J. Agric. Sci. Mansoura Univ., 30 (1): 569 - 586, 2005

PRODUCTION, PURIFICATION AND CHARACTERIZATION OF THERMOSTABLE CHITINASE AS LYTIC ENZYME FROM *Bacillus stearothermophilus* AND ITS USE FOR PREVINTING THE GROWTH AND SPORULATION OF SOME PLANT PATHOGENIC FUNGI

Shady, T. S. M.; A. A. Nasr; W. I. A. Saber and K. A. Abd El-Kawi.
Microbiol. Dept., Soils, Water and Environ. Res. Institute Agric. Res. Center, Giza, Egypt.

ABSTRACT

Chitinases (glycanohydrolases, EC 3.2.1.14) are important enzymes in various fields such as biological control, pharmaceuticals and chemical purposes as well as food and feed industries. Therefore, the present study aimed to produce chitinase enzyme and the results revealed that: Maximum enzyme activity and cell density of *B. stearothermophilus* were obtained in the fourth day on the fermentation media containing 1.5% of chitin or chitin + arabinose or/and CM – cellulose as carbon source and 1.5% yeast extract as nitrogen source, which these nutrition factors induced the enzyme biosynthesis. The shaking of culture through the fermentation period greatly enhanced the formation of this enzyme. Initial pH 8.0 and 55°C as incubation temperature were found as the best environmental factors for enzyme production. 65% is the best saturation of ammonium sulphate which gives higher enzyme activity, recovery and purification folds. The purification of enzyme with DEAE – sephadex A – 50 gives higher purification folds and specific activity of enzyme. The fractionation of enzyme preparation showed 3 peaks of enzyme. This means that, this enzyme has three fractions of chitinase, which identified as three types of endo – chitinase. pH 6.0 and 65°C were found as the optima for enzyme activity. The enzyme showed higher stability against the different pH range between 5.0 to 8.0 and completely stable at pH 6.0. This means that the enzyme protein has acidic in its nature. Also, the enzyme was stable up to 75°C and lost little activity from its maximum up to 85°C. This means that, this enzyme was a thermostable one. Some metal ions activated the enzyme activity such as Mg^{+2} and Ca^{+2}, but others such as Ag^{+} and Hg^{+2} were inhibited it. Enzyme preparations were successfully to hydrolysis different substrates with higher degree such as colloidal chitin, chitin and colloidal chitosan, but other such as dioligomer did not hydrolyze. This is may be due to its substrate specificity or substrate affinity of the enzyme. The enzyme also successfully to hydrolyse cell walls of some plant pathogenic fungi with higher degree. Also prevent the growth of these plant pathogenic as well as sporulation rate. This means that, this enzyme preparation may be used as a good antifungal agent in biological control or preventing the mould growth of pathogenic fungi.

Keywords:*Bacillus stearothermophilus*, thermostable chitinase, Production, stimulation, repression, Purification, characterization, biological control, target fungi, sporulation.

INTRODUCTION

Chitin is an unbranched polysaccharide, being the most abundant naturally occurring aminopolysaccharide and is thus abundant in nature, second only to cellulose. Besides being present on most fungal cell walls, chitin is also, the principal structural component of most biological systems

such as molluses insect exoskeletons, crustaceans (shrimp and crab), fungi, algae and marine invertebrates. Chitin a homopolymer of N – acetyl – D – glucosamine (Glu – Nac) residues linked by â – 1, 4 bonds. This component and its derivatives are of interest because they have varied biological functions, *e.g.,* as immunoadjuvants, as flocculants of waste water sludge, and as agrochemicals. For example, the addition of chitin to soil reduces populations of fungal plant pathogens and plant pathogenic nematodes. In recent years, significant research has been directed towards the use of chitin derivatives in fields of drug delivery, in the treatment of dermatitis and fungal infections, as bacteriostate and fungistate. Such biological activities of chitin oligomers are dependent on chain length and solubility (Sakai *at al*., 1998; Bhushan, 2000; Gomes *at al*., 2000; Shady *at al*., 2000; Suginta *at al*., 2000 and Shindia, 2001).

The enzymes responsible for chitin degradation and modification are chitinases (Poly (1,4 – â – 2 acetamido – 2 – deoxy – D glucoside) glycanohydrolase, EC 3.2.1.14)), which are found in a variety of organisms such as bacteria, fungi, actinomycetes, yeasts, plants, protozoans, nematodes, molluses, arthropods, and also in human beings. These enzymes are classified to three separate enzymes categorized as exochitinase (â – N – acetylhexosaminidase (EC 3.2.1.52)) which subsequently degraded the oligomers to monomers, the second group is endochitinase (EC 3.2.1.14) randomly cleaves chitin to produce chitobiose, thus, it reduces the polymer to oligomers and the third group is chitobiase (N – acetyleglucosaminidase, EC 3.2.1.30) that constitute the chitinase complex. The first one liberates soluble low molecular weight dimers; the second release multimers of N – acetyl – glucosamine (NAG) and the third completes the hydrolysis of chitobiose to NAG. Chitinolytic enzymes have been considered important in the biological control of soil borne pathogens because of their ability to degrade fungal cell walls, of which a major component of chitin. In recent years, the use of these enzymes in application fields such as biological pest control, the degradation of chitin rich wastes and the production of chitin hydrolysates for pharmaceuticals or chemical purposes and for the food / feed industries have become of increasing interest (Chen & Lee, 1995; Sakai *et al.,*1998; El – Sawah, 1999; Melent'ev & Aktuganov, 1999; Singh *et al.,* 1999; Bhushan, 2000; Gomes *et al*., 2000 and Shady *et al*., 2000).

One of the best chitinase – producing microorganisms is the bacterium *Serratia marcescens*, especially the strain QMB 1466. The chitinase hydrolyzes crystalline chitin which makes it suitable for treating shellfish waste chitin as the chitin particle for waste bioconversion process after demineralization and deprotination treatments is still highly crystalline, layered and sheetlike. Also, these enzymes are strongly induced when chitin used as carbon source in fermentation process. The hydrolysis of chitin yield soluble NAG which is consumed by bacteria for growth. Various species of *Bacillus* have been shown to secret highest amount of chitinase including *Bacillus stearothermophilus, B. lichiniformis*, *B. cereus, B. circulans, Paenibacillus pabuli* and some others. Besides the traditional use of selected strains of *Serratia marcescens* and *Streptomyces griseus* as well as *Bacillus* species for the production of chitinolytic enzymes, fungi have recently been

attracting increasing attention. However, the only fungal chitinase preparation of any commercial value comes from *Trichoderma harzianum* cultivated by submerged fermentation (Frۥndberg & Schnürer, 1994; Chen & Lee, 1995; Pleban *et al*., 1997; Fenice *et al*., 1998; Sakai *et al*., 1998 and Shady *et al.,* 2000).

The goal of the present work was to produce and purify *B. stearothermophilus* thermostable chitinase as lytic enzyme and study the properties of this purified enzyme. The antagonistic effect of these lytic enzymatic preparations against fungi parasiting on higher plants were also studied.

MATERIALS AND METHODS

Microorganisms:

Bacillus stearothermophilus used in this study for chitinase production was obtained from Microbiol. Dept. Fac. Agric. Mansoura Univ., Mansoura, Egypt. The cultures were maintained and monthly transferred on medium of Frۥndberg & Schnürer (1994). The phytopathogenic fungal strains, *Rhizoctonia solani, Aspergillus niger* and *Fusarium oxysporum* were kindly obtained from Plant pathol. Dept. Fac. Agric. Mansoura Univ., Mansoura, Egypt. These parasitic fungi were maintained and monthly transferred on potato – dextrose agar (PDA).

Fermentation technique:

The basal mineral salt solution of Frۥndberg & Schnürer (1994) was used for the production of chitinase from *Bacillus stearothermophilus*. This medium had the following composition (g/L):K_2HPO_4, 1.5; KH_2PO_4, 1.5 $MgSO_4.H_2O$, 0.7; NaCl, 0.5; KCl, 0.5; $CaCl_2.2H_2O$, 0.13 and yeast extract, 0.5. The pH was adjusted to 6.6. 50 ml portions of the fermentation medium were dispended in 250 Erlenmeyer flasks, sterilized at 121°C for 15 min. It was supplemented with 0.5 % colloidal chitin from crab shell (Sigma) as a carbon source. Chitin was sterilized separately to avoid browning. The media were inoculated with cell suspension. For preparing the inoculum, the cultures were grown on agar salnts of the above medium for 24 hrs then scraped using 5 ml sterile tap water. The inoculum was thus used to inoculate the liquid medium (in the sense of one tube for each fementation flask). After incubatioin for 8 days at 50°C, the flasks were filtered and centrifuged at 8000 rpm for 15 min. at 4°C. The clear supernatants were collected and used for crude chitinolytic enzyme source (Sakai *et al.,* 1998 and Shady *et al.,* 2000).

Assay of chitinase activity:

Chitinase activity was determined spectrophotometrically by estimating the amount of free reducing groups formed after colloidal chitin hydrolysis. The reaction mixture was composed of 1.0 ml of 1% (w/v) colloidal chitin suspended in 0.05 M phosphate buffer (pH 7.0) and 0.5 ml of the culture supernatant (El-Sawah, 1999).After 10 min of incubation at 50 °C with reciprocal shaking (60 strokes/min), (Sakia *et al.,*1998), N-acetylglucose

amine (NAGA) was estimated by the method of Reissig *et al.,* 1955). A standard curve was constructed using N-acetylglucoseamine (Sigma, St., Louis, Mo.) as a standard. A unit of chitinase activity was defined as an amount of enzyme required to produce 1 µmol of NAGA / min at 50 °C.

Ammonium sulphate precipitation:

The precipitation of *B. stearothermophilus* chitinase was carried out by adding various amounts of ammonium sulphate to the supernatant maintained at 4 °C to give percentage saturation from 20-80%. The mixture was left overnight at 4 °C and then centrifuged (10 000 rpm) under cooling. Each fraction precipitate was dissolved immediately in 20 ml (0.05 M) phosphate buffer (pH 7.5). The enzyme solution was centrifuged at 10 000 rpm for 10 min to remove the undissolved particles. The dissolved fractional precipitate was tested for both chitinase activity and protein content (Abdel-Fatah and Khella, 1995).

Enzyme purification:

To purify chitinase, the culture liquid was first separated from the biomass and the residual substrate by centrifugation (8000 rpm / 20 min). The supernatant was cooled to 5 - 10 °C and saturated to 65% with dry ammonium sulphate, which was added under continuous stirring. The precipitate was separated by centrifugation (10 000 rpm/ 30 min) and dissolved in 35 ml 50 mM tris-HCl (pH 7.1). The saturated solution of enzyme was centrifuged (3000 rpm / 10 min) to remove the insoluble residue and loaded into a 2.5 X 40 cm column, which was packed with gel filtration, sephadex G-25 (pharmacia, Sweden) and equilibrated with 50 mM tris-HCl (pH 7.1). Proteins were eluted with the same buffer solution at 3 ml / min. and fractions were collected in 15-ml aliquots. The fractions with detectable chitinase activity were pooled and loaded into a 2.5 X 40 cm column which was packed with DEAE-sephadex A-50 (pharmacia, Sweden) and equilibrated with the same buffer. The column was washed by the same buffer at an elution rate of 1 ml / min. and fractions were collected in aliquots of 10 ml. the fractions with chitinase activity were pooled and concentrated by lyophilization (Melent'ev & Aktuganov, 1999).

Protein assay:

It was determined by the method of Lowry *et al*. (1951), with bovine serum albumin as a standard.

Effect of pH on chitinase activity and stability:

Chitinase activity was assessed as above, using the following buffer (0.05M) sodium citrate (pH3.0-5.5) potassium phosphate (pH 6.0- 7.5), Tris-HCl (pH 8.0-8.5) and glycine - NaOH (pH 9.0 - 10.5).

Chitinase stability was assessed by determination the residual activity after incubating the enzyme solution in an appropriate buffer (pH 4 - 10) at 65 °C overnight.

Effect of temperature on chitinase activity and stability:

Chitinase activity was determined by incubation enzyme solution in 1.0 ml of 1% colloidal chitin suspended in 0.05M phosphate buffer (pH 6.0) at various temperatures.

Chitinase stability was determined by incubation the enzyme solution in 0.05M phosphate buffer (pH 6.0) for 60 min at various temperatures (30-90°C). the enzyme was cooled to the optimal temperature, then the residual activity was measured.

Preparation of target fungi cell wall

The fungi *R. solani, A. niger and F. oxysporium* were grown in potato dextrose agar, incubated at 30 °C and then harvested after 72h growth. Cell walls were obtained from the mycelium of the three tested fungi as described by Skujins *et al*., (1965). Mycelium of the fungus was autoclaved and homogenized at 4000 rev/min for 3 min in a tissue phosphate homogenizer. The homogenate was further subjected to ultrasonic disintegration in ice bath for 6 min in 12 cycles each of 30 s in MSE ultrasonicator. The pellet obtained by centrifugation at 5000 rev/ min for 25 min at 4 °C in a refrigerated centrifuge was washed four times with distilled water and dried.

Preparation of colloidal chitin

Colloidal chitin was prepared by the method described by Lingappa and Lockwood (1961).

Biological activity of Chitinase against fungal growth and sporulation

To determine the activity of the enzymatic Preparation in vitro of target fungi used, fungi were plated on Petri dishes with a potato – dextrose agar containing enzymatic Preparation (0.1%) and incubated at 20-22 °C for 8 days. The diameters of the fungi colonies were then determine. After 10 days of incubation, 20 ml of water was added to every dish, and a spore suspension was obtained to determine the intensity of sporulation. The suspension was homogenized for 1 min at 1500 rpm and filtered through a sterile cotton cloth. The suspension was filtered through a sterile cotton cloth. The spores were counted in a Goryaev Chamber (Tatarinova *et al*., 1996).

RESULTS AND DISCUSSION

Optimizing culture conditions for chitinase production*:*

1- Time course:

The level of secreted *Bacillus stearothermophilus* chitinase activity in cultures were measured after 1 to 8 days. The results in Fig. (1) shows that considerable variation in the levels of enzyme activity with respect to fermentation time. It is apparent that the viable cell counts were increased from 1.5 X 10^6 to 9 X 10^8 cfu/ml after incubation for 4 days thereafter, the cell density decreased slightly. The enzyme activity appeared related to the cell growth through the cultivation up to 4 days. Chitinase activity in the culture fluid was detectable after one day and increased rapidly, which reached its

maximum activity (0.87 U/ ml) in the fourth day and decreased rapidly during the prolongation of cultivation time. The late appearance of chitinase could be the result of induction as chitin eventually becomes available after consumption of protein (Mohamedin, 1993 and Shindia *et al.,*2001). Thus, the results indicated that this enzyme is not constitutive one, which induced with its substrate and its activity appeared in the surrounding media after 24 hours. Therefore, 4 days as the suitable cultivation period was chosen for the subsequent experiments. El-Sawah (1999) and Bhushan (2000) reported that chitinase reached its maximum activity after 72 hours. But, Shady *et al.* (2000) found that chitinase activity was maximized in the fifth day.

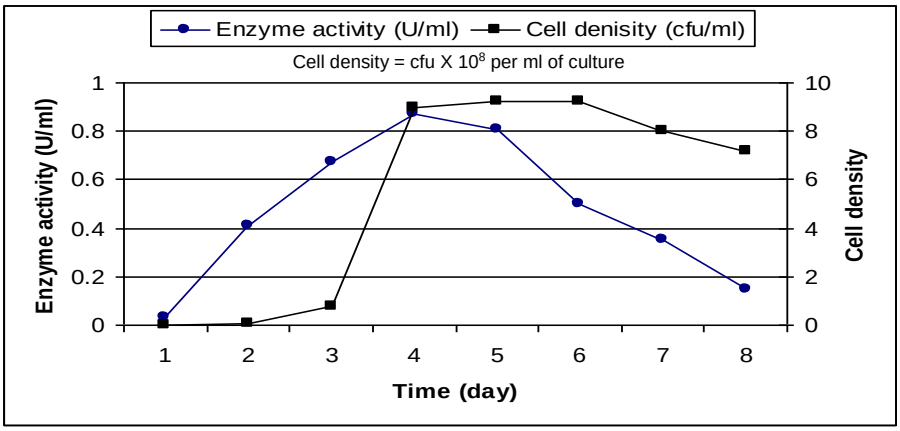

Fig. (1): Fermentation time–course of *B. stearothermophilus* chitinase production.

2- Carbon source:

It is appeared that cultivation of *B. stearothermophilus* in the presence of chitin or chitin-derivatives substrates has caused increasing in specific activity of chitinase. Results in Table (1) shows that colloidal chitin or chitin plus simple sugars induced greatly the enzyme biosynthesis.

Table (1): Effect of different carbon sources on enzyme activity.

Carbon source	Enzyme activity (U/ml)
Chitin	0.87
Chitosan	0.74
N-Acetyl glucose amine (N-AGA)	0.56
Cell wall	0.47
Glucose	0.00
Chitin + Glucose	0.57
Laminarin	0.15
Chitin + Laminarin	0.35
Chitin + N-AGA	0.67
Chitin + arabinose	0.89
Chitin + ribose	0.55
Chitin + lactose	0.49
Starch	0.10
Pectin	0.77
CM-cellulose	0.91

But, glucose and starch were repressed the biosynthesis of enzyme. This means that, this enzyme was an inducible enzyme, but not constitutive one, because it is not detectable after one day of incubation and with fermentation conditions free chitin or chitin derivatives. Also, the results reflected the importance of chitinolytic substances as an inducers for chitinase production. CM-cellulose, chitin + arabinose, and pectin were found as the best inducers and good superior for enzyme formation. Frندberg & Schnürer (1994); Gupta *et al*., (1995); El-Sawah (1999) and Shady *et al*., (2000) reported that chitinase are inducible enzymes which induced with their substrates.

3- Effect of different concentrations of the best carbon sources on enzyme production:

Chitin, chitin + arabinose and CM-cellulose were used with different concentrations to study their effective on chitinase production. Results in Table (2) showed that, chitinase was faintly active towards these substrates, also the enzyme appeared to hydrolyze high molecular mass substrate, thus they induced greatly the enzyme biosynthesis. Also, the results indicated that, the increasing of these carbon sources concentrations stimulated the synthesis of enzyme, which enzyme activity was increased gradually and reached its maximum yield (1.25, 1.35 and 1.60 units/ml) at 1.5 % chitin, 1.5% chitin + 0.5 arabinose and 1.5 % CM-cellulose, respectively, and decreased thereafter. This means that, the level of chitinase activity was 1.44, 1.52 and 1.76 times higher than in the case of the medium with chitin, chitin + arabinose and CM-cellulose, respectively. The results also indicated that, this enzyme is inducible enzyme and its activity increased greatly with the suitable carbon source as well as its concentration in the production medium. These results are in agreement with those obtained by Abdel-Fatah (1995); Sakai *et al.* (1998); El-Sawah (1999); Shady *et al*. (2000) and Shindia (2001). They reported that chitin and polymer chitin-containing substrates enhanced the enzyme formation.

Table (2): Effect of different concentrations of the carbon sources on chitinase production.

Best carbon source conc. (%)		Enzyme activity (U/ml)
Chitin	0.5	0.87
	1.0	1.05
	1.5	1.25
	0.2	1.20
Chitin + arabinose	0.5	0.89
	1.0	1.07
	1.5	1.35
	0.2	1.30
CM-cellulose	0.5	0.91
	1.0	1.17
	1.5	1.60
	0.2	1.45

4- Effect of nitrogen sources:

Yeast extract on the basal fermentation medium was replaced by different nitrogen sources (Table, 3) with equivalent nitrogen basis. The results indicated that, nitrogen sources were greatly affected on chitinase production. Yeast extract and peptone were found as the best nitrogen source, which induced the *B. stearothermophilus* chitinase formation, which being maximum enzyme activity (1.65 and 1.60 U/ml) with yeast extract or/and peptone, respectively. Also, the results showed that urea inhibited the enzyme production. This unfavourable effect of urea on chitinase formation may be due to its toxic effect in high concentration or the pH variations occurred after its addition. In general, the results also indicated that, organic nitrogen sources enhanced and promoted the enzyme synthesis than inorganic one. it is worthy mention that, in absence of an exogenous supply of nitrogen source, the tested organisms produce relatively low chitinase enzyme indicating its ability to utilize chitin or chitin containing substrates as carbon and nitrogen source. Similar findings were reported by El-Sawah (1999); Shady *et al*. (2000) and Shindia (2001).

Table (3): Effect of different nitrogen sources on chitinase production.

Nitrogen sources	Enzyme activity (U/ml)
Yeast extract	1.65
Peptone	1.60
Meat extract	1.45
Corn steep liquor	1.55
Soy bean meal	1.37
Urea	0.35
$(NH_4)_2SO_4$	1.55
$NaNO_3$	0.95
$(NH_4)_2NO_3$	1.17
Potassium nitrate	0.83
Without N. source	0.15

5- Effect of different concentrations of yeast extract on enzyme production:

It could be noticed from the data illustrated in Fig. (2) that enzyme activity increased gradually with the increasing of yeast extract concentration and reached its maximal yield (being 3.45 units/ml) in the presence of 1.5% of yeast extract and thereafter decreased sharply. This means that yeast extract induced and stimulated the formation of *B. stearothermophilus* chitinase up to 1.5% concentration, because it acts as a source of growth factors. Above this concentration of yeast extract, enzyme production repressed greatly. This sharp reduction of chitinase production may be due to yeast extract acts as carbon and nitrogen source. Also, Sakia *et al*. (1998) found that yeast extract was essential for growth and enzyme production. Abdel-Fatah (1995) reported similar observation.

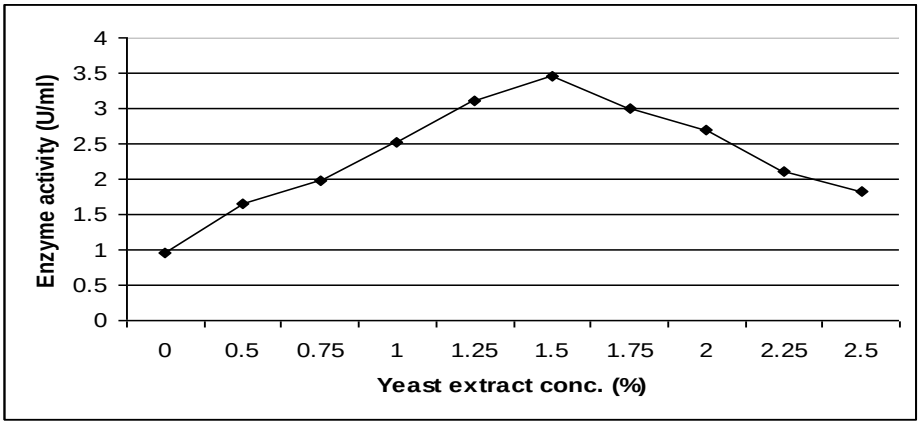

Fig. (2): Effect of addition of different concentrations of yeast extract on enzyme production.

6- Effect of shaking on enzyme production:

The effect of shaking compared with static culture was studied and the results concluded that shaking of fermentation cultures increased the productivity of the enzyme being 2.6 times than static culture. The maximum enzyme yield reached 8.97units/ml. This means that, the enzyme productivity was strongly influenced with shaking which increased aeration rate of cultures which produced sufficient oxygenation led to increasing the biosynthesis of enzyme. These observations are in agreement with those reported by Fenice *et al.*(1998) and Shady *et al.* (2000).

7- Effect of initial pH on chitinase production:

The maximum chitinase production by *B. stearothermophilus* was obtained at an initial pH 8.0 as shown in Fig. (3). This means that acidity greatly affected the biosynthesis of this enzyme. Above or below this pH level resulted sharp reduction in enzyme productivity. These results are in agreement with those reported by Shady *et al.* (2000).

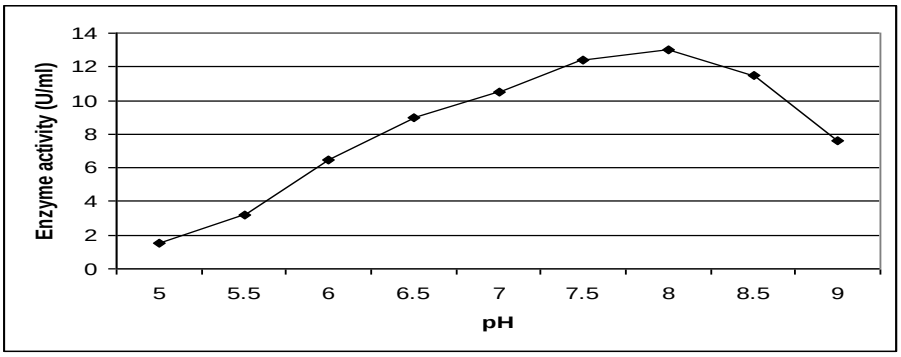

Fig. (3): Effect of initial pH on enzyme production.

8- Effect of incubation temperature:

The effect of incubation temperature (35 – 75°C) on enzyme formation was tested (Fig. 4) the highest enzyme yield was observed in the

temperature range 45 – 65°C with an optimum at 55 °C. Enzyme activity reached 13.9 units/ml at 55 °C.

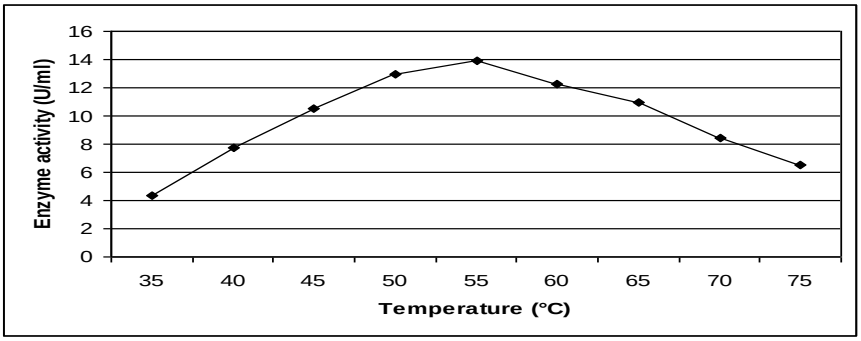

Fig. (4): Effect of incubation temperature on enzyme production.

However, by raising temperature above 55 °C, a clearly decrease in the activity was observed. Sakia *et al.* (1998) found that, 58 °C was found as the best fermentation temperature for *B. stearothermophilus* chitinase production.

Enzyme purification:

1- Ammonium sulphate precipitation:

The results in Table (4a) revealed that specific activity, recovery and purification fold in the precipitate increased with the increasing of ammonium sulphate saturation reaching their maximal values at 65% saturation being 150.57 units/mg protein, 61.38% and 6.83 folds, respectively. These parameters decreased thereafter. Therefore, enzyme at this saturation (65%) was used for further enzyme purification. Similar observations were obtained by Abdeh-Fatah & Khella (1995) and Shady *et al.* (2000).

Table (4a): Fractional precipitation of *B. stearothermophilus* chitinase with ammonium sulphate.

Ammonium sulphate saturation (%)	Volume	Enzyme activity (Units/ml)	Total activity (Units)	Total protein (mg)	Specific activity (U/mg protein)	Recovery (%)	Purification (fold)
Culture supernatant	100	13.0	1300	59.0	22.03	100.00	---
20	20	3.9	78	3.93	19.85	6.00	0.90
30	20	6.7	134	4.2	31.90	10.31	1.45
40	20	10.4	208	4.5	46.22	16.00	2.10
50	20	18.8	376	4.9	76.73	28.92	3.48
60	20	28.5	570	5.2	109.62	43.85	4.98
65	20	39.9	798	5.3	150.57	61.38	6.83
70	20	31.0	620	5.4	114.81	47.69	5.21
80	20	19.6	392	5.4	72.59	30.15	3.30

2- Enzyme purification:

The results of purification are shown in Table (4b). The enzyme was concentrated by ammonium sulphate precipitation. Gel filtration was carried out to eliminate salts and the purification proper was achieved at the stage of

Ion-exchange chromatography on DEAE-Sephadex A-50. As shown in Table (4b) the chitinase produced by *Bacillus stearothermophilus* was purified 22.06 folds with an overall yield of 16.71% of the original activity (the cell free filtrate) and specific activity 485.95 units/mg protein.

Table (4b): Enzyme purification steps.

Purification step	Volume	Enzyme activity (Units/ml)	Total activity (Units)	Total protein (mg)	Specific activity (U/mg protein)	Purificat-ion (fold)
Culture filtrate	1000	13.0	13000	590	22.03	---
After precipitation 65% saturation	50	32.72	1636.21	11.86	137.96	6.26
Gel-filtration (sephadex G-25)	50	48.35	2417.52	9.65	250.52	11.37
Gel-filtration (DEAE-sephadex A-50)	50	166.20	2172.20	4.47	485.95	22.06

3- Enzyme fractionation:

The active enzyme solution (20 ml), which was purified by using DEAE-sephadex A-50 column chromatography and dialyzed was applied at the DEAE–sephadex A-50 column. The results illustrated in Fig. (5) reveal that, the chitinase activity began to appear from the 27th fraction up to 33rd fraction. Then, another two peaks were appeared between fractions 40 – 47 and 53 – 60, respectively. Maximum enzyme activity was shown by fraction 30, 44 and 59. These results also indicated that, the isolated enzyme from *Bacillus stearothermophilus* was three types of chitinase, because three major proteins and three top peaks were observed. Therefore, three fractions (I, II and III) of *B. stearothermophilus* were recorded. Sakia *et al.* (1998) reported that, *Bacillus stearothermophilus* produced three thermostable endochitinases.

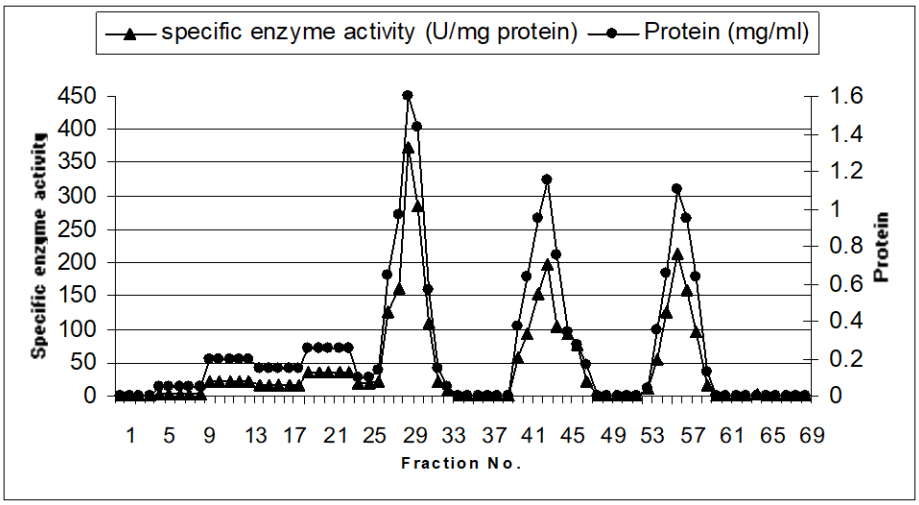

Fig. (5): Fractionations of *B. stearothermophilus* chitinase with Ion-exchange chromatography, DEAE – sephadex A-50.

The fractionation procedure of chitinase produced from *Bacillus stearothermophilus* strain showed that, the organism produced three endochitinases in its culture fluid, and the results also indicated that the three chitinases of the tested organism might be products of different genes (Sakia *et al.,* 1998).

Enzyme properties:

1- pH optimum of chitinase:

Fraction with highest enzyme activity were collected and assayed for investigated the different enzyme properties. Active – pH profiles of chitinase showed that the purified preparation of *B. stearothermophilus* chitinase exhibited optimum activity at pH 6.0, above or below, enzyme activity declined sharply (Fig. 6). These results are coincided with that reported by Sakia *et al.* (1998) and Melent'ev & Aktuganov (1999). This means that, this enzyme showed pH optimum in an acidic range. Thus the enzyme has acidic in its nature.

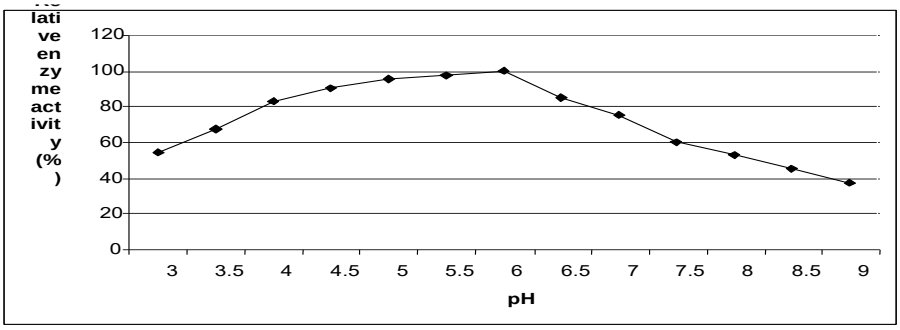

Fig. (6): Optimum pH for enzyme activity.

2- Temperature optima:

The results in Fig. (7) show that, the preparation of chitinase from *B. stearothermophilus* was most active at 65°C.

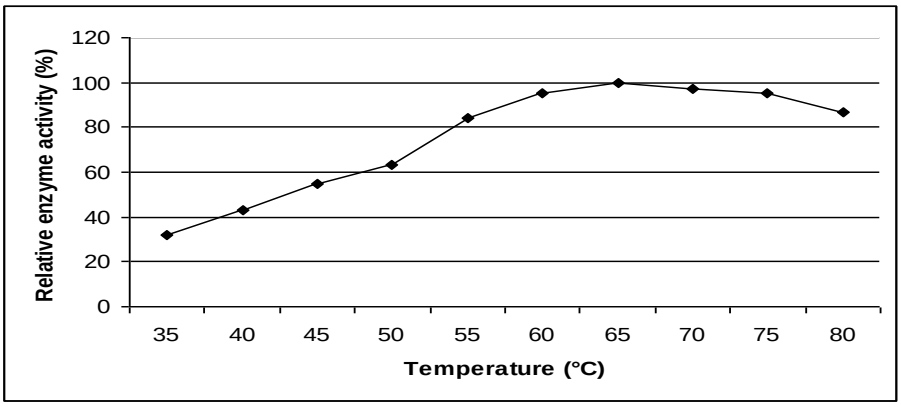

Fig. (7): Temperature optimum for enzyme activity.

This means that this chitinase showed relatively high temperature optimum and the enzyme was thermostable one. Optimum activity at 50°C was observed with the majority of extracellular hydrolytic enzymes from Bacilli (Melent'ev & Aktuganov, 1999). Similar results were reported by Sakia *et al.* (1998).

3- pH stability of the enzyme:

Data in Fig. (8) show that, the enzyme is stable within a broad pH ranging from 5.0 to 8.0. The enzyme showed complete stability towards pH 6.0 and lost little activity (15 and 17 %) at pH 5.0 and 8.0, respectively. This means that, this enzyme has many advantageous uses in biotechnology and as antifungal agent. Similar observations were reported by Trachuk *et al.* (1996); Sakia *et al.* (1998) and Shady *et al.* (2000).

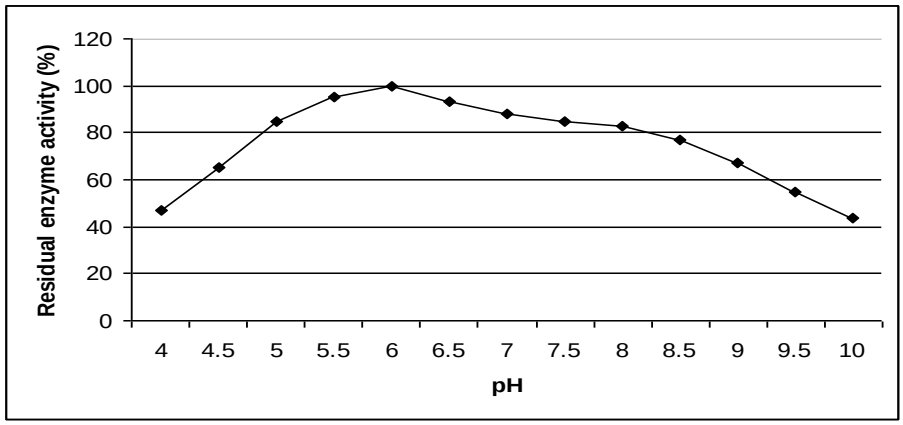

Fig. (8): pH stability of the enzyme.

4- Thermal stability of the enzyme:

Results in Fig. (9) reveal that, the enzyme was stable up to 75°C and lost little activity (7%) from its maximum activity at 80°C and lost 35% at 90°C.

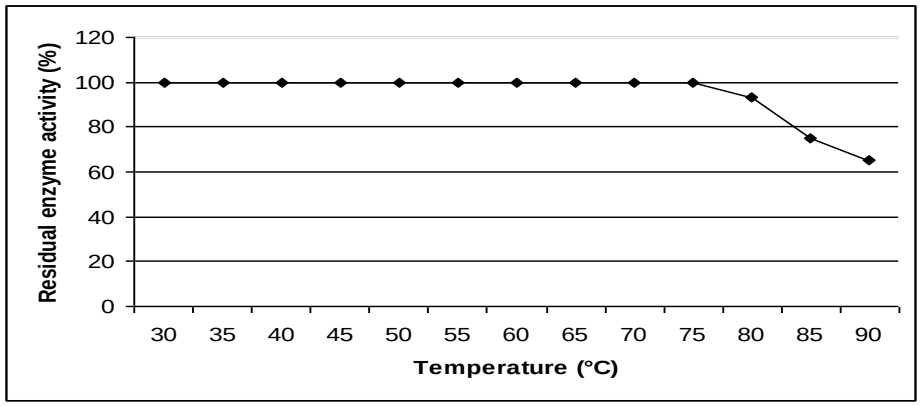

Fig. (9): Thermal stability of the enzyme.

This means that, this enzyme was thermostable chitinase, which showed higher stability against higher temperatures. Similar observations were recorded by Trachuk *et al.* (1996) and Sakia *et al.* (1998).

5- Effect of metal activators and inhibitors on enzyme activity:

Results in Table (5) reveal that metal ions (1mM) affected greatly on chitinase activity. The enzyme was stimulated by Ca^{+2} and Mg^{+2}, which they stimulated the enzyme activity with 35 and 22%, respectively. Other metal ions such as Ag^{+} and Hg^{+2} were inhibited the enzyme activity with 88 and 95%, respectively. Such inhibition may be due to the participation of a metal cation or in presence of cysteine sulfhydryl groups in the active site of this enzyme. EDTA did not influence chitinase activity. Similar observations were reported by Trachuk *et al.* (1996); Sakia *et al.* (1998) and Shady *et al.* (2000).

Table (5): Effect of some metal ions on enzyme activity.

Metal activator or inhibitor	Relative enzyme activity (%)
No addition	100
$MgCl_2$	122
$CaCl_2$	135
$MnSO_4.H_2O$	71
$ZnSO_4$	49
$CoCl_2$	67
$HgCl_2$	5
$AgNO_3$	12
NaCl	100
KCl	100
$CuSO_4$	91
EDTA	100

6- Substrate specificity of chitinase:

Bacillus stearothermophilus chitinase revealed comparable activity against colloidal chitin. It was only most active towards chitin and colloidal chitin, as well as colloidal chitosan and trisaccharide (Table, 6). The enzyme was shown to hydrolyze high molecular mass substrates and possess marginal activity against trisaccharides, but it was incapable of hydrolyzing the disaccharide to N-acetylglucoseamine, this is due to a lack of exochitinase activity and the three produced enzymes are indo-type chitinase which showed hydrolytic activities towards various high molecular mass substrates. Trachuk *et al.* (1996) and Sakia *et al.* (1998) reported similar observations.

Table (6): Substrate specificity of chitinase.

Substrate	Specificity of chitinase (ى mole of NAGA/min at 65°C)
Colloidal chitin	17.3
Chitin	19.0
Colloidal chitosan	14.4
N, N´, N´´ – Triacetyl chitotriose	3.2
N. N´ – Diacetylchitobiose	0.0

Enzyme applications:

1- Hydrolysis of cell wall of some pathogenic fungi:

Chitinase-producing bacteria can inhibit fungal growth, *e. g.,* plant-pathogenic fungi (Gomes *et al.,* 2000). One possible explanation for its inhibition is the action of chitinases and â-glucanases on chitin or glucan present in these fugal cell walls, acting as protective agents (Inbar & Chet, 1991 and Gomes *et al*., 2000). Therefore, results in Table (7) reveal that, culture supernatant containing *Bacillus stearothermophilus* chitinase as a lytic enzyme has antifungal activity against the cell walls of substrates tested (chitin containing substances) of the target fungi. The data also revealed that, this enzyme preparation was more active in degradation of cell walls, which resulted higher biodegradation of all three substrates at the end of incubation period (12 hours). But, this enzyme was more active against the cell wall of *Rhizoctonia solani* than other ones. This means that this enzyme preparations were the best suppression of these plant-pathogens as well as the best tool for biological control of many plant diseases. Similar results were obtained by Abdel-Fatah & Khella (1995); Singh *et al.* (1999) and Shady *et al.* (2000).

Table (7): Effect of *B. stearothermophilus* chitinase on specific fungal cell wall and colloidal chitin (3:1) on the hydrolysis of cell walls of different target fungi.

Cell wall substrates	Enzyme activity (ىg NAGA/ml)						
	1 h	2 h	3 h	4 h	5 h	6 h	12 h
Rhizoctonia solani	105	135	180	225	275	330	370
Aspergillus niger	90	120	170	215	270	310	355
Fusarium oxysporium	85	115	165	200	255	295	325

2- Prevent of fungal growth and its sporulation rates by chitinase preparations:

The addition of this enzyme preparation to the nutrient medium (potato-dextrose agar) decreased the growth rate as well as fugal sporulation rate of the three fungal colonies by 45 – 59% and 70 – 73%, respectively (Table, 8). This is may be due to its hydrolysis of cell walls (Table, 7), thus affecting greatly on fungal growth. This maximum antifungal activity was observed aganst *Fusarium oxyspoium* growth. From these results, this enzyme may be play an important role in the defence against parasitic fungi on higher plants, and prevent mould growth in stored vegetable materials. Similar to the inhibition of *Trichoderma viride* growth by plant chitinases and the inhibition of wheat fusariosis by *Streptomyces kurssonovii* chitinase. (Tatarinova *et al*., 1996; Singh *et al.,* 1999 and Shady *et al*., 2000).

Table (8): preventing effect of enzyme preparations on fungal growth and sporulation rates on PDA containing 0.1% *B. stearothermophilus* chitinase.

Target fungi	Growth rate (%)	Control	Sporulation (%)	Control
Rhizoctonia solani	48	100	27	100
Aspergillus niger	55	100	30	100
Fusarium oxysporium	41	100	29	100

REFERENCES

Abdel–Fatah, M. K. (1995). Studies on the Chitinolytic enzymes of *Streptomyces*: 1- culture conditions for the production of chitinase enjyme cellulosae chitin – degrading *Streptomyces* cellulosae (F-2) strain. Egypt. J. Microbiol., 30: 53-65.

Abdel–Fatah, M. K. and Y. E. Khella (1995). Studies on the chitinolytic enzymes of *Streptomyces*: Purification and characterization of chitinase enzyme from a chitin-degrading *Streptomyces cellulosae* (F-2). Egypt. J. Microbiol., 30: 67 – 83.

Bhushan, B. (2000). Production and characterization of a thermostable chitinase from a new alkalophilic *Bacillus* sp. BG- II. J. Appl. Microbiol., 88: 800 – 808.

Chen, J-P. and M-S. Lee (1995). Enhanced production of *Serratia marcescens* chitinases in PEGI dextran aqueous two – phase systems. Enz. Microbiol. technol., 17: 1021 – 1027.

El–Sawah, M.M.A.(1999). Can immobilization of *Paenibacillus pabuli* cells or its chitinases provide an effective method to its potential application in chitin degradation? Annals Agric. Sci., Ain shams Univ., Cairo, 44: 83 – 99.

Fenice, M.; J. L. Leuba and F. Federici (1998). Chitinolytic enzyme activity of *Penicillium janthinellum* Pg in Bench – Top Bioreactor. J. Ferment. Bioeng., 86: 620 – 623.

Fr◌ndberg, E. and J. Schnürer (1994). Chitinolytic properties of *Bacillus pabuli* KI. J. Appl. Bacteriol., 76: 361 – 367.

Gomes, R. C, L. T. A. S. Semedo; R. M. A. Soares; C. S. Alviano; L. F. Linhares and R. R. R. Coelho (2000). Chitinolytic activity of actinomycetes from a cerrado soil and their potential in biocontrol. Lett. Appl. Microbiol., 30: 146 – 150.

Gupta, R.; R. K. Saxena; P. Chaturvedi and J. S. Virdi (1995). Chitinase production by *Streptomyces virdificans*: its potential in fungal cell wall lysis. J. Appl. Bacteriol., 78: 378 – 383.

Inbar, J. and I. Chet (1991). Evidence that Chitinases produced by *Aeramonas cavie* involved in the biological central of soil – borne plant pathogens by this bacterium. Soil Biol. Biochem., 23: 973 – 978.

Lingappa, Y. and J. L. Lockwood (1961). A chitin medium for isolation, growth and maintenance of actinomycetes. Nature (London), 184: 158 – 165.

Lowry, O. H.; N. G. Rosebrough; A. L. Farr and R. J. Randall (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem., 193: 265 – 275.

Melent'ev, A. I. and G. E. Aktuganov (1999). Isolation, purification and characterization of chitinases from *Bacillus* sp. 739. Appl. Biochem. Microbiol., 35: 556 – 560.

Mohamedin, A. H. A. (1993). Studies on the biological activities of bacterid with particular reference to *Streptomyces*. Ph. D. Thesis, Fac. of Sci., Mansoura Univ., Egypt.

Pleban, S., L. Chernin and I. Chet (1997). Chitinolytic activity of an endophytic strain of *Bacillus cereus*. Lett. Appl. Microbiol., 25: 284 – 288.

Reissig, J. L.; J. L. Strommger and L. F. Leloir (1955). A modified colorimetric method for the estimation of N – acetylamino sugars. J. Biol. Chem., 27: 959 – 966.

Sakai, K.; A. Yokota; H. Kurokawa; M. Wakayama and M. Morhguchi (1998). Purification and characterization of three thermostable endochitinases of a noble *Bacillus* strain , M.H –I, islated from chitin-containing compost. Appl. Environ. Microbiol., 64: 3397 – 3402.

Shady,T. S. M.; M. M. A. EL-Sawah and M. A. Demerdash (2000). Some properties and factors affecting chitinase production by *Bacillus licheniformis* and its use as antifungal agent. J.Agric. sci .Masnsoura univ., 25: 5195 – 5209.

Shindia, A. A. (2001). Chitinases production by same thermotolerant and thermophilic fungi Isolated from composts. Egypt. J. Microbiol., 36: 39 – 57.

Shindia, A. A., M. I. Abou El Hawa and K. El. S. M. Shalaby (2001). Fungal degradation of shrimp shell chitin waste: I–Effects of culture conditions on the production of chitinase by a potent *Trichoderma viride.* Egypt. J. Microbiol., 36: 119 – 134.

Singh, P. P., Y. C. Shin, C. S. Park and Y. R. Chung (1999). Biological control of Fusarium wilt of Cucumber by Chitinalytic bacteria. Phytopoth., 89: 92 – 99.

Skujins, J. J., H. J. Pogieter and M. Alexander (1965). Dissolution of fungal walls by a Streptomycete – chitinase and glucanase. Archives Biochem. Biophys., 111: 358 – 364.

Suginta, W.; P. A. W. Robertson; B. Austin, S. C. Fry and L. A. Fothergill – Gilmore (2000). Chitinases from *Vibrio*: activity screening and purification of chiA from *Vibrio carchariae*. J. Appl. Microbiol., 89: 76 – 84.

Tatarinova, N. Y.; O. S. Grincheuko; V. P. Varlamoov; G. V. Paspekhov; I. S. Vasikova and V. G. Dzhavakhiya (1996). A complex of chitinolytic enzymes from *Streptomyces kurssanovii* and its use as treatment for *Fusarium culmorum*, wheat pathogen. Appl. Biochem. Microbiol., 32: 223 – 226.

Trachuk, L. A., L. P. Revina, T. M. Shemyakina; G. G. Chestukhina and V. M. Stepanov (1996). Chitinases of *Bacillus licheniformis* B.6839: isolation and properties. Con. J. Microbiol., 42: 307 – 315.

إنتاج وتنقيه ودراسة خواص إنزيم الكيتينيز الثابت حرارياً والمنتج من بكتريا الـ *Bacillus stearothermophilus* واستخدامه في منع نمو وتجرثم بعض الفطريات الممرضة للنبات

توفيق سعد محمد شادي- عبد المنعم عبد الله نصر- وسام الدين إسماعيل على صابر - خالد عبد الفتاح عبد القوي.

قسم الميكروبيولوجي- معهد بحوث الأراضي والمياه والبيئة- مركز البحوث الزراعية- الجيزة - مصر

نظراً لتعدد استخدامات إنزيمات الكيتينيز سواء في مجال المقاومة الحيوية أو الصناعات الدوائية أو لاستخداماتها في مجال الصناعات الغذائية والأعلاف الحيوانية، فقد تركزت الأبحاث في الآونة الأخيرة للاهتمام بإنتاج هذه الإنزيمات وبخاصة الميكروبية منها. ولذلك تركزت هذه الدراسة وتنقية ودراسة خصائص إنزيم الكيتينيز واستخدامه في مقاومة بعض الفطريات الممرضة للنبات وقد انتهت الدراسة للنتائج التالية:-

١- تم الحصول على أعلى نشاط للإنزيم وأعلى تركيز للخلايا في اليوم الرابع من النمو على بيئة محتوية على ١,٥% كيتين أو كيتين + أرابينوز أوكربوكس ميثيل سليلوز مع مستخلص الخميرة كمصدر للنيتروجين لأن هذه العوامل الغذائية تحث على الإنتاج العالي من الإنزيم في بيئة النمو.

٢- هز المزارع أثناء عملية التخمير لها تأثير حثي عالي على إنتاج الإنزيم.

٣- درجة الـpH ٨ ,٥٥ م° هما أنسب الظروف البيئية للإنتاج العالي من الإنزيم.

٤- استخدام درجة ٦٥ % تشبع من كبريتات الأمونيوم أدى إلى ترسيب الإنزيم بكفاءة عالية حيث أعطي أعلى نشاط نوعي للإنزيم وتنقيه الإنزيم باستخدام DEAE-sephadex A-50 أعطي معدل عالي من التنقية ونشاط نوعي عالي جداً من الإنزيم وبعمل تفريدة لبروتين الإنزيم الناتج من الخطوة السابقة أظهر أن الإنزيم عبارة عن نظام يحتوى علي ٣ أنواع من الإنزيم تم تعريفها علي أنها من النوع – endo chitinase.

٥- كانت درجه الـ pH ٦ ,٦٥ م° هما المثاليين لنشاط الإنزيم.

٦- أظهر الإنزيم درجة ثبات عالية تجاه درجات الـ pH في المدى من ٥-٨ في حين أنه احتفظ بكامل نشاطه عند درجه pH ٦ وهذا يعني أن بروتين الإنزيم ذو طبيعة حامضية في نشاطه.

٧- أظهر الإنزيم أيضاً ثبات كامل تجاه الحرارة حتى ٧٥م° ثم نقص نشاطه بمعدل قليل حتي ٨٥م° وهذا يعني أن الإنزيم من النوع الثالث حرارياً.

٨- كان لبعض المعادن مثل الماغنيسيوم والكالسيوم تأثير حثي كبير على نشاط الإنزيم حيث تم تنشيط سطح البروتين الإنزيمي الداخل في التفاعل مع مادة تفاعله في حين كان للبعض الآخر مثل أيون الفضة والزئبقيك تأثير تثبيطي كبير لنشاط الإنزيم.

٩- كان المستحضر الإنزيمي المفصول من هذا الميكروب درجة نجاح عالية في تحليل مواد تفاعل مختلفة ولكن بدرجات متفاوتة مثل الكيتين والكيتين الغروي والكيتوزان الغروي في حين أن الإنزيم لم ينجح في تحليل الداي أوليجوميرز مما يعني أن هناك تخصص عالي لهذا الإنزيم تجاه مواد التفاعل ذات الوزن الجزيئي العالي.

١٠- أيضا نجح الإنزيم في تحليل مستحضر الجدر الخلوية لبعض الفطريات الممرضة للنبات وبدرجه تحلل عالية فضلاً عن أنة نجح في منع نمو هذه الفطريات الممرضة بدرجة كبيرة جداً ومنعها من عملية التجرثم أي أنه يستطيع القضاء عليها مما يعنى أهميته في المقاومة الحيوية ومنع نمو الفطريات الممرضة باستخدامه كماده مضادة للفطريات.

وفي النهاية توصي الدراسة بإمكانية استخدام المستحضر الإنزيمي لهذا الميكروب والمحتوي علي إنزيمات الكيتينيز في عمليات المقاومة الحيوية سواء للفطريات الممرضة أو الحشرات وغيرها من الكائنات المحتوية علي الكيتين وذلك من أجل القضاء عليها بالنظم البيولوجية أو الحيوية صديقة البيئة والتي ليس لها آثار ضارة علي صحة الحيوان أو الإنسان.