predicted to be near optimum was (g/l): fish scales waste, 15; glucose, 5; $MgSO_4.7H_2O$, 5; $FeSO_4.7H_2O$, 8; $MnSO_4.2H_2O$, 3.2; $ZnSO_4.7H_2O$, 0.7; $CoCl_2$, 4.0 and inoculum size (79 x $10^7$ spores/100 ml). A verification experiment demonstrated that the enzyme activity

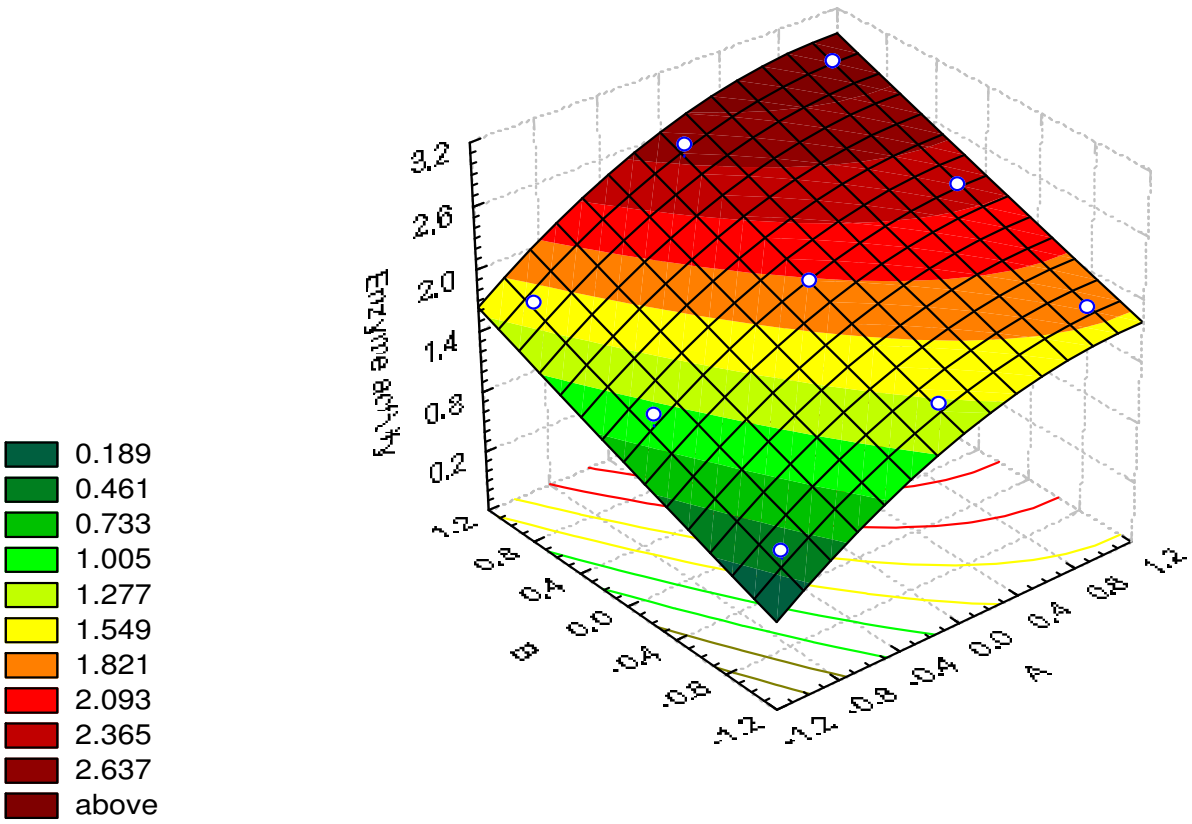


**Figure 6.** Effect of $FeSO_4.7H_2O$ (A) and glucose (B) and their interactive effect on chitinase production.

expressed in this medium was increased by 71% (2.376 u/min) when compared to the basal condition. The results of the Plackett-Burman experiment revealed that the initial concentrations and levels of $FeSO_4.7H_2O$, glucose and $MnSO_4.2H_2O$, the cell experience during fermentation is of importance. As certain factors were eliminated due to lack of significance, $FeSO_4.7H_2O$, glucose and $MnSO_4.2H_2O$ were retained and examined more thoroughly by applying Box-Behnken design.

## 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 ($FeSO_4.7H_2O$, glucose and $MnSO_4.2H_2O$) 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 9) are (g/l): glucose, 6.5; $FeSO_4.7H_2O$, 9.5 and $MnSO_4.2H_2O$, 4.7, with a predicted chitinase activity of 4.230u/min after 95 h of fermentation. It was reported by El-Katany and Gubitz, (2000) that 0.5% glucose addition to chitin substrate repressed chitinase production by *T. harzianum*. Ghanem (1992) found that addition of glucose was of repressive action on chitinase production by *Bacillus amyloliqefaciens*, while $FeCl_3.6H_2O$ highly induced chitinase production. Lopes et al. (2008) found that chitinase production by *Moniliophora perniciosa* was repressed by the addition of glucose.

In order to verify the optimization results, an experiment was carried out under the predicted optimal condition, where the extracellular protein and chitinase activity were monitored at different time intervals of 85, 90 and 95 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 4.309 u/min which is with 1.81 fold increase when compared to the pre-optimized medium control 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, $FeSO_4.7H_2O$ and $MnSO_4.2H_2O$) and response (chitinase activity). A verification experiment was accomplished and revealed approximately 99% MODEL validity. The great similarity between predicted and observed results in this application and others (Al-Ahmadi et al., 2008; Lopes et al., 2008; Akhir et al., 2009; Ghanem et al., 2009) confirm

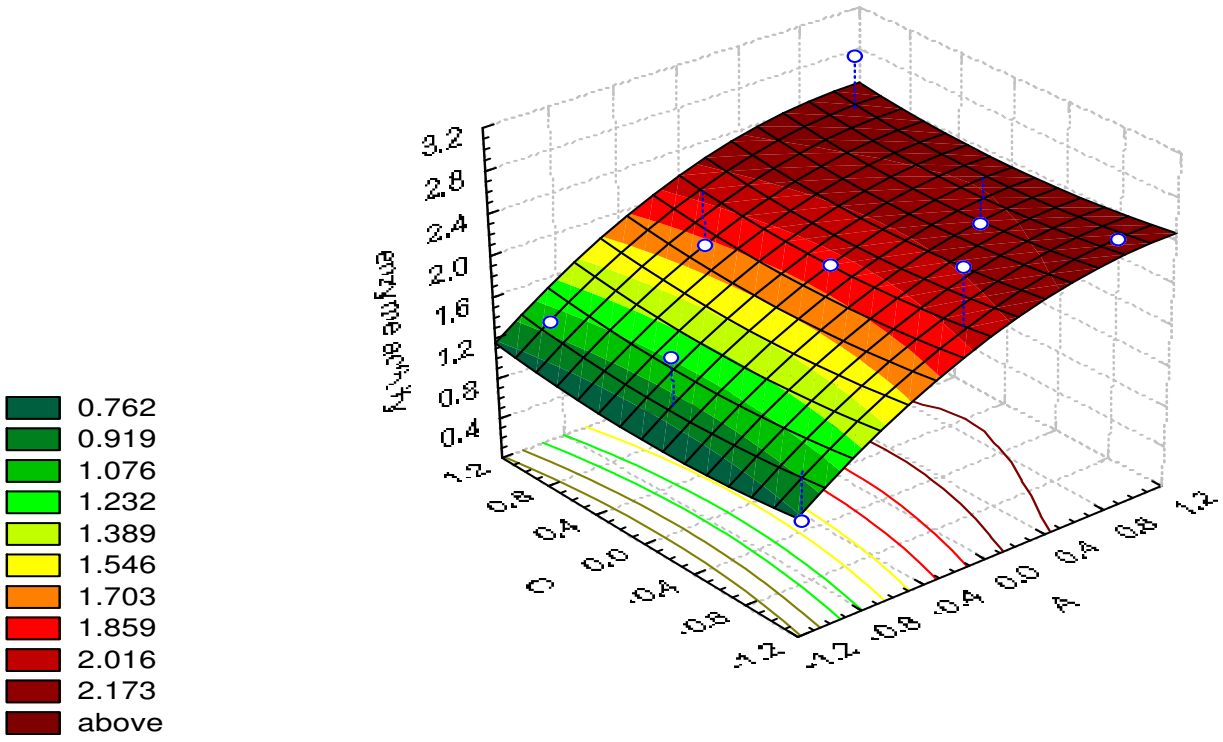


**Figure 7.** Effect of $FeSO_4.7H_2O$ (A) and $MnSO_4.2H_2O$ (C) and their interactive effect on chitinase production.

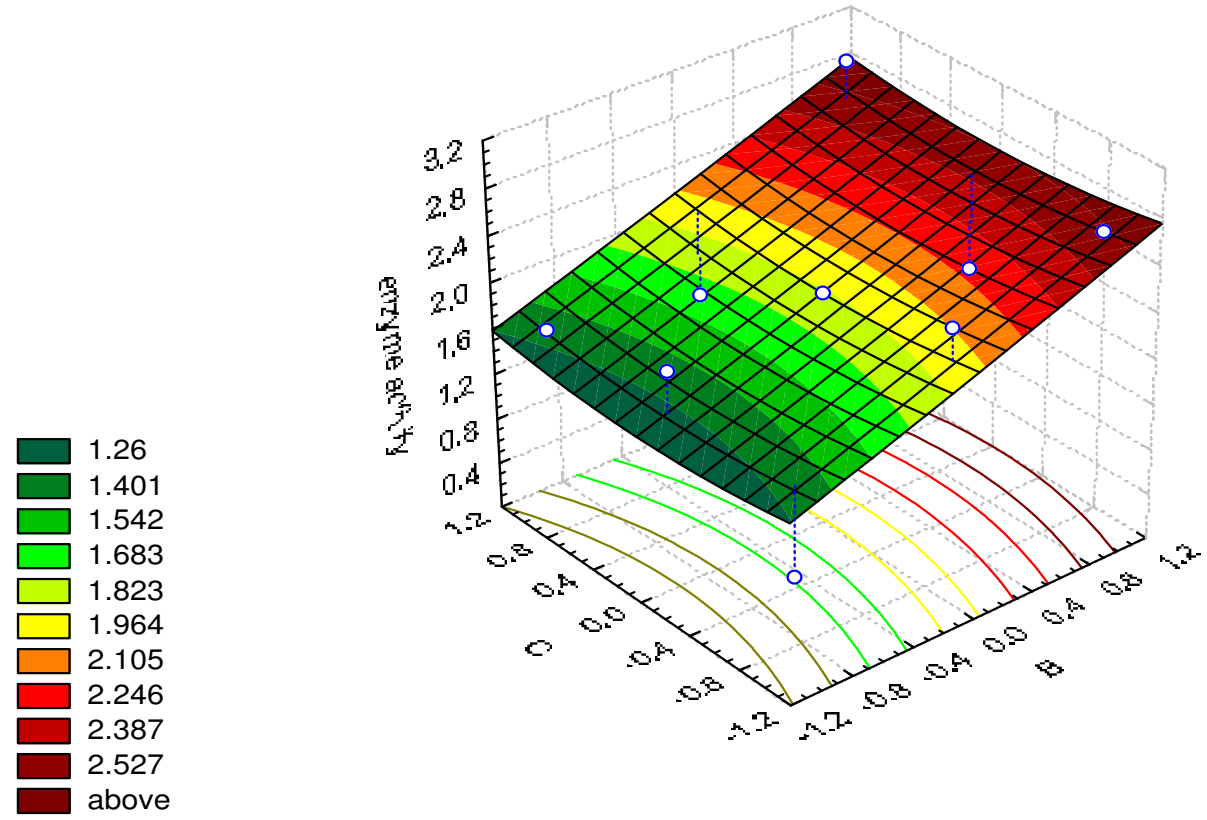


**Figure 8.** Effect of glucose (B) and $MnSO_4.2H_2O$ (C) and their interactive effect on chitinase production.

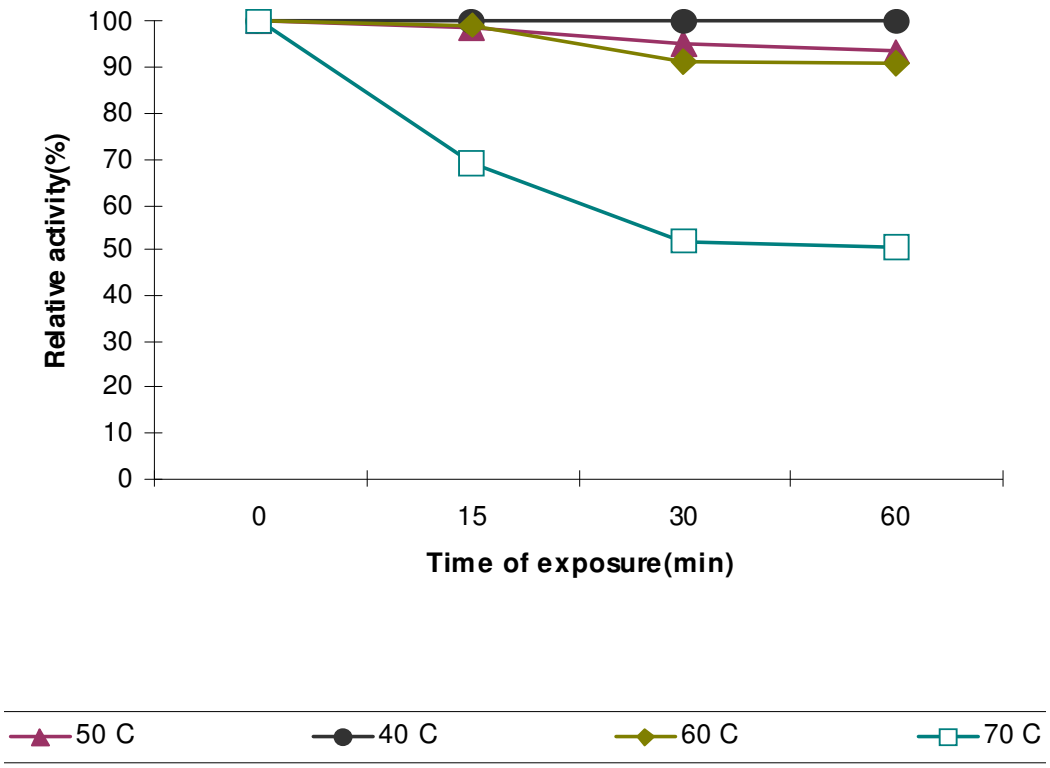


**Figure 9.** Thermal stability of crude chitinase produced by *A. terreus* from fish-scales waste.

the high accuracy and applicability of the Box-Behnken model in optimization processes. It is also likely that the optimized culture condition accelerated the rate of chitinase expression in *A. terreus* as it showed maximum enzyme activity within 90 h instead of 96 h in the case of control culture.

## Characterization of crude chitinase

The crude chitinase enzyme produced by *A. terreus* cultivated for 90 h under the optimized factors for maximum chitinase production using Box-Behnken design, was obtained by centrifugation at 5000 rpm for 15 min in cooling centrifuge. The supernatant culture filtrate is used as 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, 2 ml of crude enzyme and 0.5 ml of colloidal chitin (1% chitin) and pH 6 for 10 min of reaction time. These results are comparable to those obtained with *P. janthinellum* (Fenice et al., 1998), *P. aculeatum* (Bindo et al., 2005), *P. chrysogenum* (Patidar et al., 2005), *Metarhizium anispliae* (Nahar et al., 2004) and *T. harzianum* (Sandhya et al., 2004) for the tested charachteristics, but higher or lower than those of *Aspergillus* sp. (Rattanakit et al., 2002) and *A. terreus* on shrimp-shell chitin (Al-Nusaire, 2007).

Crude chitinase is thermostable (Figure 9) and lost only less than 10% of its activity when heated in the absence of its substrate at 60°C for 60 min. This finding is comparable to that of *Bacillus* sp. (Wang and Chang, 1997) and is superior to that of *Mucor rouxii* (Pedraza-Reyes and Lopez, 1989), *A. terreus* (Al-Nusaire, 2007) and *Sphingomonas* sp.(Xu-fen et al., 2007).

The effect of $CuSO_4.5H_2O$, NaCl, KCl, $MgSO_4.7H_2O$, $CaCl_2$, $ZnSO_4.7H_2O$, $CdCO_3$, $HgCl_2$, $PbCl_2$ and EDTA at 1 and 10 mM, on the activity of chitinase revealed that *A. terreus* crude chitinase appear to have specific requirement of Cu, Ca, Zn and Mn ions for its activity, while the other tested were of inhibitory action for chitinase. In harmony with these findings, Wang and Chang (1997) and Al-Nusaire (2007) found that Cu ions increase chitinase activity of *P. aeruginosa* and *A. terreus*, and Mn ions likewise increase the activity of the enzyme of *Alteromonas* sp. (Tsuijibo et al., 1992). However, the inhibitory action of the other tested ions was in harmony with other microbial chitinases (Hunphreys and Gooday, 1984; Pegg, 1988; El-Aassar et al., 1992; Al-Nusaire, 2007).

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