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Purification and Properties of Chitinase Enzyme Produced by *Bacillus licheniformis*

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Partial purification of the crude chitinase obtained from culture filtrate of *Bacillus licheniformis* was carried out by fractional precipitation with ammonium sulphate, acetone, and ethanol. Ethanol (50-75%) showed the highest recovered protein and chitinase activity. Further purification of ethanol fraction, was achieved by gel filtration chromatography on Sephadex G-100, 2 protein peaks were obtained. The second peak showed a high recovered activity and was considered as the pure chitinase enzyme. Also, properties of the purified chitinase enzyme obtained from *Bacillus licheniformis* cultures were studied. The results showed that the optimum enzyme and substrate concentrations were 0.4 mg enzyme/reaction mixture and 0.6 mg chitin/reaction mixture, respectively. The optimum reaction temperature was 37ºC, and the pure enzyme showed a maximum activity at a reaction pH 7.0. In addition, the activity of immobilized chitinase enzyme by adsorption on different immobilization materials was studied. Carboxymethyl cellulose showed a higher enzyme activity (1.524 U/ml) as compared to the other immobilization materials, however, it was lower than the free enzyme activity. By reusing the immobilized enzyme adsorbed on carboxymethylcellulose, the chitinase activity increased till the 3rd run and reached 1.6 U/ml, and showed a gradual decrease up to the 6th run at which the adsorbed enzyme showed the lowest enzyme activity. In conclusion, chitinase enzyme obtained from *Bacillus licheniformis* may be a good candidate for application in different biotechnological fields especially in bioremediation of chitin wastes, the production of protoplasts of algal cells, and bio-control of fungal phytopathogens.

Key words: *Bacillus lichenifomis*; chitinase; purification; immobilized enzyme.

Chitinases are a group of hydrolytic enzymes that catalyze depolymerization of chitin. Chitinases occur in various organisms and play important physiological and ecological roles. Chitinases (EC 3.2.1.14) cleave the β-1,4-glycosidic bonds of chitin and are capable of hydrolyzing chitin to its oligomers and monomer, *N*-acetyl-[2]-Dglucosamine GlcNAc. Based on amino acid sequences of glycosyl hydrolases, chitinases and *N*-acetyl-hexosaminidases were grouped into three families – 18, 19 and 20. The families 18 and 19 comprised endochitinases from different sources such as viruses, bacteria, fungi, insects and plants[1].

Bacteria have a large role in chitin degradation but not all species are able to hydrolyze chitin. Bacteria have been shown to produce extracellular chitinases. Bacteria which can produce chitinases include Gram-positive bacteria of the genus *Arthrobacter, Bacillus, Clostridium, Nocardia,* and *Steptomyces*, and Gram-negative *Aeromonas, Chromobacterium, Photobacterium,*

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Psuedomonas, Serratia, and *Vibrio*. Also, chitinase production was reported in different species of *Bacillus* such as *B. amyl-oliquefaciens*[2], *B. cereus*[3], *B. circulans*[4], and *B. licheniformis*[5]. The distribution of chitinase was studied in 29 *Bacillus* species and 10 species were found to produce endochitinase, including *B. alvei, B. chitinospours, B .licheniformis*, and *B. pulvifaciens*, while 15 species produced exochitinase, including *B. circulans* and *Serratia marcescens*. When an exochitinase from *Serratia marcescens* was expressed in *E. coli*, it was secreted into the periplasm[6]. This chitinase has a high specific activity. Because of its stability, wider pH optimum and linear kinetics over a wide range than many other chitinases, the *S. marcescens* chitinase has been recommended for analytical use. For these reasons, it is also a good candidate for the large scale degradation of chitin for industrial purposes.

Purification and characterization of chitinase producing bacteria is important due to the microbial chitinases application in agricultural and pollution control. Purification process is mean by separation of interest protein from mixture of protein while maintain its biological function. This can be conduct by controlling the pH, temperature, and ionic strength of the interest enzyme. All purification method is designed based on molecular size, electrostatic properties, and relative solubility in salts until purification homogeneity achieved[7].

For biotechnological interests, in addition to the potential applications of chitinase itself, the chito-oligosaccharides [(GlcNAc)*n*] have been found to function as antibacterial agents, elicitors, lyzozyme inducers, immuno-enhancers[8], and chitinase inhibitors are used to inhibit growth of chitin-containing plant- pathogens and plague insects that need chitinases for normal development[9]. Chitinases are reported to dissolve cell walls of various fungi, a property that has been used for the generation of fungal protoplasts. Chitinase-producing organisms are effectively used in the bioconversion process to treat shellfish waste and also to obtain value-added products from such wastes[10].

MATERIALS AND METHODS

Samples

The shrimp-and crab shell powder used in these experiments was prepared in the laboratory. The shrimp and crab shells collected from the marine food processing industry were washed with tap water and raw shrimp wastes were dried under sun for several days and then dried in oven at 70°C for 3 to 4 days. Then, the dried shrimp shell was blended using blender. The solid material so obtained was dried milled, and sieved to powder.

Microorganisms

In the present study, six bacterial strains showing chitinolytic activity were isolated from sea water and sediment samples collected from Alexandria coasts at Abou-Qir Bay and EL-Shatby beach. The bacterial strain which was used in the present experiments was identified in the Center for Identification of Bacteria and Yeasts, Elazhar University, Cairo. The isolated strain was identified using different characteristic according to Bergye's Manual of Determinative Bacteriology[11].

Culture media

A chitin medium was used as an initial growth medium for the purpose of observing colony morphology and developing a pure culture and also for chitinase production. The chitin agar medium was composed of (g/L): Chitin, 20; $(NH4)_2SO_4$, 1; KH_2PO_4, 0.5; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.5; $FeCL_3.6H_2O$, 400 ML; $CaCL_2.2H_2O$, 400; $ZnHSO_4.7H_2O$, 400 ML; agar, 20. An optimized culture medium (50 ml medium) in 250 ml Erlenmeyer flasks was inoculated with 4 ml bacterial suspension. The flasks were incubated at 37°C for 48 h. The pH value was initially adjusted to 7.0, and the flasks were incubated under static conditions.

Preparation of the crude enzyme

The bacterial culture was centrifuged at 6000 rpm for 20 min in a cooling centrifuge (Chilspin, made in England) at 4ºC. The clear supernatant was assayed for enzymatic activity. The samples were considered as the source of the crude enzyme. The protein content of the enzyme preparations was determined as previously described[12]. Chitinase activity was determined according as previously described[13] using colloidal chitin which was prepared as described before[14].

Partial purification of the enzyme

This was achieved by salting out with ammonium sulphate and precipitation and by fractional precipitation with acetone and ethanol. The crude culture supernatant grown under

optimal conditions was precipitated at different concentrations of each precipitant in a sequential manner.

Fractionation by salting-out with ammonium sulphate

A certain volume of the crude enzyme was collected from the cultivation medium. The Protein content of the enzyme solution was exactly measured as well as its activity. The whole enzyme solution was then kept for about 30 min in an ice bath. Fractional Precipitation then began after adding solid ammonium sulphate slowly while stirring to the ice-cold enzyme solution until the required saturation of ammonium sulphate was reached. The solution was left for 2 h and rotated for 15 min at 6000 rpm in a refrigerated centrifuge. Further ammonium sulphate was added to the supernatant fluid and the process repeated until the desired final saturation of ammonium sulphate reached 90%. Several enzyme fractions were thus obtained at 25, 50, 70 and 90% saturation of ammonium sulphate. Each precipitate was dissolved in certain Amount of distilled water and dialyzed against Distilled water in a refrigerator until the water outside the dialyzing bag gave no precipitate with 1% calcium chloride solution indicating that the enzyme solution became free of sulphate. This was achieved by changing the water outside the dialyzing bag several times. After complete dialysis, the protein content and enzyme activities of each fraction were determined.

Fractional precipitation with acetone

Acetone was cooled at 4°C one day before starting this experiment. A certain amount of the crude enzyme was rotated in a refrigerated centrifuge to remove the muddy matter. The protein content and enzyme activity of the clear enzyme solution were determined and the solution was then kept in an ice-salt bath for 30 min. Fractional precipitation then began after adding slowly while stirring a certain volume of cold acetone to the ice-salt to the ice-salt cold enzyme solution until the required concentration of acetone was reached. After removing the precipitated fraction by refrigerated centrifugation at 6000 rpm for 20 min, further acetone was added to the supernatant fluid and the process repeated for supernatant fluid, and the process repeated until the desired final concentration of acetone was reached. Several enzyme fractions were thus obtained at 25, 50, 75 and 90% concentration of acetone. The Protein content and enzyme activity of each fraction were determined.

Fractional precipitation with ethanol

Absolute ethanol was used and cooled at 4°C one day before starting this experiment. A certain amount of the crude enzyme was rotated in a refrigerated centrifuge to remove the muddy matter. The protein content and enzyme activity of the clear enzyme solution were determined and the solution was then kept in an ice- salt bath for 30 min. Fractional Precipitation then began after adding slowly while stirring a certain volume of cold absolute ethanol to the ice-salt to the ice-salt cold enzyme solution until the required concentration of ethanol was reached . After removing the precipitated fraction by refrigerated centrifugation at 6000 rpm for 20 min, further ethanol was added to the supernatant fluid and the process repeated until the desired final concentration of ethanol was reached. Several enzyme fractions were thus obtained at 35, 50, 75 and 90% concentration of ethanol. The protein content and enzyme activity of each fraction were determined.

Column chromatography

Gel filtration, sample loading and elution

The primary objective of gel filtration is to achieve rapid separation of molecules based on size[15]. The enzyme preparation to be separated was dissolved in 0.05 M phosphate buffer pH 7.0, and loaded into the column pre-equilibrated with the same buffer. The non-adsorbed proteins were eluted with phosphate buffer, pH 7. The elution of adsorbed proteins was carried out using a 200 ml linear gradient 0 to 1 M of NaCl in phosphate buffer, pH 7.0. The flow rate was adjusted to 1.0 ml/min, and 5 ml fraction were collected using a fraction collector and a peristaltic pump at a flow rate of 40 ml/h. the protein content of each fraction and the enzyme activity were determined by the methods indicated before[12,13].

SDS-Polyacrylamide gel electrophoresis

The method was carried out according to the method described previously by Laemmli and Fever[16].

Properties of the purified chitinase enzyme

The pure enzyme was tested in a reaction containing different concentrations of enzyme. Every enzyme solution was incubated for 1 h at

45ºC. Controls were made at each experiment concentration using heat denaturated enzyme**.** Also, the effect of substrate concentration on the activity of the purified chitinase enzyme was studied using different concentration of chitin ranging from 0.1 to 0.9 mg/ml reaction mixture which incorporated 0.4 mg enzyme protein and was adjusted to pH 0.7 with a phosphate buffer (0.05 M). In addition, the effect of temperature of the reaction on the activity of the purified enzyme was investigated. The enzymatic reaction was carried out for 1 h at 30, 35, 37, 40, 50, 60 and 70ºC using an enzyme protein and substrate concentration 0.4 mg and 0.5 mg respectively per ml reaction mixture. Besides, the influence of pH of the purified chitinase enzyme was determined. The activity was measured at under a standard assay condition with the following buffer system: 0.01 M sodium acetate buffer, pH 4.0 to 6.0; 0.01 M sodium phosphate buffer, pH 7.0 to 9.0. Moreover, the effect of incubation period of the reaction on the activity of the purified chitinase enzyme was studied. The enzymatic reaction was carried for different times 15, 30, 45, 60, 72, and 90 min at 50°C using an enzyme protein and substrate concentration of 0.4 mg and 0.5 mg per reaction mixture, respectively.

Adsorption of purified chitinase on different supports

Different supports were used for simple adsorption of the purified chitinase without the addition of any cross-linking agent. This was fulfilled by taking 200 mg (dry basis) of each support was stirred in 0.1 M acetate buffer pH 5.2 in a refrigerator for 6 h, with 5 mg of the purified enzyme protein in a total volume 5 ml. the supports were then removed by centrifugation at 6000 rpm for 10 min in a refrigerated centrifuge, washed well with a small amount of distilled water to remove free enzyme, and finally protein adsorbed was calculated by the difference of protein taken for immobilization and the protein left in combined supernatant liquor and washing. For measuring the enzyme activity of immobilized enzyme about 50 mg loaded support were added to the reaction mixture.

RESULTS

Purification of chitinase produced by *Bacillus licheniformis*

Purification and characterization of the chitinase enzyme produced in *B*acillus *licheniformis* cultur is done. Combined culture filtrates containing the crude enzyme were at first partially purified by fractional precipitation with different agents. The different precipitated fractions were tested for their protein content and chitinase activity. The fraction showing the highest specific

Table 1. Fractional precipitation of chitinase from *Bacillus licheniformis* cultures with ammonium sulphate, acetone, and ethanol

		Protein content (mg/ml)	Recovered protein (%)	Chitinase activity (U/mg protein)	Recovered activity (%)
Culture filtrate		120	100	105.2	100
Ammonium	25	3.8	3.16	3.75	3.56
sulphate	50	4.75	3.95	5.6	5.32
saturation	75	9	7.5	11.25	10.7
(%)	90	8.75	7.29	8.2	7.8
	Total	26.3	21.9	28.8	27.38
Acetone	25	3.55	2.96	4.4	4.18
Concentration	50	8.7	7.25	8.25	7.84
(%)	75	12.3	10.25	15.75	14.97
	90	11.25	9.38	11.05	10.5
	Total	35.8	29.84	39.45	37.49
Ethanol	25	6.25	5.2	11.25	10.7
Concentration	50	9.4	7.83	17.3	16.4
(%)	75	15.2	12.6	29.5	28.04
	90	10.8	9	15.3	14.54
	Total	41.65	34.63	73.35	69.7

Table 2. Purification steps of chitinase enzyme produced in *Bacillus licheniformis* cultures

Purification step	Protein content (mg)	Chitinase activity (U/mg)	Specific activity (U/mg)	Purification (field)
Culture filtrate (crude enzyme)	120	1o5.2	0.87	1
Ethyl alcohol precipitation (75%)	15.2	29.5	1.94	2.23
Sephadex G-100	2.11	7.77	3.68	4.23

Table 3. Effect of immobilization of chitinase enzyme produced by *Bacillus licheniformis*

Immobilization material	Protein content (mg/ml)	Chitinase activity (U/ml)
Free enzyme	1.63	1.797
Carboxymethylcellulose	1.224	1.524
Tricalcium phosphate	1.075	1.315
Active carbon	0.955	1.043
Silica gel	1.12	1.44
Starch	0.776	0.962

chitinase activity was further purified by gel filtration and some characters of the purified enzyme were studied.

Partial purification

Several protein fractions were obtained by adding different concentrations of ammonium sulphate, acetone, and ethanol, separately to a certain volume of the clear crude enzyme solution. The protein content and chitinase activity of each fraction were determined.

Fractional precipitation with ammonium sulphate

Table 1 indicates that fractional precipitation with ammonium sulphate brought about 21.33% of total protein. The recovered protein showed the highest peak value at the fraction precipitation with 75% ammonium sulphate saturation. This fraction represented about 34.24% of the total recovered protein. The activity of this fraction was about 39.04% of the total recovered activity. Higher or lower ammonium sulphate saturations showed lower chitinase activity. The total recovered activity obtained by ammonium sulphate fractions was 27.38% of the activity present in the crude enzyme solution.

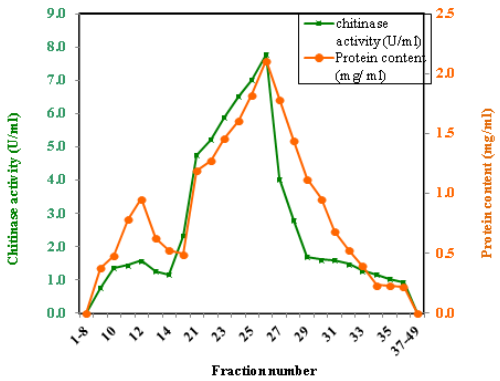

Fig. 1. Gel filteration in Sephadex G-100 of the partially purified chitinase preparation (75% ethanol fraction) obtained from the culture filtrate of *Bacillus Licheniformis*

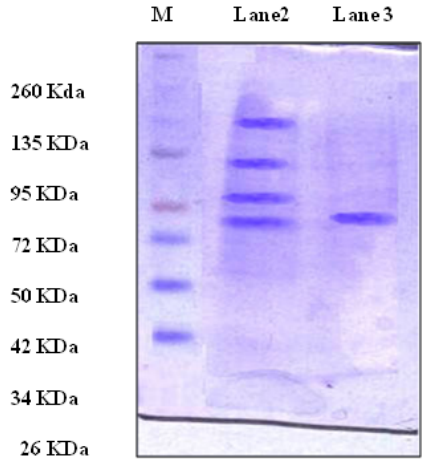

Fig. 2. SDS PAGE of chitinase purification from *Bacillus licheniformis*, lane 1: the molecular protein weight markers (KDa), lane 2: 75% ethyl alchol precipitate, lane3: purified enzyme

Fractional precipitation with acetone

Table 1 shows that fractional precipitation with acetone brought about 29.8 % of total protein. The highest peak value for recovered protein was observed at 75% acetone saturation. This fraction represented about 34.35% of the total recovered protein. The highest enzyme activity was also observed at 75% fraction. It represented about 39.3% of the total recovered activity. The total recovered activity obtained by acetone fractions was 37.5% of the activity present in the crude enzyme solution.

Fractional precipitation with ethanol

The results recorded in Table 1 indicate that fractional precipitation with ethanol brought about 34.6% of total protein. The recovered protein showed the highest peak value at the fraction precipitation with 75% ethanol concentration. This fraction represented about 36.44% of the total recovered protein. All other fractions showed a relatively lower protein recovery. The chitinase activity of the fraction precipitated with different ethanol concentration increased gradually up to 75% fraction where the maximum activity was obtained. The activity of this fraction was about 40.23% of the total recovered activity. Higher or lower ethanol concentration showed lower chitinase activity. The total recovered activity obtained by ethanol fractions was about 69.7% of the activity present in the crude enzyme solution.

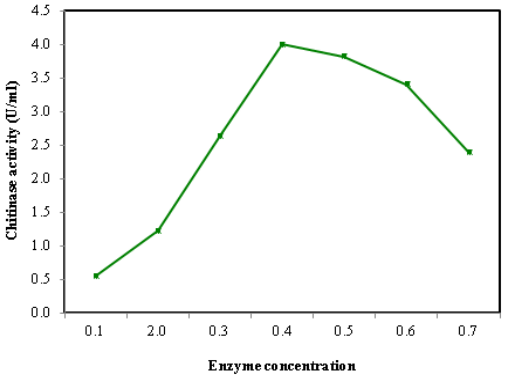

Fig. 3. Effect of enzyme concentration on the activity of the purified chitinase produced by *Bacillus licheniformis*

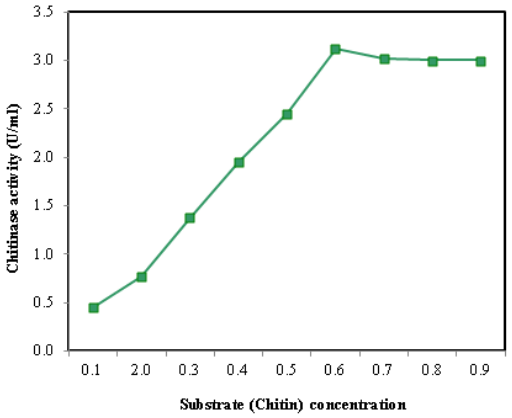

Fig. 4. Effect of substrate concentration on the activity of the purified chitinase produced by *Bacillus licheniformis*

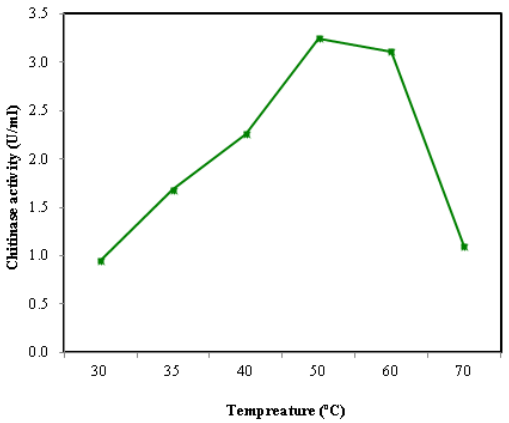

Fig. 5. Effect of reaction temperature on the activity of purified chitinase produced by *Bacillus licheniformis*

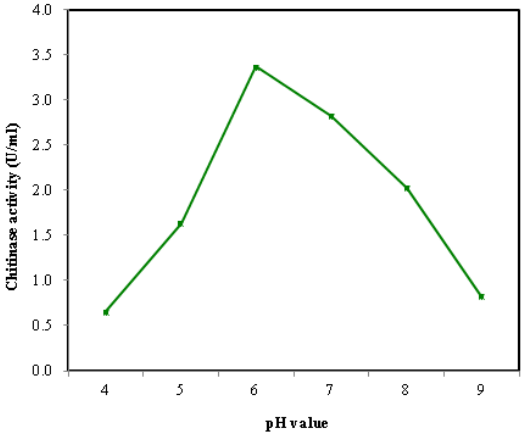

Fig. 6. Effect of pH of the reaction on the activity of the purified chitinase produced by *Bacillus licheniformis*

The total recovered activity by ethanol fractionation was the highest compared to the other agents, it reached about 69.7% of the activity present in the crude enzyme solution. According to the previous results, it was shown that among 12 fractions obtained with all the tested agents, the 75% ethanol fraction contained the highest chitinase activity. Therefore, it was selected as the chitinolytic enzyme fraction for further purification.

Column chromatography of the partially purified chitinase enzyme

In the preceding part of the work, fractional precipitation of the crude enzyme obtained from the culture filtrates of *Bacillus licheniformis* was performed. It has been found that ethyl alcohol is the most suitable agent and provided a fraction containing the highest chitinase activity. The results shown in Figure 1 indicated that the gel filtration of the partially purified chitinase yielded 49 fractions. The protein recovered by the obtained fractions reached about 117.24% of the applied sample. The total chitinase activity recovered from the Sephadex G-100 column represented about 74.08% of the original activity. It was noticed that the protein was separated into 2 components in the column as one major and the other one was minor. The minor protein peak was covered by fractions 9 to 16, and represented about 17.84% to the total recovered protein of the column. The second protein peak was a major one and was covered by fractions 17 to 34. The protein recovered by this peak represented about 80.30% of the total protein recovered from the column. The chitinase activity was also fractionated in the Sephadex column into 2 peaks. The first peak was a minor one and was present with the first protein component. Fraction numbers 9 to16 and the activity recovered by this peak represented about 11.78% of the total chitinase activity recovered from the column. The second chitinase peak was a major one and was covered by fractions 17 to 29. The activity recovered by this peak represented about 86.53% of the total chitinase activity recovered from the column. The purification steps of the chitinase were summarized in the Table 2.

SDS-Polyacrylamide gel electrophoresis

Chitinolytic active fractions thus collected during gel filtration were concentrated and analyzed by SDS-PAGE (12%) for the determination of molecular weight of chitinase as shown in Figure 2. SDS-PAGE analysis of the purified enzyme showed mainly one protein band in the molecular mass of 45 KDa.

Properties of the purified chitinase enzyme

Figure 3 shows that the maximum chitinase activity (4.0 U/mg) was obtained at an enzyme concentration of 0.4 mg/reaction mixture. Higher or lower enzyme concentration showed a decrease in the enzymatic activity. Therefore, this enzyme concentration was concerned in the next experiments. Also, Figure 4 shows that the maximum chitinase activity (3.12 U/mg) was obtained at an enzyme protein concentration of (0.6 mg/reaction mixture). Further increase of the substrate concentration yielded slightly lower enzyme activity. According to the previous results, a

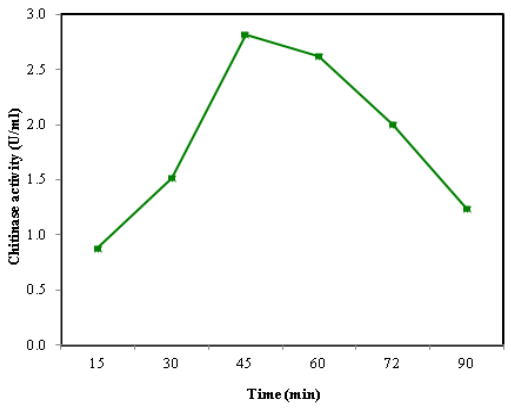

Fig. 7. Effect of the time of the reaction on the activity of the purified chitinase produced by *Bacillus licheniformis*

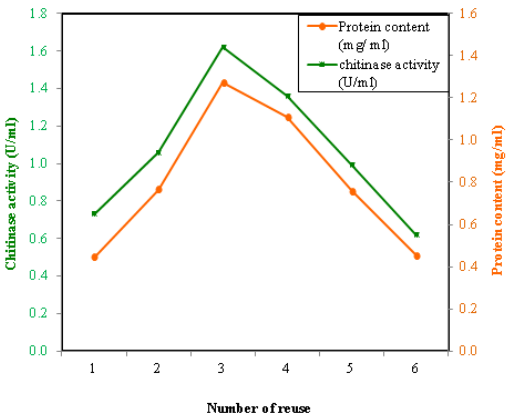

Fig. 8. Semi-continuous production of chitinase by *Bacillus licheniformis* cells adsorbed on carboxymethylcellulose

substrate (chitin) concentration 0.6 mg/reaction mixture was used in the next experiment. In addition, Figure 5 demonstrates that the chitinase activity increased gradually by increasing the reaction temperature .where the maximum chitinase activity was obtained 3.25 U/mg at 50ºC. Further rise of temperature resulted in a gradual decrease in activity. Therefore, in the next experiment, the temperature of the reaction was adjusted at 50ºC. Moreover, Figure 6 shows that the optimum pH value for the pure enzyme was 6 where the highest specific activity was obtained (3.37 U/mg protein). Higher or lower pH values showed a minor effect on the activity and the lowest activity was obtained at pH 4. However, the enzyme showed a relatively high activity in a pH range from 6.0 to 7.0. Besides, Figure 7 shows that an increase in chitinase activity accompanied with an increase in time of incubation up to 45 min. at which the activity of pure enzyme yielded its highest value (2.82 U/ml). Further increase of the incubation period showed a gradual decrease of the activity.

Effect of immobilization of chitinase enzyme

Carboxymethylcellulose showed a maximum binding of the chitinase and the results given in Table 3 indicate that the maximum chitinase activity for the pure enzyme was (1.524 U/ml), which is lower than the free enzyme activity.

Semi-continuous activity of purified chitinase adsorbed on carboxymethylcellulose

Figure 8 demonstrates that by reusing the enzyme adsorbed on carboxymethylcellulose, chitinase activity increased till the 3rd run and reached 1.6 U/ml. Then, the reused enzyme showed gradual decreases up to the 6th run where the adsorbed enzyme showed lowest activity.

DISCUSSION

Recently, chitinases have gained interest in different biotechnological applications due to their ability to degrade chitin in the fungal cell wall and insect exoskeleton, leading to their use as antimicrobial or insecticidal agents[17,18]. Another interesting application of chitinase is for bioconversion of chitin, a cheap biomaterial, into pharmacological active products, namely *N*-acetylglucosamine and chito-oligosaccharides[19,20]. Production of chitin derivatives with suitable enzymes is more appropriate for sustaining the environment than using chemical reactions. Other interesting applications include the preparation of protoplasts from filamentous fungi[21], bio-control of insects and mosquitoes as well as the production of single cell protein. Thus, there have been many reports on expression and characterization of chitinases from various organisms, including bacteria, fungi, plant and animals[22].

Partial purification of the crude chitinase enzyme produced by *Bacillus lic*heniformis was fulfilled by fractional precipitation with ammonium sulphate, acetone and ethyl alcohol. A total of 31 fractions were obtained, and the highest recovered protein was present in the fractions precipitated with ethyl alcohol yielding a total of (36.44%), followed by acetone (34.35%) and ammonium sulphate (34.2%). The highest total recovered activity was also obtained by ethyl alcohol (40.23%) followed by acetone (39.3%) and ammonium sulphate (39.04%). Some of the obtained fractions showed a specific activity higher than that of the crude enzyme indicating the presence of purification. Among all the obtained fractions, the 75% ethyl alcohol fraction showed the highest chitinase activity, protein recovery. Therefore, this fraction was selected for further purification.

Further purification of the 75% ethyl alcohol preparation was carried out by subjecting the partially purified enzyme to gel filtration on Sephadex G-100. The protein was separated to 2 peaks. The second peak was a major one and contained most of the recovered enzyme activity from the column, while the 1st peak showed a much lower recovered activity. These results indicate that more than one chitinase component were present in the partially purified sample. The activity recovered by the major peak represented about 86.53% of the total chitinase activity recovered from the column.

The properties of the pure chitinase isolated from *Bacillus licheniformis* cultures were studied. The optimum enzyme concentration was 0.4 mg/reaction mixture. It was also showed that the optimum substrate concentration was 0.6 mg/ reaction mixture. The optimum pH value for having a maximum chitinase activity was obtained at 6.0. Besides, the enzyme showed a relatively high activity in a pH range from 6.0 to 7.0. These results are compatible to those of chitinase purified from other microorganisms. In a previous study,

chitinase from marine *Psudomonas aeruginos*-like bacterium had a high activity at a pH 7.0[23], while another study revealed that ChiA of *Streptomyces marcescens* has a broad pH optimum around 5.0 to 6.0[24]. Taken together, it appears that chitinases from different sources are active within a broad pH range. Also, the activity of the enzyme increased by increasing the reaction temperature and reached a maximum activity value at 50ºC. The enzyme showed also a good activity at 37ºC. It could be assumed that the purified chitinase enzyme of *Bacillus licheniformis* has a temperature range from 37-50ºC. Similarly, it was previously reported that the optimum temperature for the activity of chitinase produced by *Aeromonas* was 50ºC[25]. Moreover, the activity of the purified enzyme of *Bacillus licheniformis* was increased by increasing the time of the reaction, reaching a maximum value after 45 to 60 min. Longer incubation periods of the enzyme reaction showed an adverse effect on the activity. This may be attributed to a partial inhibition of the chitinase enzyme by the products of the reaction at certain concentration.

Experiment of immobilization of the purified enzyme using entrapment technique for chitinase production from *Bacillus licheniformis* using different supports was examined. Carboxymethylcellulose (CMC) was the best one for entrapping the bacterial cells. It gave the highest enzyme activity (1.524 U/ml). The immobilization by entrapment using CMC as the support material was slightly lower than of free cell cultures. In addition, the results of the activity of purified enzyme by entrapment were studied in semi-continuous batch cultures. The CMC support material was reused for 6 successive cycles. Reuse of the enzyme adsorbed on CMC enhanced the enzyme in the third reuse, and a higher activity value was recorded. However, a gradual decrease was observed in the next reuse.

Studies on chitinolytic microorganisms have yielded a large increase in knowledge regarding their role in inhibition of growth of fungal plant pathogens. Still this knowledge is not sufficient enough to formulate a preparation based on these agents that can work efficiently in all different environmental conditions. The bio-control agents are also affected due to the geological and environmental conditions. Studying the environmental conditions of the area where biological control agent has been employed and statistical based formulation approach techniques will increase life span of microorganisms in different ecological conditions. Moreover, extensive studies are required on the maximum utilization of chitinous wastes for production of chitinases and biomass.

Therefore, chitinases, the hydrolytic enzymes that specifically degrade chitin, are gaining much attention worldwide, not only because of its wide spectrum of applications but also for the lacuna of an effective production method[26-29]. The present study revealed the presence of a relatively high chitinase activity in the culture supernatant of *Bacillus licheniformis*. This organism was isolated from a marine sediment. There were few reports on the production of chitinase from *Bacillus licheniformis*. Cell immobilization by adsorption gave also some preliminary information for the production of the enzyme by such methods to obtain better results. Purification and characterization of the enzyme showed the good microbial chitinase preparation and its relatively wide range of pH and temperature of the reaction.

CONCLUSIONS

The results of the present study indicate that the chitinase enzyme obtained from *Bacillus licheniformis* may represent a feasible candidate for application in different biotechnological fields especially in bioremediation of chitin wastes, the production of protoplasts of algal cells, bio-control of fungal phytopathogens. The enzyme may be also useful in food industry and medical applications.

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