Immobilization and Solid-State Fermentation Methods for Chitinase Production from *Bacillus licheniformis*

Mervat F. El-Sherif [1], Amany S. Youssef [2], Maha A. Hassan [1], Hanim M.G. Hassan [1], Samy A. El-Aassar [2]

[1.] Biological and Geological Department, Faculty of Education, Alexandria University, Alexandria, Egypt
[2.] Botany and Microbiology Department, Faculty of Science, Alexandria University, Alexandria, Egypt
amanyyoussef70@yahoo.com

Abstract: The aim of this work was to study the ability of locally isolated bacterial strains to utilize chitin as a marine polysaccharide to produce chitinase enzyme. *Bacillus licheniformis* produced the highest chitinase activity (1.052 U/ml) under static conditions. Also, *B. liceniformis* cells adsorbed on clay particles showed a higher enzyme activity (3.63 U/ml) when compared to that obtained from free cell cultures. In addition, the results showed that by reusing cells adsorbed on clay particles the chitinase production increased till the 2nd run and reached (4.25 U/ml), then the reused cultures showed gradual decreases up to 6th run were the adsorbed cells showed the lowest chitinase activity. Moreover, *B. lichenifomis* cells immobilized in agar 2% concentration produced the highest chitinase activity (1.25 U/ml) when compared with free cells. The results revealed that the reuse of cells entrapped in 10 ml gel-cell cubes increased chitinase production till the 3rd run (1.52 U/ml) which was higher than that of batch fermentation culture by free cell, and then decreased gradually till the 5th run. Also, *B. licheniformis* cells were fermented on different solid subsrates. Fermentation by using crab as a solid substrate showed the highest chitinase production (1.85 U/ml). The results of the present investigation collectively indicate the possibility of using *B. licheniformis* cells for the production of a cheap and highly active chitinase preparation which ca be used in many industrial and agricultural purposes.

[El-Sherif MF, Youssef AS, Hassan MA, Hassan HMG, El-Assar SA. **Immobilization and Solid-State Fermentation Methods for Chitinase Production from *Bacillus licheniformis*.** *Life Sci J* 2013;10(4):3036-3043]. (ISSN:1097-8135). http://www.lifesciencesite.com. 404

Keywords: Chitinase; *Bacillus licheniformis*; immobilization; solid-state fermentation

1. Introduction

Chitin, an insoluble linear polymer of *N*-acetyl-D-glucosamine (GlcNAc), is the second most abundant biopolymer in nature after cellulose (Duo-Chuan, 2006). Chitin degradation products, either in the form of oligomer or monomer, have received increasing attention because of their broad applications in the fields of medicine, agriculture, food, pharmaceutical and biotechnology. Chitin oligosaccharides are known to have activities as elicitors, antimicrobial and antifungal agents (Wang et al., 2008), immunoinhancers (Aam et al., 2010; Muzzarelli, 2010), and antitumor activity (Faramarzi et al., 2009; Shen et al., 2009). The monomers, GlcNAc and D-glucosamine (GlcN) are amino sugars having therapeutic potential for the treatment of osteoarthritis (Tamai et al., 2003), inflammatory bowel disease (Salvatore et al., 2000). The accumulation of the chitin waste in both marine environments seems to be a serious challenge for most of shellfish-producing countries. The utilization of this waste for the purpose of chitinase production seems to be of highly considerable interest.

Bioconversion of chitinous materials has been proposed as a waste treatment alternative to the disposal of shellfish wastes. In details, shrimp waste contains 10-20% calcium, 30-40% protein, and 8-10% chitin throughout the tropical region (Legaretta et al., 2000). It was reported that chitin and its derivative chitosan have many applications in the pharmaceutical, cosmetic, food, and textile industries (Hirano, 1997). Chitin can be degraded by chitinase enzyme through which called chitinolytic process. In this case, chitin is being utilized naturally by many organisms as a source of carbon or nitrogen. The utilization of shellfish waste not only solves environmental problems but also decreases the production costs of microbial chitinases. Chitinases are a group of hydrolytic enzymes that catalyze depolymerization of chitin. 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. Microbial production of chitinase had great attention of both industrial and scientific environments (Ahmadi et al., 2008). Bacteria have a large role in chitin degradation but not all species are able to hydrolyze chitin. Bacteria which can produce extracellular chitinases include different *Bacillus spp.* such as *B. cereus* (Chang et al., 2007), *B. circulans* (Chen et al., 2004), and *B. licheniformis* (Waldeck et al., 2006).

Cell immobilization technology is particularly suited for production of extracellular enzymes and there is a growing interest in applying cell immobilization techniques for continuous production of enzymes (Ramakrishna and Prakasham, 1999). This

offers several advantages over the conventional processes, which include the easy separation and reuse of cells, high cell concentrations, flexibility in reactor design, and operation as well as stabilization of several cell functions (Dervakos and Webb, 1991). Immobilized whole cell systems have been successfully employed in the production of several extracellular enzymes by terrestrial microbes e.g. α-amylase (Duran-Paramo et al., 2000), lipase (Ferrer and Sola, 1992), protease (El-Aassar et al., 1990), and cellulase (Xin and Kumakura, 1993).

Also, solid-state fermentation is another method used for the production of enzymes which involves the cultivation of microorganisms on moist solid materials. The solid substrates act as a source of carbon, nitrogen and minerals as well as growth factors, and they have a capacity to absorb water, which meets the vital requirement for the microorganism. Solid-state fermentation processes can be conducted under controlled conditions, which are useful for producing valuable products like enzymes or secondary metabolites (Hesseltine, 1977; Ulmer et al., 1981).

Therefore, the present study was performed to investigate the ability of locally isolated bacterial strains to utilize chitin as a marine polysaccharide to produce chitinase enzyme by immobilized and solid-state fermentation methods.

2. Material and Methods

Chitin Source

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. The dried shrimp shell was blended using blender. The solid material obtained was dried, milled, and sieved to powder.

Microorganisms

Six bacterial strains with chitinolytic activity were isolated from the 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, El-Azhar University, Cairo, according to Bergye's Manual of Determinative Bacteriology (Hensyl, 1994). Also, fungal strains were used in the solid state culture experiment such as *Aspergillus niger*, and *Penicillium* were kindly obtained from Microbiology department, Faculty of Science, Alexandria University.

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 is composed of (g/L): production the chitin agar Chitin,20; $(NH_4)_2SO4$,1; KH_2PO_4,0.5; K_2HPO_4,1; $MgSO_4.7H_2O$,0.5; $FeCL_3.6H_2O$,400µl; $CaCl_2.2H_2O$,400µl; $ZnHSO_4.7H_2O$,400µl; and agar,20. The 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. Also, glucose peptone medium was used for the cultivation and maintenance of fungi. It was composed of (g/L): Glucose,20; peptone,5; KH_2PO_4,1; $MgSO_4$,0.5; and agar,20.

Sample Collection and Screening of Chitin-Degrading Microorganisms

Bacterial strains were isolated from seawater or sediment using enrichment technique. The following selective mineral was used (g/L): Chitin,20; $(NH_4)_2SO_4$,1; KH_2PO_4,0.5; K_2HPO_4,1; $MgSO_4.7H_2O$,0.5; $FeCL_3.6H_2O$,400µl; $CaCL_2.2H_2O$,400µl; $ZnHSO_4.7H_2O$,400µl; and agar,20. Pour plate technique was used to isolate the chitinolytic microorganisms where one gram of each soil sample was dispensed in 9 ml sterilized distilled water. Serial dilution from the original suspensions was prepared down to 10. One ml of each dilution was placed on a selective medium for each strain. The chitin agar plates were incubated for 5 days at 30˚C.

Purification of Isolated Strains

Chitinolytic bacterial colonies forming clear zones were purified by streaking on a solid medium of the same composition and then isolated on chitin-agar slants. The slants were kept as stock cultures at 4ºC and sub-cultured each 4 weeks or before use.

Primary Screening for Chitinase Production

Each of the isolated chitinolyitic bacterial strains was inculated in 250 Erlenmeyer flask filled with 50 ml of a sterile mineral medium (of the same composition as that used for isolation), supplemented with chitin as a carbon source at a final concentration of 20 g/L. Each flask was inoculated with one ml bacterial suspension prepared from a freshly prepared slant. The set of flasks were incubated under static condition. The cells were then separated by centrifugation at 6000 rpm for 20 min, and freezed for further processing.

Preparation of the Crude Enzyme

The bacterial culture was centrifuged at 6000 rpm for 20 min in a cooling centrifuge (Chilspin, England) at 4ºC. The clear supernatant was assayed for enzymatic activity. The samples were considered as the source of the crude enzyme.

Estimation of Protein Content, Preparation of Colloidal Chitin and Determination of Chitinase

Activity

The protein content of the enzyme preparations was determined according to method of Lowry et al. (1951). Cholloidal chitin was prepared as described by Monreal and Reese (1969), and chitinase activity was determined according to the method of Hood and Meyers (1977).

Cell Immobilization for Chitinase Production by Adsorption

The *B. licheniformis* cells were adsorbed on sponge cubes, luffa pulp and clay particle. An optimized culture medium containing the porous supports (about 15 particles/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. Scanning electron microscope photographs of adsorbed *B. licheniformis* cells on different solid supports were prepared using a Joel electron microscope (Japan), Electron Microscope Center, Faculty of Science, Alexandria University.

Semi-continuous Production of Chitinase by Adsorbed *B. licheniformis*

The semi-continuous production of chitinase by *B. licheniformis* cells adsorbed on clay particles was investigated. For this purpose, 50 ml fractions of the optimized production medium containing clay particles were used. Semi-continuous production of chitinase enzyme was carried out by batch wise reuse of the adsorbed cells at the end of each reuse the culture media were decanted and fresh media were added under aseptic conditions to the supports. The time interval for the reuse was 48 h. Incubation was carried out at 37°C in a static incubator.

Immobilization of *B. licheniformis* Cells by Entrapment in Ca-alginate and Agar

The *B. licheniformis* cells were entrapped in 2% calcium alginate gel beads as described by Eikmeier et al. (1984), and in agar as described by Chapatwala et al. (1993).

Semi-continuous Production of Chitinase by *B. licheniformis* Entrapped in Agar

The production of chitiase by the reuse of *B. licheniformis* cells entrapped in agar was investigated as previously mentioned with the adsorbed cells.

Chitinase Production from *B. licheniformis* by Solid-State Fermentation

The *B. Licheniformis* cells were fermented on dried shrimp, dried crab, wheat bran dried *Aspergillus* and dried *Penicillium*. The different dried solid substrate (1g) were taken in 250 Erlenmeyer conical flasks and mixed with 2 ml medium. The flasks were sterilized at 121□C for 15 min and cooled at room temperature. The solid media prepared were inoculated with 4 ml inoculum suspension per flask. The inoculated flask was incubated at 37□C for 2 days. At the end of incubation period, the flasks were taken out and the content of each flask was extracted with 20 ml of sterile distilled water and kept on a rotary shaker and agitated at 150 rpm for 20 min. The slurry was then squeezed through cheese cloth and the filtrate centrifuged at 6000 rpm for 20 min. The clear supernatant was collected and used as crude enzyme preparation for assay of enzymes.

3. Results

Isolation and Purification of Chitinolytic Bacteria

Table 1 shows that the isolates from the enriched culture were able to degrade chitin and produce chitinase enzyme with different levels. *B. licheniformis* produced the highest chitinase activity (1.052 U/ml). Therefore, *B. licheniformis* was selected as the test organism in this study.

Table 1. Chitinase activity of the tested isolates grown on chitin agar medium at static culture

Isolated Strains	Protein content (mg/ml)	Chitinase activity (U/ml)
Bacillus licheniformis	1.2	1.052
***Bacillus sp.* 1**	0.95	0.88
***Bacillus sp.* 2**	0.86	0.80
Staphylococcus	1.21	0.73
***Bascillus sp.* 3**	1.34	0.80
***Bascillus sp.* 4**	0.76	0.64

Chitinase Production by *B. licheniformis* cells adsorbed on different solid supports

Table 2 shows that a high cell adsorption occurred on all the used supports. However, clay particles showed a higher cell adsorption when compared to the other supports. Cultures containing adsorbed cells on sponge and clay particles showed a relatively high chitinase activity, while luffa pulp showed the lowest activity. Cultures containing clay particles showed a higher chitinase activity than that of the free cells. According to the previous results, clay particles were selected as a solid support for adsorption of *B. licheniformis* cells and production of chitinase enzyme in the next experiment.

Table 2. Production of chitinase by *Bacillus licheniformis* absorbed on different soild porous supports

Immobilization material	Protein content (mg/ ml)	Chitinase activity (U/ml)
Free cells	1.2	1.052
Sponge	2.26	1.36
Luffa pulp	1.78	1.06
Clay	3.38	3.63

Also, electron microscopic photographs (Figure 1) show the adsorption of *B. licheniformis* cells on the pores of clay particles, sponge, and luffa pulp.

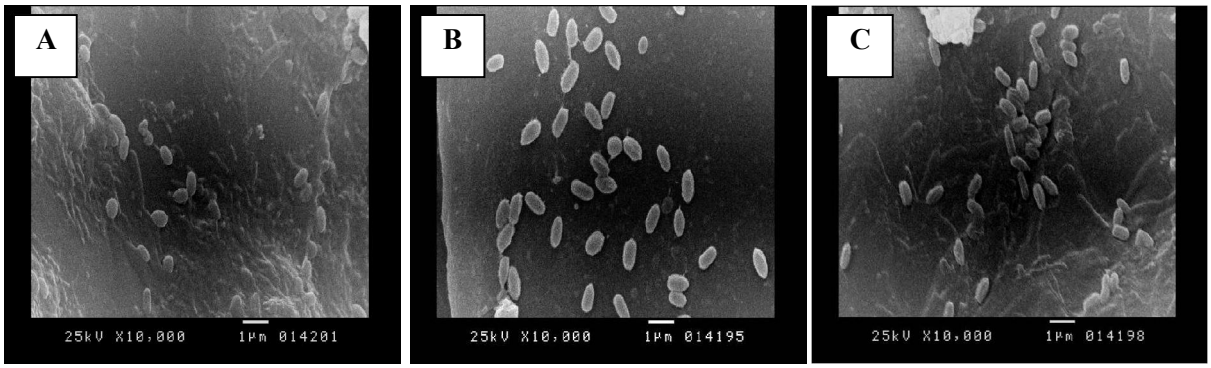

Figure 1. Electron microscopic micrograph (10.000×) showing adsorbed *Bacillus licheniformis* cells on: (A) clay particles, (B) sponge particles, and (C) luffa pulp.

Semi-continuous Production of Chitinase by Adsorbed *B. licheniformis* Cells

Table 3 shows that by reusing cells adsorbed on clay particles, the production of chitinase increased till the 2nd run and reached 4.25 U/ml, then the reused cultures showed a gradual decreases up to 6th run were the adsorbed cells showed the lowest chitinase activity. However, the 4th run was still higher than that of batch fermentation cultures by free cells.

Table 3. Semi-continuous production of chitinase by *Bacillus licheniformis* cells absorbed on clay particles

Number of runs	Protein content (mg/ ml)	Chitinase activity (U/ml)
1st	3.38	3.63
2nd	2.51	4.25
3rd	2.23	2.52
4th	1.46	1.62
5th	0.48	0.96
6th	0.33	0.96

Chitinase Production by Entrapped *B. licheniformis* Cells

Table 4 demonstrates that immobilization by entrapment using agar as the gel material produced the highest chitinase activity (1.25 U/ml) which was higher than this of free culture (1.025 U/ml).

Table 4. Effect of different gel materials on the production of chitinase by entrapped *Bacillus licheniformis* cells

Gel materials	Protein content (mg/ ml)	Chitinase activity (U/ml)
Free cells	1.2	1.052
Agar	1.65	1.25
Alginate	0.88	0.64
Alginate + chitin	0.95	0.85

Semi-continuous Production of Chitinase by *B. licheniformis* Cells Entrapped in Agar

Table 5 demonstrates that the reuse of 10 ml gel-cell cubes lead to an increase in chitinase production from the 2nd till the 3rd run and then decreased in the 4th and 5th runs. However, chitinase activities obtained in the 2nd and 3rd runs were higher than that of batch fermentation culture by free cell.

Table 5. Semi-continuous production of chitinase by *Bacillus licheniformis* cells entrapped in agar

Number of runs	Protein content (mg/ ml)	Chitinase activity (U/ml)
1st	1.65	1.25
2nd	1.50	1.32
3rd	1.61	1.52
4th	1.23	0.93
5th	0.85	0.55

Chitinase Production from *B. licheniformis* by Solid-State Fermentation

Figure 2 shows that the fermentation by using crabs as a solid substrate produced the highest chitinase production (1.85 U/ml). However, *Aspergillus* and *Penicillium* showed lower chitinase production when compared to the other solid substrates.

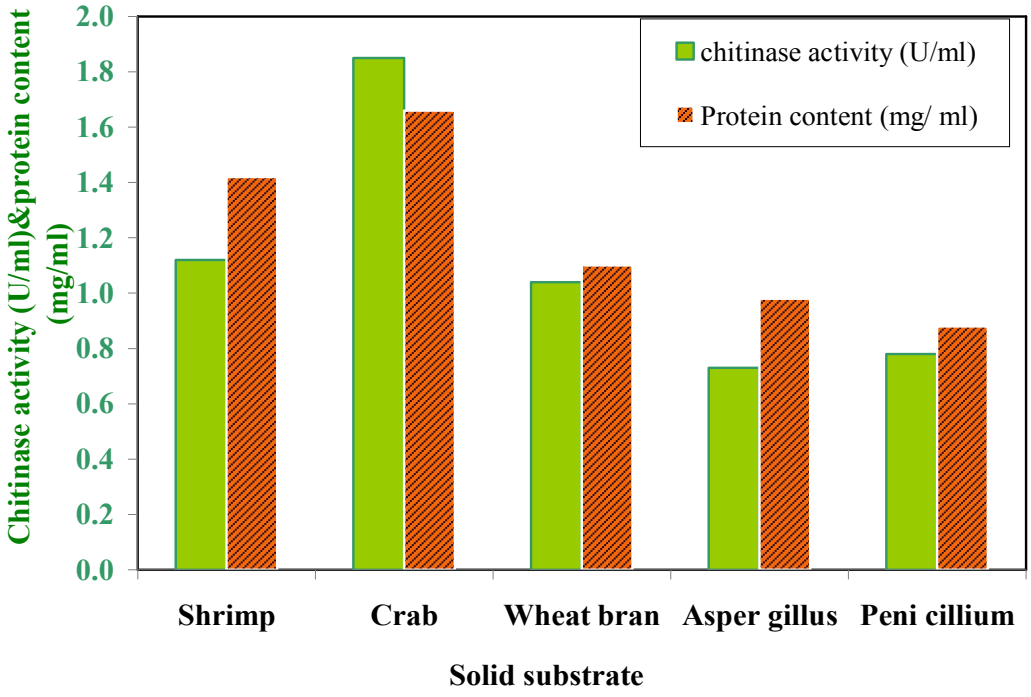

Figure 2. The production of chitinase from *Bacillus licheniformis* by solid-state fermentation using different substrates.

4. Discussions

An interesting application of chitinase is for bioconversion of chitin, a cheap biomaterial, into pharmacological active products, namely *N*-acetylglucosamine and chito-oligosaccharides (Bhattacharya et al., 2007; Hayes et al., 2008) 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 (Dahiya et al., 2006), bio-control of insects and mosquitoes as well as the production of single cell protein. There are many reports on expression and characterization of chitinases from various organisms, including bacteria, fungi, plant and animals (Howard et al., 2003). Therefore, chitinases, the hydrolytic enzymes that specifically degrade chitin, are gaining much attention worldwide (Makino et al., 2006; Wang et al., 2006).

In the present work, the bacteria were isolated from marine environment (sea water and sediment) by an enrichment technique using chitin as a sole carbon source. One *staphylococcus spp.* and five *Bacillus spp.* were obtained. From these six bacterial isolates, *B. licheniformis* showed the highest chitinase activity (1.052 U/ml). Many investigators reported the production of chitin degrading enzymes from *B. pabuli* (Frandberg and Schnurer, 1994), *B. stearothermophilus* (Sakai et al., 1994), *B. subtilis* (Wang et al., 2006), *B. thuringiensis* sub *sp. aizawai*

(De la Vega et al., 2006), and *B. thuringiensis* sub. *kurstaki* (Driss et al., 2005), In the present work, *B. licheniformis* cultures showed the highest growth and a high protein content, therefore, it was selected as a test organism for further investigations.

Several methods of immobilization have been attempted and by far the commonest methods used in immobilization of whole cells is gel entrapment in alginate or carrageenan and physical adsorption on solid supports, probably because these methods are less harsh on the living cells. The supports used for cell adsorption are wide and varied, which includes ceramics (Shiraishi et al., 1989), polyurethane foam (Haapala et al., 1995), and luffa sponge (Ogbonna et al., 1994). *B*acillus cells were immobilized by entrapment into different gel materials such as calcium alginate, κ-carrageenan, polyacrylamid, cellulose and agarose and then used as biocatalysts for fermentative production of various enzymes (Landau et al., 1992). The biosynthesis of chitinase by *B. licheniformis* cells immobilized by adsorption on different porous solid supports was studied. The highest chitinase activity was given by supporting *B. licheniformis* cells on clay particle within medium. The value obtained was 3.63 U/ml. this activity was about 1.2 times more than that obtained from cell-free medium. This may be due to the better contact between the cells and chitinase which were both adsorbed on the support material. On the other hand, sponge and luffa pulp cultures showed a slightly lower chitinase activity. Enhancement of enzyme production by adsorbed bacteria cells on porous solid support was also reported by other investigators (Groom et al., 1988).

The production of chitinase enzyme by adsorbed *B. licheniformis* cells was studied in semi-continuous batch cultures. The support was reused for 6 successive cycles covering a period of 216 h. Reuse of the adsorbed cells enhanced the production of the enzyme in the second reuse and higher activity value were recorded. However, a gradual decrease was observed in the next reuse. The good cell adsorption obtained on clay particle and the relative stability of the adsorbed cells when reused in batch mode, together with the simplicity of the immobilization procedure and the low price of the used support, present some advantage for the possibility of using adsorbed *B. licheniformis* cells for chitinase production. Also, experiment of immobilization using entrapment technique for chitinase production from *B. licheniformis* using different gel materials was examined. Agar was the best one for entrapping the bacterial cells. It gave the highest enzyme activity 1.25 U/ml this activity was about 1.5 times more than that obtained from free cells. This could be due to the fact that 2% agar was the most appropriate concentration for good gel porosity which was responsible for diffusion and transfer of oxygen, nutrients and metabolite products to and from the gel beads. It seemed that low gel concentration probably allowed better release of the enzyme to surrounding medium. In addition, the production of chitinase enzyme by entrapment of *B. licheniformis* cells was studied in semi-continuous batch cultures. The gel material was reused for 5 successive cycles. Reuse of the entrapped cells enhanced the production of the enzyme in the third reuse and higher activity value was recorded. However, a gradual decrease was observed in the next reuse.

The production of chitinase by solid-state fermentation in which *B. licheniformis* cells were fermented on different solid subsrates was examined. The fermentation by using crabs as solid substrate showed the highest chitinase production (1.85U/ml). San-Lang and Jau-Ren (2001) studied chitinase production using shellfish wastes from different *Bacillus* sp. Also, Wen et al. (2007) reported chitinase with antifungal activity from *B. cereus* using shrimp and crab shell waste powder. Also, experiment of immobilization of the purified enzyme using entrapment technique for chitinase production from *B. licheniformis* using different supports was examined. Carboxymethylcellulose was the best one for entrapping the bacterial cells. It gave the highest enzyme activity (1.524 U/ml). The immobilization by entrapment using carboxymethylcellulose as the support material was slightly lower than of free cell cultures.

In conclusion, these characters collectively indicate that the chitinase enzyme obtained from *B. 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, bio-control of fungal phytopathogens. The enzyme also plays a vital role in many fields such as food industry and medical applications. The results of the present investigation also indicate the possibility of using *B. licheniformis* cells for the production of a highly active chitinase preparation using cheap carbon source (chitin) as well as a cheap material for immobilization (clay particles for adsorption and agar for entrapment).

Corresponding Author:
Dr. Amany S. Youssef
Department of Botany and Microbiology, Faculty of Science, Alexandria University, Alexandria, Egypt
E-mail: amanyyoussef70@yahoo.com
Phone: +2-01222835093

References

1. Duo-Chuan L. Review of fungal chitinases. Mycopathologia 2006;161:345-60.

2. Wang SL, Lin HT, Liang TW, Chen YJ, Yen YH, Guo SP. Reclamation of chitinous materials by bromelain for the preparation of antitumor and antifungal materials. Bioresour Technol 2008;99:4386-93.
3. Aam BB, Heggset EB, Norberg AL, Sorlie M, Varum KM, Eijsink VGH. Production of chitooligosaccharides and their potential applications in medicine. Mar Drugs 2010;8:1482-517.
4. Muzzarelli RAA. Chitins and chitosans as immunoadjuvants and non-allergenic drug carriers. Mar Drugs 2010;8:292-312.
5. Faramarzi MA, Fazeli M, Tabatabaei M, Yazdi S, Adrangi K, Al-Ahmadi J, Tasharrofi N, Aziz MF. Optimization of cultural conditions for production of chitinase by a soil isolate of *Massilia timonae*. Biotechnology 2009;8:93-9.
6. Shen KT, Chen MH, Chan HY, Jeng JH, Wang YJ. Inhibitory effects of chitooligosaccharides on tumor growth and metastasis. Food Chem Toxicol 2009;47:1864-71.
7. Tamai YK, Miyatake Y, Okamoto Y, Takamori K., Sakamoto S, Minami, S. Enhanced healing of cartilaginous injuries by N-acetyl-D-Glucosamine and glucuronic acid. Carbohydr Polym 2003;54:251-62.
8. Salvatore SR, Heuschkel S, Tomlin SE, Edwards S. A pilot study of *N*-acetyl glucosamine, a nutritional substrate for glycosaminoglycan synthesis, in paediatric chronic inflammatory bowel disease. Aliment Pharmacol Ther 2000;14:1567-79.
9. Legaretta GI, Zakaria Z., Hall GM. Lactic fermentation of prawn waste: comparison of commercial and isolated starter culture. Adv Chitin Sci 2000;1:399-406.
10. Hirano S. Applications of chitin and chitosan in the ecological and environmental fields. In: Goosen MFA, ed. Applications of chitin and chitosan. Technomic Publishing Company Inc., USA. 1997; pp 31-50.
11. Ahmadi KA, Yazdi MT, Najafi MF, Shahverdi AR, Faramarzi MA, Zarrini G, Behravan J. Optimization of medium and cultivation conditions for chitinase production by the newly isolated Aeromonas species. Biotechnology 2008;7:266-72.
12. Chang WT, Chen YC, Jao CL. Antifungal activity and enhancement of plant growth by *Bacillus cereus* grown on shellfish chitin wastes. Bioresour Technol 2007;98:1224-30.
13. Chen CY, Wang YH, Huang CJ. Enhancement of the antifungal activity of *Bacillus subtilis* F29-3 by the chitinase encoded by *Bacillus circulans* chiA gene. Can J Microbiol 2004 ;50:451-4.
14. Waldeck J, Daum G, Bisping B, Meinhardt F. Isolation and molecular characterization of chitinase deficient *Bacillus licheniformis* strains capable of deproteinization of shrimp shell waste to obtain highly viscous chitin. Appl Environ Microbiol 2006;72:7879-85.
15. Ramakrishna SV, Prakasham RS. Microbial fermentations with immobilized cells. Curr Sci 1999;77:87-100.
16. Dervakos GA, Webb C. On the merits of viable cell immobilization. Biotechnol Adv 1991;9:559-612.
17. Duran-Paramo E, Garcia-Kirchner O, Hervagault JF, Thomas D, Barbotin JN. α- amylase production by free and immobilized *Bacillw subtilis*. Appl Biochem Biotechnol 2000;84-86:479-85.
18. Ferrer P, Sola C. Lipase production by immobilized *Candida* cells. Appl Microbiol Biotechnol 1992;37:737-41.
19. El-Aassar SA, El-Badry HM, Abdel-Fattah AF. The biosynthesis of proteases with fibrinolytic activity in immobilized cultures of *Penicillium chrysogenum* H9. Appl Microbiol Biotechnol 1990;33:26-30.
20. Xin LZ, Kumakura M. Immobilization of *Trichoderma reesei* cells on paper cvered by hydrophilic and hydrophobic co-polymers generated by irradiation. Enzyme Microb Technol 1993;15:300-3.
21. Hesseltine CW. L-glutaminase production by marine *Vibrio costicola* under solid state fermentation using. different substrates. J Mar Biotechnol 1977;4:176-9.
22. Ulmer DC, Tengerdy RP, Murphy VG. Investigation of some factors important for solid-state fermentation of sugarcane bagasse for animal feed production. Enzyme Microb Technol 1981;12:808-11.
23. Hensyl WR. (ed.): Bergey's Manual of Determinative Bacteriology, 9th edn. Williams and Wilkins, Baltimore, USA. 1994; pp 559-80.
24. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265-75.
25. Monreal J, Reese ET. The chitinase of *Serratia marcescens*. Can J Microbiol 1969;15:689-96.
26. Hood MA, Meyers SP. Microbiolgical and chitino castic activities associated with *Penaeus setiferus*. J Ocean Soc Japan 1977;33:235-41.
27. Eikmeier H, Westmeier F, Rehm HJ. Morphological development of Aspergillus niger immobilized in Ca-alginate and K-carrageenan. Appl Microbiol Biotechnol 1984;19:53-7.
28. Chapatwala KD, Babu GRV, Wolfram JH, Woese CR. Screening of encapsulated microbial

cells for the degradation of inorganic cyanides. J Ind Microbiol 1993;11:69-72.
29. Bhattacharya D, Nagpure S, Gupta RK. Bacterial chitinases: properties and potential. Crit Rev Biotechnol 2007;27:21-8.
30. Hayes M, Carney B, Slater J, Bruck W. Mining marine shellfish wastes for bioactive molecules: chitin and chitosan--Part B: applications. Biotechnol J 2008;3:878-89.
31. Dahiya N, Tewari R, Hoondal GS. Biotechnological aspects of chitinolytic enzymes: a review. Appl Microbiol Biotechnol 2006;71:773-82.
32. Howard MB, Ekborg NA, Weiner RM, Hutcheson SW. Detection and characterization of chitinases and other chitin-modifying enzymes. J Ind Microbiol Biotechnol 2003;30:627-35.
33. Makino A, Kurosaki K, Ohmae M, Kobayashi S. Chitinase catalyzed synthesis of alternatingly *N*-deacetylated chitin: a chitinchitosan hybrid polysaccharide. Biomacromolecules 2006;7:950-7.
34. Frandberg E, Schnurer J. Citinolytic properties of Bacillus pabuli K1. J Appl Bacteriol 1994;76:361-7.
35. Sakai K, Narihara M, Kasama Y, Wakayama M, Moriguchi M. Purification and characterization of thermostable beta-N-acetylhexosaminidase of *Bacillus stearothermophilus* CH-4 isolated from chitin-containing compost. Appl Environ Microbiol 1994;60:2911-5.
36. Wang SL, Lin TY, Yen YH, Liao HF, Chen YJ. Bioconversion of shellfish chitin wastes for the production of *Bacillus subtilis* W-118 chitinase. Carbohydr Res 2006;341:2507-15.
37. De la Vega LM, Barboza-Corona JE, Aguilar-Uscanga MG, Ramirez-lepe M. Purification and characterization of an exochitinase from *Bacillus thuringiensis* subsp. *aizawai* and its action against phytopathogenic fungi. Can J Microbiol 2006;52:651-7.
38. Driss F, Kallassy-Awad M, Zouari N, Jaoua S. Molecular characterization of a novel chitinase from *Bacillus thuringiensis* subsp. *kurstaki*. J Appl Microbiol 2005;99:945-53.
39. Shiraishi F, Kawakami K, Kono S, Tamura A, Tsuruta S, Kunsunoki K. Characterisation of production of free gluconic acid by *Gluconobacter suboxydans* adsorbed on ceramic honeycob monolith. Biotechnol Bioeng 1989;33:1413-8.
40. Haapala R, Parkkkinen E, Suominen P, Linko S. Production of extracellular enzymes by immobilized *Trichoderma reesei* in shake flask cultures. Appl Microbiol Biotcchnol 1995;43:815-21.
41. Ogbonna JC, Lin YC, Lin YK, Tanaka H. Loofa *(Luffa cylindrica*) sponge as a carrier for microbial cell immobilization. J Ferment Bioeng 1994;78:437-42.
42. Landau NS, Egorov NS, Gornova IB, Krasovaskaya SB, Virnik AD. Incorporation of *Bacillus firmus* cells in triacetate cellulose fibres and films and their use in proteinase biosynthesis. Prikl Biokhim Mikkrobiol 1992;28:108-13.
43. Groom CA, Daugulis AJ, White BN. Continuous alpha-amylase production using *Bacillus amyloliquefaciens* adsorbed on an ion exchange resin. Appl Envion Microbiol 1988;28:8-13.
44. San-Lang W, Jau-Ren H. Microbial reclamation of shellfish waste for the production of chitinases. Enzyme Microb Tichnol 2001;28:376-82.
45. Wen CM, Tseng CS, Cheng CY, Li YK. Occurrence of chitinolytic bacteria in water and bottom sediment of eutrophic lakes in Iławskie Lake District. Pol J Environ Stud 2007;10:331-6.

12/12/2013