ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 10 (3): 292-297, 2011
ISSN 1682-296X / DOI: 10.3923/biotech.2011.292.297
© 2011 Asian Network for Scientific Information

Enzymatic Production of N-Acetyl-D-Glucosamine from Chitin Using Crude Enzyme Preparation of *Aeromonas* sp. PTCC1691

[1]K. Jamialahmadi, [2]J. Behravan, [3]M. Fathi Najafi, [4]M. Tabatabaei Yazdi, [4]A.R. Shahverdi and [4]M.A. Faramarzi
[1]Department of Modern Sciences and Technologies, Faculty of Medicine,
Mashhad University of Medical Sciences, Mashhad, Iran
[2]Biotechnology Research Center and Faculty of Pharmacy,
Mashhad University of Medical Sciences, Mashhad, Iran
[3]Department of Veterinary Research and Biotechnology,
Razi Vaccine and Serum Research Institute, Ahmadabad Ave-Mashhad, Iran
[4]Biotechnology Research Center, Department of Pharmaceutical Biotechnology, Faculty of Pharmacy,
Tehran University of Medical Sciences, Tehran, Iran

Abstract: The present study was directed to the production of N-acetyl-D-glucosamine (GlcNAc) by partial hydrolysis of colloidal α-chitin using the crude enzyme preparation of *Aeromonas* sp. PTCC 1691. The selective production of GlcNAc was observed during continuous hydrolysis of chitin and at the same time period, the production of GlcNAc oligomers was negligible (below 0.5%). The maximum production of GlcNAc, a yield of 79%, was obtained after 24 h of incubation at the enzyme-substrate ratio of 2 U mg^{-1}. These results suggest that the multi-chitinolytic enzyme complex produced by *Aeromonas* sp. PTCC 1691 could be useful for bioconversion of chitin waste into N-acetyl-D-glucosamine for industrial applications.

Key words: *Aeromonas* sp. PTCC 1691, chitin, crude enzyme, enzymatic hydrolysis, N-acetyl-D-glucosamine

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 (Xia *et al*., 2011), antimicrobial and antifungal agents (Wang *et al*., 2008; Tsai *et al*., 2000), immunoinhancers (Muzzarelli, 2010; Aam *et al*., 2010) and antitumor activity (Shen *et al*., 2009; Faramarzi *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) and gastritis (Gindzienski *et al*., 1971). GlcNAc is more suitable than GlcN for oral administration, because of its sweet taste (Sashiwa *et al*., 2001). Generally, GlcNAc is produced by acid hydrolysis of chitin, a process that is mainly performed in concentrated acid at high temperature. This procedure, however, has some problems such as high cost, low yield (below 65%) and acidic wastes. Therefore, much attention has been focused on enzymatic hydrolysis for production of GlcNAc from chitin (Ramirez-Coutino *et al*., 2006; Donzelli *et al*., 2003; Sashiwa *et al*., 2003). Chitinases (EC 3.2.1.14) are a group of complex enzymes that catalyze the degradation of chitin. These enzymes have been found in many organisms including viruses, bacteria, fungi, insects, plants and animals (Akhir *et al*., 2009; Dahiya *et al*., 2006). Although, many chitinases produced from *Serratia* sp. (Haynes *et al*., 1999), *Bacillus* sp. (Thamthiankul *et al*., 2001), *Aeromonas* sp. (Kuk *et al*., 2005b), *Clostridium* sp. (Li *et al*., 2002), *Trichoderma* sp. (Abd-Aziz *et al*., 2008) and *Aspergillus* sp. (Rattanakit *et al*., 2003) have been reported for the production of GlcNAc from chitin, only a few biocatalysts useful for the industrial production of GlcNAc are currently available and limited research in this field has been made.

In our previous report, a strong chitinolytic bacterium, *Aeromonas* sp. PTCC 1691, was isolated from the waste of shrimp shells sample collected from local area; Poonel, Gilan Province, Iran (Jami Al Ahmadi *et al*., 2008a). The present study describes the effective production of N-acetyl-D-glucosamine from chitin by the crude enzyme preparation from the newly isolate (*Aeromonas* sp. PTCC 1691).

Corresponding Author: Dr. Khadijeh Jamialahmadi, Department of Modern Sciences and Technologies, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran Tel: +98-511-8002293 Fax: +98-511-8002960

MATERIALS AND METHODS

The study was conducted in 2006-2008 by Mashhad and Tehran Universities of Medical Sciences, in Iran.

Microorganism and cultivation conditions: The bacterial strain was isolated from the waste of shrimp shells sample collected from the north part of Iran using the agar medium containing 0.5% colloidal chitin as the selective medium. This strain was further identified as *Aeromonas* sp. using the morphological and biochemical properties along with 16S rRNA partial sequence analysis and deposited as PTCC 1691 in Persian Type Culture Collection (Tehran, Iran).

Preparation of colloidal chitin: Colloidal chitin was prepared according to the method of Roberts and Selitrennikoff (1988) with a few modifications. Five grams of chitin powder from crab shells (Sigma-Aldrich Co., USA) was added slowly into 90 mL of concentrated HCl under vigorous stirring for 2 h. The mixture was added to 500 mL of ice-cold EtOH (95%) with rapid stirring and kept overnight at 25°C and then stored at -20°C until use. When in need, the precipitant was collected by centrifugation at 6000 rpm for 10 min at 4°C and washed several times with 0.1 M sodium phosphate buffer (pH 7.0) until the pH was natural. The colloidal chitin was kept at 4°C until it was used.

Culture condition for chitinase production: For the production of enzyme, *Aeromonas* sp. PTCC 1691 was grown in a liquid medium containing (g L^{-1}) colloidal chitin, 7.5; Na_2HPO_4, 0.65; KH_2PO_4, 1.5; NaCl, 0.25; $(NH_4)_2SO_4$, 1.5; $MgSO_4$, 0.12; $MgCl_2$, 1.52; $CaCl_2$, 0.005 and Triton X-100, 0.2% (v/v) in shaken flasks at 30°C, pH 8.0 and 150 rpm. After incubation for 2 days, the culture broth was centrifuged at 4°C for 10 min at 8000 rpm and the clear supernatant was collected and stored at -20°C for further experiments.

Enzymatic activities: Chitinase activity was determined with colloidal chitin as the substrate. Enzyme solution (0.3 mL) was added to 0.3 mL of substrate solution which contained 1% colloidal chitin in a sodium phosphate buffer (0.1 M, pH 8.0). The mixture was incubated at 37°C for 45 min. The reducing sugar released was measured by the Dinitrosalicylic acid (DNS) method (Miller, 1959) at 540 nm using N-acetyl-D-glucosamine (GlcNAc) as standard. One unit of chitinase activity was defined as the amount of enzyme producing 1 μmoL of GlcNAc per hour under the specified assay conditions.

For determination of β-N-acetylglucosaminidase activity, the reaction mixture contained 100 μL p-nitrophenyl-N-acetylglucosaminide (5 mM), 100 μL crude enzyme and 200 μL 0.1 M sodium phosphate buffer (pH 8.0). After incubation at 37°C for 1 h, 1 mL glycine-NaOH (0.2 M, pH 10.5) was added to stop the reaction. The amount of p-nitrophenol released was determined by measuring the absorbance at 405 nm. One unit of β-N-acetylglucosaminidase activity was defined as the amount of enzyme necessary to liberate 1 μmoL p-nitrophenol per hour under assay conditions.

Enzymatic hydrolysis of chitin: A typical enzymatic reaction for production of GlcNAc was carried out by incubating a mixture of a known concentration of crude enzyme and the chitin substrate in 0.1 M phosphate buffer (pH 8.0) at 37°C in the presence of sodium azide (200 ppm) as a preservative. The exact set of conditions for each experiment was specified in the footnote of each figure. At each time point, a portion of the reaction mixture was sampled, diluted with water and then mixed with CH_3CN at the ratio of 30: 70 (v/v), filtered and analyzed by HPLC (column, Shodex Asahipak NH2P-50; mobile phase, acetonitrile/water (70:30, v/v); flow rate, 1.0 mL min^{-1}; detection at 210 nm). The amounts of GlcNAc, $(GlcNAc)_2$ and $(GlcNAc)_3$ in the reaction mixtures were estimated from the calibration curve of the corresponding standards. The HPLC data were analyzed by a pre-set program (Class-VP software, SHIMADZU).

RESULTS AND DISCUSSION

Chitinolytic bacterium selection and crude enzyme preparation: In our previous reports (Jami Al Ahmadi *et al*., 2008a, b), five chitinolytic strains; JK1, JK2, JK3, JK4 and JK5 which displayed he highest chitinolytic activities in the shortest time were purified and tested for β-N-acetylglucosaminidase activity after growth in chitin liquid medium. Among them,isolate JK1 which showed the highest β-N-acetylglucosaminidase (exochitinase) activity and relatively high chitinase activity was chosen for further studies.

On the basis of morphological, biochemical and molecular studies, JK1 was identified as *Aeromonas* sp. and deposited in Persian Type Culture Collection (PTCC 1691). Enzyme activity of the culture supernatants of this strain was maximal at 2d after inoculation (9.2 U mL^{-1}) and decreased thereafter (data not shown). Therefore, culture supernatants from 2d-old cultures were used in preparation of crude enzyme for hydrolysis of colloidal

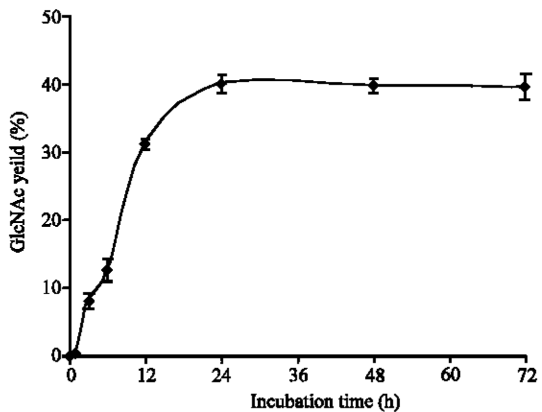
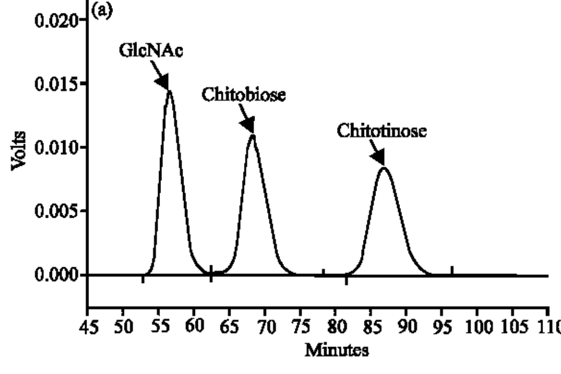


Fig. 1: Time courses on the GlcNAc production from colloidal chitin by crude enzyme produced by *Aeromonas* sp. PTCC 1691. Chitin: (S) = 20 mg mL^{-1}; Crude enzyme: 4 U; pH 8.0 (0.1 M phosphate buffer); 37°C; 100 rpm

chitin and GlcNAc production. α-chitin powder from crab shells was used as substrate for digestion by crude bacterial chitinase because of its more abundance and its relatively low cost compared with β-chitin.

Enzymatic hydrolysis of chitin for GlcNAc production: The time course for production of N-acetyl-D-glucosamine from chitin by crude enzyme is shown in Fig. 1. The production of GlcNAc increased rapidly with increasing the incubation time in the first 24 h, while that of GlcNAc oligomers was less. No noticeable production took place in the subsequent 48 h. This result is in agreement with the results of Ilankovan *et al.* (2006) who reported that the most of the N-acetylchitobiose produced by pepsin treatment of chitin in 72 h was produced in the first 24 h and no noticeable production took place in the subsequent 48 h. In the research presented here, the maximum yield of GlcNAc production was 42% after 24 h of incubation but during the same time period, the yield of chitobiose and chitotriose was negligible (below 0.5%).

The decrease in the rate of hydrolysis after 24 h might be due to enzyme denaturation during the reaction and/or GlcNAc product inhibition. The stability studies showed that the chitinases produced from *Aeromonas* sp. PTCC 1691 had a good stability at 37°C for more than 48 h but the investigation of GlcNAc product inhibition revealed that the high initial concentration of GlcNAc can slightly inhibit the hydrolysis reaction (data not shown). This result suggested that the feedback inhibition of the end product maybe the minor cause of decreasing in the production rate. So we supposed that the structure of α-chitin substrate itself possibly the main reason for the



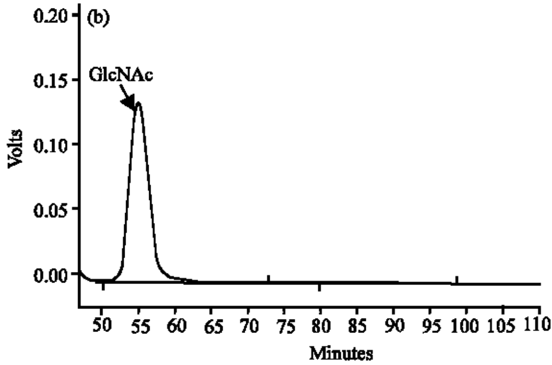


Fig. 2: HPLC chromatograms of the hydrolyzates of chitin after 24 h of incubation with crude enzyme (20 U) produced by *Aeromonas* sp. PTCC 1691 (a) standards and (b) Hydrolysates from chitin

reduction in the GlcNAc production rate. According to the results, in the beginning of the enzyme(s) activity on chitin substrate, the rate of GlcNAc production increased rapidly, suggesting that the high concentration of the substrate and accessibility of nearly all available sites of the decrystallized chitin particles for the enzyme hydrolysis are the main reason for production of GlcNAc. But after that, because of limited accessibility of the β-glycosidic bonds in the interior of the chitin crystal particles for enzymatic attack the rate of hydrolysis was slowed down. The same suggestions have been given by different researches for the absence of further enzymatic hydrolysis of chitin after 24 h (Chen *et al.*, 2010; Klaikherd *et al.*, 2004).

The HPLC chromatogram in Fig. 2a and b show quite selective production of GlcNAc during continuous hydrolysis of chitin and at the same time, the production of GlcNAc oligomers (G2-G3: dimer-trimer) was negligible. This fact suggests that both endo- and exotype chitinases exist in crude enzyme of *Aeromonas* sp. PTCC 1691 which produce GlcNAc selectively by cooperation. The chitinase is responsible for hydrolyzing polymeric chitin chains into smaller chitooligosaccharides which are in turn further hydrolyzed by β-N-acetylglucosaminidase to

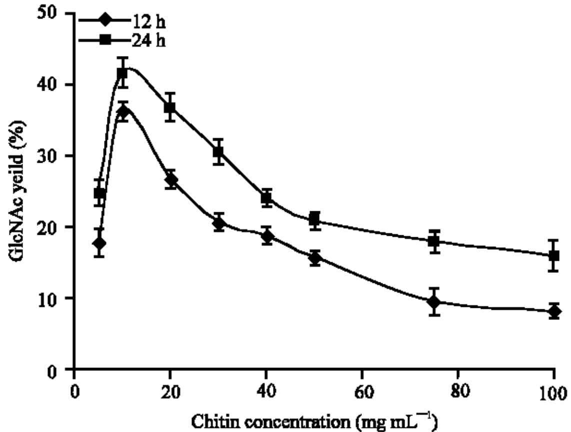


Fig. 3: The effect of concentration of α-chitin on GlcNAc production. Crude enzyme: 4 U; pH 8.0 (0.1 M phosphate buffer); 37°C; 100 rpm

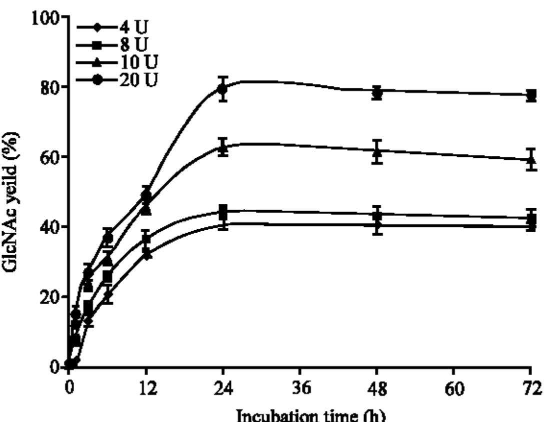


Fig. 4: The effect of concentration of enzyme on GlcNAc production. Chitin: (S) = 10 mg mL^{-1}; Crude enzyme: 4, 8, 10 and 20 U; pH 8.0 (0.1 M phosphate buffer); 37°C; 100 rpm

GlcNAc. A similar result was reported by Sashiwa *et al.* (2002) who found that the selective production of GlcNAc from chitin by using crude enzymes obtained from *Aeromonas hydrophila* H-2330 and chitin oligomers (dimer-heptamer) was negligible. Binod *et al.* (2007) used the endochitinase and chitobiase from *Penicillium aculeatum* NRRL 2129 and *Trichoderma harzianum* TUBF 927, respectively, for production of GlcNAc under solid-state fermentation. Their study showed that for one-step hydrolysis of chitin to GlcNAc, the enzyme preparation must contain both endochitinase and chitobiase.

Effect of substrate concentration on GlcNAc production: Figure 3 shows the effect of substrate concentration on the production of N-acetyl-D-glucosamine. When the colloidal chitin concentration was raised from 5 to 10 mg mL^{-1}, while keeping the enzyme concentration constant, the percentage yield of GlcNAc was increased significantly. But, further increasing the substrate concentration resulted in lower percentage yield of GlcNAc, so that in 100 mg mL^{-1} of colloidal chitin after 12 and 24 h of incubation, GlcNAc was produced with yields of 11 and 21.5%, respectively. Sukwattanasinitt *et al.* (2002) investigated the effect of substrate concentration on GlcNAc production by using the mixed enzyme, 9:1 cellulase Ac and lipase An. The increase of β-chitin concentration, while keeping the enzyme concentration constant, resulted in lower percentage yield of GlcNAc. When the concentration of substrate was increased from 10 to 40 mg mL^{-1}, the percentage yield of GlcNAc dropped by half, from 61% to 28%, respectively. They suggested that the reduction of the percentage yield of GlcNAc at high concentration of substrate may be attributed to a poor mixing as the reaction media became very viscous at this concentration.

Effect of enzyme concentration on GlcNAc production: To investigate the effect of enzyme concentration on GlcNAc production, colloidal chitin (10 mg mL^{-1}) was incubated with various amounts of crude enzyme at 37°C and the yield of GlcNAc was estimated. As can be seen in Fig. 4, percent yield of GlcNAc increased with the increase in enzyme amount and higher concentrations of the enzyme leads to more production of the GlcNAc. There was no significant difference in GlcNAc production between 10 mg/ 4 U and 10 mg/ 8 U. The maximum production of GlcNAc, a yield of 79%, was obtained after 24 h of incubation at the enzyme-substrate ratio of 2 U mg^{-1}. The yield of GlcNAc increased rapidly over 24 h but thereafter the production of GlcNAc was almost stopped.

Jung *et al.* (2007) reported the production of N-acetyl-D-glucosamine and N-acetylchitooligosaccharides by use of crude enzyme obtained from *Paenibacillus illinoisensis* KJA-424. The production rate of GlcNAc increased continuously during incubation, while that of GlcNAc oligomers declined. The maximum production of GlcNAc was 62.2% after 24 h of incubation while during the same time period; the yield of N-acetylchitooligosaccharides was below 10%. Kuk *et al.* (2005a) reported the selective production of GlcNAc and $(GlcNAc)_2$ using the crude enzyme of *Aeromonas* sp. GJ-18, with yields of 74 and 35% at 45 and 55°C, respectively, after 5 days of incubation. GlcNAc was

produced from α- and β-chitin by crude enzymes of *Burkholderia cepacia* TU09 and *Bacillus licheniformis* SK-1. Chitinase from B. cepacia TU09 produced GlcNAc in 85% yield from α- and β-chitin within 1 and 7 days, respectively. B. licheniformis SK-1 chitinase completely hydrolyzed β-chitin within 6 days, giving a final GlcNAc yield of 75%, along with 20% of chitobiose (Pichyangkura *et al*., 2002).

CONCLUSION

The results of this study indicates that the crude enzyme preparation of the newly chitinolytic isolate, *Aeromonas* sp. PTCC 1691, was effective in enzymatic production of N-acetyl-D-glucosamine from α-chitin in good yield under simple and quite mild procedure. These results suggest this strain as a valuable enzyme source for selective production of GlcNAc from chitin.

ACKNOWLEDGMENTS

The results described in this paper were part of K. Jamialahmadi Ph.D. thesis proposal. Authors would like to thank the Research Councils of Mashhad and Tehran Universities of Medical Sciences, Iran for financial support of this project.

REFERENCES

Aam, B.B., E.B. Heggset, A.L. Norberg, M. Sorlie, K.M. Varum and V.G.H. Eijsink, 2010. Production of chitooligosaccharides and their potential applications in medicine. Mar. Drugs., 8: 1482-1517.

Abd-Aziz, S., L.S. Teoh, N. Alitheen, N. Shahab and K. Kamaruddin, 2008. Microbial degradation of chitin materials by *Trichoderma virens* UKM1. J. Biol. Sci., 8: 52-59.

Akhir, S.M., S. Abd-Aziz, M.M. Salleh, R.A. Rahman, R.M. Illias and M.A. Hassan, 2009. Medium optimisation of chitinase enzyme production from shrimp waste using *Bacillus licheniformis* TH-1 by response surface methods. Biotechnology, 8: 120-125.

Binod, P., C. Sandhya, P. Suma, G. Szakacs and A. Pandey, 2007. Fungal biosynthesis of endochitinase and chitobiase in solid state fermentation and their application for the production of N-acetyl-D-glucosamine from colloidal chitin. Bioresour. Technol., 98: 2742-2748.

Chen, J.K., C.R. Shen and C.L. Liu, 2010. N-acetylglucosamine: Production and applications. Mar. Drugs, 8: 2493-2516.

Dahiya, N., R. Tewari and G.S. Hoondal, 2006. Biotechnological aspects of chitinolytic enzymes. Appl. Microbiol. Biotechnol., 71: 773-782.

Donzelli, B.G.G., G. Ostroff and G.E. Harman, 2003. Enhanced enzymatic hydrolysis of langostino shell chitin with mixtures of enzymes from bacterial and fungal sources. Carbohydr. Res., 338: 1833-1923.

Duo-Chuan, L., 2006. Review of fungal chitinases. Mycopathologia, 161: 345-360.

Faramarzi, M.A., M. Fazeli, M. Tabatabaei Yazdi, S. Adrangi, K. Jami Al-Ahmadi, N. Tasharrofi and F. Aziz Mohseni, 2009. Optimization of cultural conditions for production of chitinase by a soil isolate of *Massilia timonae*. Biotechnology, 8: 93-99.

Gindzienski, A., K. Zwierz and A. Rozanski, 1971. The activity of N-acetylglucosamine kinase from human gastric mucosa. Biochem. Med., 5: 188-194.

Haynes, C.A., P. Aloise and A.L. Creagh, 1999. Process for producing N-acetyl-D-glucosamine. US Patent No.5998173.

Ilankovan, P., S. Hein, C.H. Ng, T.S. Trung and W.F. Stevens, 2006. Production of N-acetyl chitobiose from various chitin substrates using commercial enzymes. Carbohydr. Polym., 63: 245-250.

Jung, W.J., A. Souleimanov, R.D. Park and D.L. Smith, 2007. Enzymatic production of N-acetyl chitooligosaccharides by crude enzyme derived from *Paenibacillus illioisensis* KJA-424. Carbohydr. Polym., 67: 256-259.

Jami Al Ahmadi, K., M. Tabatabaei Yazdi, M. Fathi Najafi, A.R. Shahverdi, M.A. Faramarzi, G. Zarrini and J. Behravan, 2008a. Optimization of medium and cultivation conditions for chitinase production by the newly isolated: *Aeromonas* sp. Biotechnology, 7: 266-272.

Jami Al Ahmadi, K., M. Tabatabaei Yazdi, M. Fathi Najafi, A.R. Shahverdi, M.A. Faramarzi, Gh. Zarrini and J. Behravan, 2008b. Isolation and characterization of a chitinolytic enzyme-producing microorganism, *Paenibacillus chitinolyticus* JK2 from Iran. Res. J. Microbiol., 3: 395-404.

Klaikherd, A., M.L.S. Jayanta, J. Boonjawat, S.I. Aiba and M. Sukwattanasinitt, 2004. Depolymerization of α-chitin to mono-and disaccharides by the serum fraction from the para rubber tree, *Hevea brasiliensis*. Carbohydr. Res., 339: 2799-2804.

Kuk, J.H., W.J. Jung, G.H. Jo, J.S. Ahn, K.Y. Kim and R.D. Park, 2005a. Selective preparation of N-acetyl-D-glucosamine and N,N'-diacetylchitobiose from chitin using a crude enzyme preparation from *Aeromonas* sp. Biotechnol. Lett., 27: 7-11.

Kuk, J.H., W.J. Jung, G.H. Jo, Y.C. Kim, K.Y. Kim and R.D. Park, 2005b. Production of N-acetyl-β-D-glucosamine from chitin by *Aeromonas* sp. GJ-18 crude enzyme. Appl. Microbiol. Biotechnol., 68: 384-389.

Li, H., K. Morimoto, N. Katagiri, T. Kimura, K. Sakka, S. Lun and K. Ohmiya, 2002. A novel β-N-acetylglucosaminidase of Clostridium paraputrificum M-21 with high activity on chitobiose. Appl. Microbiol. Biotechnol., 60: 420-427.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal. Chem., 31: 426-428.

Muzzarelli, R.A.A., 2010. Chitins and chitosans as immunoadjuvants and non-Allergenic drug carriers. Mar. Drugs., 8: 292-312.

Pichyangkura, R ., S. Kudan, K. Kuttiyawong, M. Sukwattanasinitt and S. Aiba, 2002. Quantitative production of 2-acetamido-2-deoxy-D-glucose from crystalline chitin by bacterial chitinase. Carbohydr. Res., 337: 557-559.

Ramirez-Coutino, L., M.D.C. Marin-Cervantes, S. Huerta, S. Revah and K. Shirai, 2006. Enzymatic hydrolysis of chitin in the production of oligosaccharides using *Lecanicillium fungicola* chitinases. Process Biochem., 41: 1106-1110.

Rattanakit, N., S. Yang, M. Wakayama, A. Plikomol and T. Tachiki, 2003. Saccharification of chitin using solid-state culture of *Aspergillus* sp. S1-13 with shellfish waste as a substrate. J. Biosci. Bioeng., 95: 391-396.

Roberts, W.K. and C.P. Selitrennikoff, 1988. Plant and bacterial chitinases differ in antifungal activity. J. Gen. Microbiol., 134: 169-176.

Salvatore, S., R. Heuschkel, S. Tomlin, S.E. Davies and S. Edwards *et al*., 2000. A pilot study of N-acetyl glucosamine, a nutritional substrate for glycosaminoglycan synthesis, in paediatric chronic inflammatory bowel disease. Aliment Pharmacol. Ther., 14: 1567-1579.

Sashiwa, H., S. Fujishima, N. Yamano, N. Kawasaki, A. Nakayama, E. Muraki and S.I. Aiba, 2001. Production of N-acetyl-D-glucosamine from β-chitin by enzymatic hydrolysis. Chem. Lett., 4: 308-309.

Sashiwa, H., S. Fujishima, N. Yamano, N. Kawasaki and A. Nakayama *et al*., 2002. Production of N-acetyl-D-glucosamine from alpha-chitin by crude enzymes from *Aeromonas hydrophila* H-2330. Carbohydr. Res., 337: 761-763.

Sashiwa, H., S. Fujishima, N. Yamano, N. Kawasaki and A. Nakayama *et al*., 2003. Enzymatic production of N-acetyl-D-glucosamine from chitin: Degradation study of N-acetyl-chitooligosaccharide and the effect of mixing of crude enzymes. Carbohydr. Polym., 51: 391-395.

Shen, K.T., M.H. Chen, H.Y. Chan, J.H. Jeng and Y.J. Wang, 2009. Inhibitory effects of chitooligosaccharides on tumor growth and metastasis. Food. Chem. Toxicol., 47: 1864-1871.

Sukwattanasinitt, M., H. Zhu, H. Sashiwa and S. Aiba, 2002. Utilization of commercial non-chitinase enzymes from fungi for preparation of 2-acetamido-2-deoxy-D-glucose from β-chitin. Carbohydr. Res., 337: 133-137.

Tamai, Y., K. Miyatake, Y. Okamoto, Y. Takamori, K. Sakamoto and S. Minami, 2003. Enhanced healing of cartilaginous injuries by N-acetyl-D-Glucosamine and glucuronic acid. Carbohydr. Polym., 54: 251-262.

Thamthiankul, S., S. Suan-Ngay, S. Tantimavanich and W. Panbangred, 2001. Chitinase from *Bacillus thuringiensis* subsp. Pakastani. Appl. Microbiol. Biotechnol., 56: 395-401.

Tsai, G.I., Z.Y. Wu and W.H. Su, 2000. Antibacterial activity of a chitooligosaccharide mixture prepared by cellulase digestion of shrimp chitosan and its application to milk preservation. J. Food Prot., 63: 747-752.

Wang, S.L., H.T. Lin, T.W. Liang, Y.J. Chen, Y.H. Yen and S.P. Guo, 2008. Reclamation of chitinous materials by bromelain for the preparation of antitumor and antifungal materials. Bioresour. Technol., 99: 4386-4393.

Xia, W., P. Liu, J. Zhang and J. Chen, 2011. Biological activities of chitosan and chitooligosaccharides. Food Hydrocolloids, 25: 170-179.