Journal of Zhejiang University SCIENCE B
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Analysis of both chitinase and chitosanase produced by *Sphingomonas* sp. CJ-5*

ZHU Xu-fen†, ZHOU Ying, FENG Jun-li
(*College of Life Science, Zhejiang University, Hangzhou 310058, China*)
†E-mail: zhuxufen@mail.hz.zj.cn

Received Apr. 28, 2007; revision accepted Sept. 19, 2007

Abstract: A novel chitinolytic and chitosanolytic bacterium, *Sphingomonas* sp. CJ-5, has been isolated and characterized. It secretes both chitinase and chitosanase into surrounding medium in response to chitin or chitosan induction. To characterize the enzymes, both chitinase and chitosanase were purified by ammonium sulfate precipitation, Sephadex G-200 gel filtration and DEAE-Sepharose Fast Flow. SDS-PAGE analysis demonstrated molecular masses of chitinase and chitosanase were 230 kDa and 45 kDa respectively. The optimum hydrolysis conditions for chitinase were about pH 7.0 and 36 °C, and these for chitosanase were pH 6.5 and 56 °C, respectively. Both enzymes were quite stable up to 45 °C for one hour at pH 5~8. These results show that CJ-5 may have potential for industrial application particularly in recycling of chitin wastes.

Key words: *Sphingomonas* sp., Chitinase, Chitosanase
doi:10.1631/jzus.2007.B0831 **Document code:** A **CLC number:** Q55

INTRODUCTION

Chitin, chitosan and cellulose consist of β-(1,4) linked glucopyranoses and their differences are in functional groups at the C-2 position of their constituent sugar, i.e., the acetamido, amino, and hydroxyl groups, respectively. Except cellulose, chitin, a homo-polysaccharide of *N*-acetyl-D-glucosamine (GlcNAc), is the most abundant biopolymers on earth with an estimated annual production in the order of 10^{10} to 10^{11} tons (Gooday, 1990). It is a principal structural component of most fungi and algae cell walls, insect exoskeletons, the shells of crustaceans, and the microfilarial sheath of nematodes (Flach *et al*., 1992). Chitin is a fibrous and extremely insoluble material, whereas chitosan is a water-soluble and much more tractable material with a broad range of applications (Hirano, 1989). Chitosan is a partially or totally deacetylated derivative of chitin. Chitin and chitosan are distinguished by the amount of acetylation of the D-glucosamine (GlcN) residues. Polymers containing more than 70% acetylating are considered chitin, while those with less than 30% are called chitosan. The enormous amounts of chitin and chitosan produced annually in biosphere are degraded by chitinase and chitosanase.

Chitinase (EC 3.2.1.14) and chitosanase (EC 3.2.1.132) are two members of the glycoside hydrolyse family, and are characterized by their ability to catalyze the hydrolytic cleavage of chitin and chitosan, respectively. Chitinase catalyzes the degradation of chitin to give biotechnologically valuable chitooligomers, and they have been detected in a wide variety of organisms including bacteria, fungi, viruses, plants and insects. Based on amino acid sequence of their catalytic domains, chitinases from all organisms are classified into two phylogenetically distinct families 18 and 19 among the 87 families of glycosyl hydrolases (Gooday, 1997; Henrissat and Bairoch, 1993; Henrissat and Davies, 1997; Saito *et al*., 1999). Family 18 chitinases are distributed in wide range of

* Project supported by the Marine Bureau of Zhejiang Province, China

organisms, including bacteria, fungi, plants (classes III and V), mammals, and viruses. Structurally they consist of several different domains including the catalytic domains, chitin-binding domain, fibronectin type III-like, and cadherin-like domains. In contrast, family 19 chitinases have been found only in plants and some bacterial strains (Watanabe *et al*., 1999; Kawase *et al*., 2004). On the other hand, chitosanases are found in microorganisms and plants (Somashekar and Joseph, 1996), and belong to five glycosyl hydrolase families: 5, 8, 75, 80 and 46. The distinction between chitinases and chitosanases is that chitinase specifically cleaves the *N*-acetyl-D-glucosaminidic bonds while chitosanase cleaves the β-D-glucosaminidic bonds. Chitosanases are able to hydrolyze all kinds of linkages in chitosan except the GlcNAc-GlcNAc bond (Zhu *et al*., 2003). However, some chitinases hydrolyze GlcN-GlcNAc bonds in addition to GlcNAc-GlcNAc ones.

Chitinase plays an important physiological and ecological role in recycling chitin in nature (Tsujibo *et al*., 1993; Park *et al*., 1997). It is thought that bacteria produce chitinases primarily to provide carbon and nitrogen as a nutrient (Cohen-Kupiec and Che, 1998). Recently, chitinase and chitosanase have received considerable attention because chitinase might play a role in plant defense systems against chitin-containing pathogens and mosquito control (Mendonsa *et al*., 1996), as well as chitosanase has been used to obtain chitosan oligomers for clinical applications as wound-healer, blood anticoagulants and haemostatic materials (Hirano, 1996; Sandorf, 1989).

Sphingomonas sp. CJ-5 is a Gram-negative flagellated and aerobic rod-shaped bacterium of shrimp shell-enriched soil origin. This chitinolytic strain not only excretes chitinase, but also chitosanase into the growth medium in the presence of chitin or chitosan. In this paper, we report the characterization of both chitinase and chitosanase produced by *Sphingomonas* sp. CJ-5 strain.

MATERIALS AND METHODS

Reagents and chemicals

Chitin (10% deacetylated) and chitosan (70%~90% deacetylated) were products of Funakoshi Co., Ltd., Tokyo, Japan. Colloidal chitin and colloidal chitosan were prepared by the method of Yabuki *et al*. (1988). The molecular mass marker of standard proteins was purchased from Songon Co., Ltd., Shanghai, China. The standard samples of GlcNAc and GlcN were purchased from Sigma, USA. Other reagents were all of analytical grade.

Screening and identification of bacterial strain

A chitinolytic bacterium CJ-5, screened by using chitin as a sole carbon source, was isolated from shrimp shell-enriched soil collected in Zhejiang, China. Preliminary identification of strain CJ-5 was performed using morphological and physiological tests (Dong and Cai, 2001). The corresponding 16S rDNA of the strain CJ-5 was amplified by PCR using primers, FP9 (5′-GAGTTTGATCATGGCTCAGA TTG-3′) corresponded to *Escherichia coli* positions 9~31 and RP1480 (5′-TTCCTTGTTACGACTTCA CCC-3′) corresponded to *E. coli* positions 1460~1480. The thermal cycling program included initial at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min. This was followed by a final extension step at 72 °C for 7 min. The sequencing of PCR product was carried out using primers FP9, RP1480 and 450F (5′-AATAAGCT CCGGCTAACTCC-3′).

Culture condition

Strain CJ-5 was grown on minimal medium (0.07% K_2HPO_4, 0.03% KH_2PO_4, 0.05% $MgSO_4$, 0.03% peptone and 0.03% yeast extract), containing 1%~3% chitin or 1%~3% chitosan, and initial pH of medium was 6.0~8.0. The 50~150 ml sterilized medium in 500-ml flask was inoculated with 1%~3% of 24 h pre-grown cells, and incubated aerobically at 27~33 °C for 7 d under vigorous shaking. The culture solution was centrifuged, and its supernatant was used to measure the enzymes activity. Effects of substrate concentration (chitin or chitosan), culture temperature, capacity of medium, initial pH of medium and amount of inoculation for chitinase and chitosanase production were analyzed, respectively. The optimal condition of the strain fermentation was determined by flask culture.

Enzyme assay

The activities of chitinase and chitosanase were

determined by quantitative estimation of the reducing sugars produced with the colloidal chitin or chitosan as appropriate substrate of enzyme assay (Sun *et al*., 2006). Briefly, the reaction mixture consisted of 0.5 ml enzyme solution and 0.5 ml of 1% colloidal chitin or colloidal chitosan in 1 ml McIlvaine buffer (100 mmol/L citric acid, 200 mmol/L sodium phosphate) at the indicated pH. The mixture was incubated for 30 min at 36 °C for chitinase or 56 °C for chitosanase using a shaking water bath, and the reaction was stopped in boiling water for 10 min. The amount of reducing sugars released in the supernatant was measured by a method that uses dinitrosalicylic (DNS) acid reagent, and the absorbance was measured at 540 nm. One unit (U) of chitinase or chitosanase activity was defined as the amount of enzymes that liberated 1 μmol of the reducing sugar per minutes at the same condition using either GlcNAc or GlcN as the standard.

Enzyme purification

The enzyme was purified from culture supernatant of strain CJ-5. Cell free culture broth was saturated with ammonium sulfate between 30% and 65% concentrations. The solution was left overnight at 4 °C, centrifuged, and the precipitates were dissolved in McIlvaine buffer (pH 7.0). The crude enzyme solution was dialyzed against the same buffer for 24 h, and then subjected to Sephadex G-200 gel filtration column (1.6 cm×60 cm) equilibrated with the same buffer. The protein was eluted with the same buffer at a flow rate of 24 ml/h. Each fraction was collected, and the chitinase and chitosanase activities were measured. The fractions with chitinase or chitosanase activity were separately pooled, concentrated and further applied on DEAE-Sepharose Fast Flow column (1.6 cm×20 cm) pre-equilibrated with 10 mmol/L Tris-HCl (pH 7.6). The absorbed chitinase or chitosanase was eluted by a linear gradient of NaCl from 0 to 0.5 mol/L in the same buffer. The flow rate was maintained at 0.25 ml/min. All purification steps were done at 4 °C. The active fractions corresponding to chitinase and chitosanase were collected and used as source enzymes for enzyme analysis.

SDS-PAGE was performed on 8% separation gels as described by Laemmli (1970). Gels were stained with Coomassie Brilliant Blue.

Enzyme characterization

McIlvaine buffer (pH 3~8) and 50 mmol/L Tris-HCl buffer (pH 8~9) were used for studying the effects of pH and temperature on the activity of chitinase and chitosanase by the standard assay method.

The pH stability of chitinase or chitosanase was assayed from the residual activity after the incubation at 36 °C or 56 °C in the buffer of various pH (3~9) for 60 min. The temperature stability of this enzyme was assayed from the residual activity after the incubation at various temperatures (20~70 °C) at pH 7.0 for 60 min.

The effect of various metal ions on the chitinase or chitosanase activity was verified by adding different metal ions (K^+, Na^+, Ag^+, Ca^{2+}, Mg^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}) at 12.5 mmol/L. The relative activity was calculated with respect to the control where the reaction was carried out in the absence of any additive.

RESULTS

Identification of the chitinolytic-producing strain

A chitinolytic strain CJ-5 was identified, and it showed that the strain is Gram-negative rod-shaped with single polar flagellum, no spore formation, aerobic, oxidase-positive and catalase-positive. A similarity search for the nucleotide sequence of 16S rDNA was carried out online at http://www.ncbi.nlm.nih.gov using the BLAST search program for the nucleotide database maintained in GenBank. The result of this search showed that the 16S rDNA of the strain CJ-5 has a high level of nucleotide sequence identity (98%) with several members of *Sphingomonas* genus, and placed the strain CJ-5 in genus *Sphingomonas*.

Fermentation conditions

We first analyzed the optimal conditions for chitinase and chitosanase production, and it was determined that 2% pro-grown cells of *Sphingomonas* sp. CJ-5 were inoculated into 125 ml medium containing 2.5% substrate of chitin (initial pH 7.0 of medium) or chitosan (initial pH 6.5 of medium) in 500 ml flask, and incubated at 30 °C under vigorous shaking.

Based on above condition, the changes of chitinase, chitosanase, pH, and reducing sugar of culture supernatant were analyzed for 7 d in chitin medium or chitosan medium, respectively. It showed that the chitinase and chitosanase production in both substrates medium increased at first 4 d, and the reducing sugar increased at first 5 d, then declined (Fig.1), while the pH of culture increased from about 7.0 up to above 9.2. The chitinase production was higher in chitin medium than that in chitosan medium, whereas the chitosanase production was higher in chitosan medium than that in chitin medium.

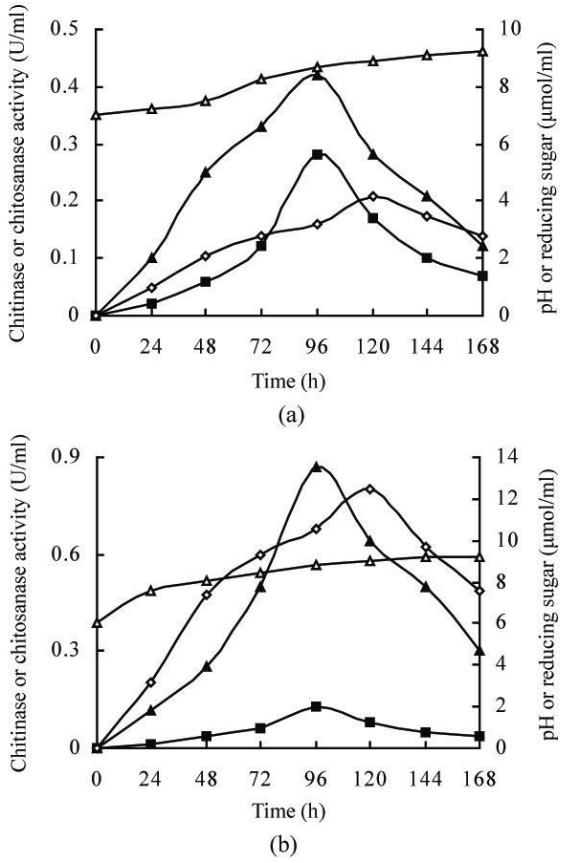

Fig.1 Chitinase and chitosanase activities of *Sphingomonas* sp. CJ-5 incubated in chitin or chitosan medium. *Sphingomonas* sp. CJ-5 was grown in minimum medium containing 2.5% chitin (a) or chitosan (b) at 30 °C. The pH, reducing sugar, chitinase and chitosanase activities of the culture supernatant were analyzed during 6 d of culture

Δ: pH; ◊: Reducing sugar; ■: Chitinase; ▲: Chitosanase

Molecular mass of enzymes

The chitinase and chitosanase of the strain CJ-5 were purified which was described as methods. Both fractions of chitinase and chitosanase were applied to SDS-PAGE. As shown in Fig.2, some bands were observed in precipitate of ammonium sulfate, and one band was in purified chitinase fraction or chitosanase fraction. The molecular mass was estimated to be 230 kDa and 45 kDa for chitinase and chitosanase, respectively.

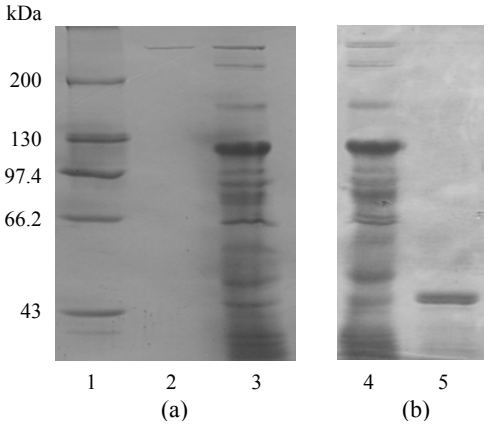

Fig.2 SDS-PAGE analysis of the chitinase (a) and chitosanase (b) secreted by *Sphingomonas* sp. CJ-5

Lane 1: Marker protein (200, 130, 97.4, 66.2, 43 kDa); Lane 2: Chitinase purified from Sephadex G-100 and DEAE-Sepharose Fast Flow; Lanes 3 and 4: Supernatant after precipitation with 65% of saturated concentration of ammonium sulfate; Lane 5: Chitosanase purified from Sephadex G-100 and DEAE-Sepharose Fast Flow

Enzymes properties

The effect of pH and temperature on chitinase and chitosanase activities is shown in Fig.3. The optimal reaction temperatures of the chitinase and chitosanase were determined as 36 and 56 °C, respectively. The optimal pH were 7.0 for chitinase and 6.5 for chitosanase.

The thermal stability of chitinase and chitosanase activity showed that both enzymes were stable below 45 °C (Fig.4a). When the chitinase and chitosanase were kept at 45 and 50 °C for 60 min respectively, the 50% enzyme activity was left, and when the enzymes were kept at 60 °C for 60 min, only a low activity (1.6% for chitinase and 0.6% for chitosanase respectively) could be detected. The thermal stability of the chitosanase was higher than that of the chitinase.

The pH stability of enzymes activity showed that the chitinase activity was stable over a broad pH (Fig.4b), more than 70% of activity retained in the pH

range from 5 to 8, and above 50% in the pH range from 4 to 9, whereas the chitosanase activity was stable in a narrow pH range from 5.5 to 7.5. Below pH 5.0 and above pH 8.0, chitosanase activity fell rapidly, with less than 15% activity detected below pH 5.0 and above pH 9.0.

The effect of different ion on chitinase and chitosanase activity showed that ions such as Mg^{2+} and Mn^{2+} could stimulate the chitinase and chitosanse activity, whereas Cu^{2+}, Co^{2+}, Zn^{2+}, and Ag^{+} ions could inhibit the enzymes activity apparently. Fe^{3+} ion stimulated the chitinase activity but strongly inhibited the chitosanase activity.

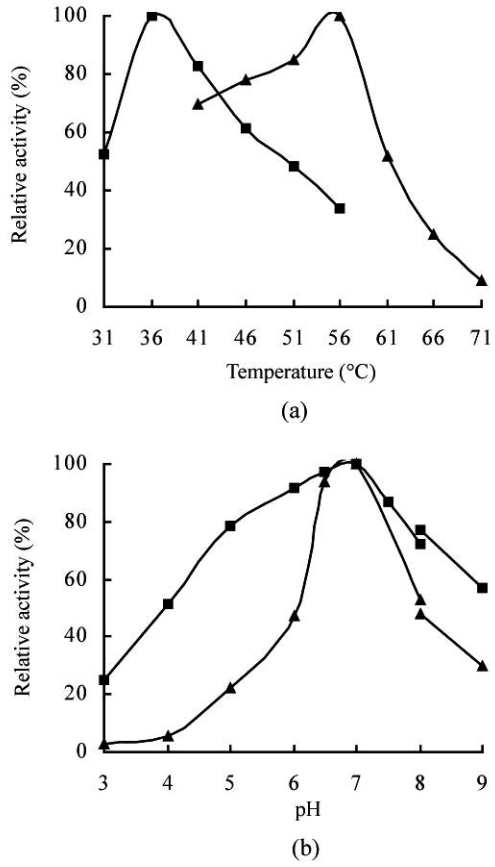

Fig.3 Effects of temperature or pH on chitinase and chitosanase activity. The enzyme activity assays were carried out in McIlvain buffer (pH 7.0) at various temperatures between 31 and 56 °C for chitinase, and between 41 and 71 °C for purified chitosanase. The chitinase and chitosanase activities were measured (a). The enzymatic reactions were performed at various pH between 3.0 and 9.0 (pH 3~8, McIlvaine buffer; pH 8~9, 50 mmol/L Tris-HCl buffer), and the chitinase and chitosanase activities were measured at 36 and 56 °C, respectively (b)

■: Chitinase; ▲: Chitosanase

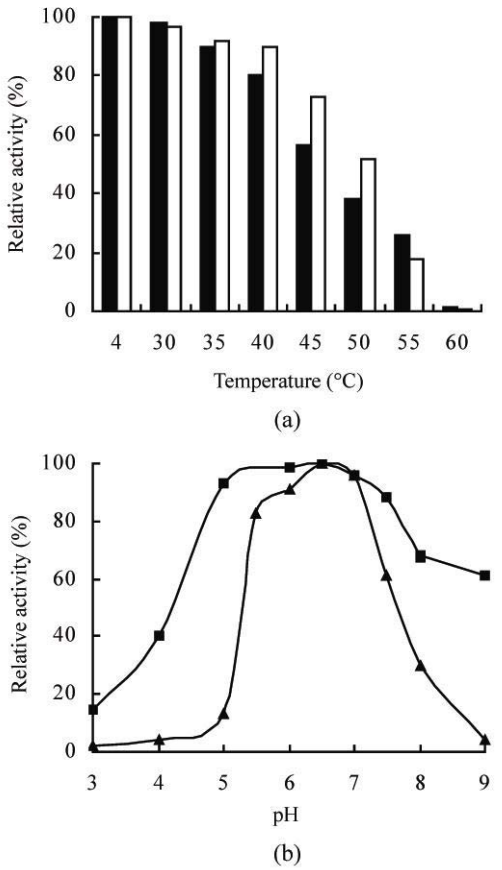

Fig.4 Thermal (a) or pH (b) stability of the chitinase and chitosanase of *Sphingomonas* sp. CJ-5. The temperature stability was investigated by keeping the purified enzymes at various temperatures for 1 h. The pH stability was investigated by keeping the purified enzymes at various pH (pH 3~8, McIlvaine buffer; pH 8~9, 50 mmol/L Tris-HCl buffer) for 1 h. The remaining activities of chitinase and chitosanase were measured in McIlvaine buffer (pH 7.0) at 36 and 56 °C, respectively

(a) ■: Chitinase; □: Chitosanase; (b) ■: Chitinase; ▲: Chitosanase

DISCUSSION

In this study, the purification and biochemical characterization of the chitinase and chitosanase, produced by CJ-5 in a chitin or chitosan medium, have been described. The strain CJ-5 was identified as a novel chitinolytic and chitosanolytic bacterium of genus *Sphingomonas* on the basis of its 16S rDNA sequence, morphology, chemical, and physiological properties. To our knowledge, chitinase or chitosanase from the genus *Sphingomonas* has not yet been reported. This is the first study of both chitinase and

chitosanase secreted by *Sphingomonas* sp.

There have been many reports that microorganisms produce chitinase. The chitinases were discovered in species of the genera, such as bacteria: *Aeromonas* (Lan *et al*., 2006), *Alteronomas* (Tsujibo *et al*., 1993), *Bacillus* (Rivas *et al*., 2000), *Enterobacter* (Chernin *et al*., 1997), *Janthinobacterium* (Gleave *et al*., 1995), *Microbacterium* (Sun *et al*., 2006), *Pseudoalteromonas* (Techkarnjanaruk and Goodman, 1999), *Pseudomonas* (Folders *et al*., 2001), *Serratia* (Gal *et al*., 1998), *Xanthomonas* (Sakka *et al*., 1998), *Vibrio* (Morimoto *et al*., 1993), *Coccidioides* (Pishko *et al*., 1995), *Clostridium* (Morimoto *et al*., 1997), and *Streptomyces* (Kawase *et al*., 2006); fungi: *Candida* (McCreath *et al*., 1995), *Aspergillus* (Xia *et al*., 2001), *Metarhizium* (Kang *et al*., 1998), *Rhizopus* (Yanai *et al*., 1992), *Trichoderma* (Hayes *et al*., 1994) and *Saccharomyces* (Carstens *et al*., 2003). *Sphingomonas* sp. CJ-5 is not only producing chitinase but also chitosanase in the presence of chitin. The reports of bacterium producing both chitinase and chitosanase are only found in *Enterobacter* sp. G-1 (Yamasaki *et al*., 1992), and *Bacillus* sp. X-b (Helistö *et al*., 2001).

Most chitinases are reported inducible. Inoculating *Sphingomonas* sp. CJ-5 in a series of media with various carbon sources, such as glucose, glucosamine, and *N*-acetyl-D-glucosamine, resulted in no significant chitinase activity, although the strain grew well. These suggested that the chitinase and chitosanase of *Sphingomonas* sp. CJ-5 were induced by chitin. An attempt to induce chitinase and chitosanase activity by chitosan was also performed, and our results showed that *Sphingomonas* sp. CJ-5 could produce chitinase and chitosanas in chitosan medium. Moreover, the chitosanase activity in chitosan medium was higher than in chitin medium, and the reducing sugar of culture supernatant was 3-fold of that in chitin medium. It is interesting that *Sphingomonas* sp. CJ-5 produced chitinase in presence of chitosan.

Although it has been reported the chitinase and chitosanase from several sources of hydrolyse carboxymethyl cellulose and chitin to a different extent (Somashekar and Joseph, 1996), the chitinase or chitosanase of *Sphingomonas* sp. CJ-5 cannot hydrolyse carboxymethyl cellulose (date not shown).

The chitinase and chitosanase of *Sphingomonas* sp. CJ-5 are fairly stable and active, and degrade chitin efficiently, although the enzyme yield is not high. Moreover, the ability of *Sphingomonas* sp. CJ-5 to produce maximum chitinase activity under mild conditions of agitation and aeration could be an important advantage in view of further process development. The chitinase and chitosanase from *Sphingomonas* sp. CJ-5 may be potentially valuable for industrial application and recycling of chitin wastes particularly.

References

Carstens, M., Vivier, M.A., Pretorius, I.S., 2003. The *Saccharomyces cerevisiae* chitinase, encoded by the CTS1-2 gene, confers antifungal activity against *Botrytis cinerea* to transgenic tobacco. *Transgenic. Res.*, **12**(4):497-508. [doi:10.1023/A:1024220023057]

Chernin, L.S., De la Fuente, L., Sobolev, V., Haran, S., Vorgias, C.E., 1997. Molecular cloning, structural analysis and expression in *Escherichia coli* of a chitinase gene from *Enterobacter agglomerans*. *Appl. Environ. Microbiol.*, **63**(3):834-839.

Cohen-Kupiec, R., Che, I., 1998. The molecular biology of chitin digestion. *Curr. Opin. Biotechnol.*, **9**(3):270-277. [doi:10.1016/S0958-1669(98)80058-X]

Dong, X.Z., Cai, M.Y., 2001. Determinative Manual of Common Bacterial Systems. Scientific Press, Beijing (in Chinese).

Flach, J., Pilet, P.E., Jolles, P., 1992. What's new in chitinase research. *Experientia*, **48**(8):701-716. [doi:10.1007/BF02124285]

Folders, J., Algra, J., Roelofs, M.S., Loon, L.C., Tommmassen, J., Bitter, W., 2001. Characterization of *Pseudomonas aeruginosa* chitianase, a gradually secreted protein. *J. Bacteriol.*, **183**(24):7044-7052. [doi:10.1128/JB.183.24.7044-7052.2001]

Gal, S.W., Choi, J.Y., Kim, C.Y., Cheong, Y.H., Cheong, Y.J., Choi, Y.J., Bahk, J.D., Lee, S.Y., Cho, M.J., 1998. Cloning of the 52-kDa chitinase gene from *Serratia marcescens* KCTC2172 and its proteolytic cleavage into an active 35-kDa enzyme. *FEMS Microbiol. Lett.*, **160**(1):151-158. [doi:10.1111/j.1574-6968.1998.tb12905.x]

Gleave, A.P., Taylor, R.K., Morris, B.A.M., Greenwood, D.R., 1995. Cloning and sequencing of a gene encoding the 69-kDa extracellular chitinase of *Janthinobacterium lividum*. *FEMS Microbiol. Lett.*, **131**(3):279-288. [doi:10.1111/j.1574-6968.1995.tb07788.x]

Gooday, G.W., 1990. The Ecology of Chitin Degrationtic. *In*: Marshall, K.C. (Ed.), Advance in Microbiol Ecology. New Plenum Press, NY, Vol. 11, p.387-430.

Gooday, G.W., 1997. The many uses of chitinases in nature. *Chitin. Chitosan. Res.*, **3**:233-243.

Hayes, C.K., Klemsdal, S., Lorito, M., Di, P.A., Peterbauer, C., Nakas, J.P., Tronsmo, A., Harman, G.E., 1994. Isolation and sequence of an endochitinase-encoding gene from a

cDNA libray of *Trichoderma harzianum*. *Gene*, **138**(1-2): 143-148. [doi:10.1016/0378-1119(94)90797-8]

Helistö, P., Aktuganov, G., Galimzianova, N., Melentjev, A., Korpela, T., 2001. Lytic enzyme complex of an antagonistic *Bacillus* sp. X-b: isolation and purification of components. *J. Chromatogr. B Biomed. Sci. Appl.*, **758**(2): 197-205. [doi:10.1016/S0378-4347(01)00181-5]

Henrissat, B., Bairoch, A., 1993. New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.*, **293**(3):781-788.

Henrissat, B., Davies, G., 1997. Structural and sequence-based classification of glycoside hydrolases. *Curr. Opin. Struct. Biol.*, **7**(5):637-644. [doi:10.1016/S0959-440X(97)80072-3]

Hirano, S., 1989. Production and Application of Chitin and Chitosan in Japan. *In*: Chitin and Chitosan. Elsevier, Essex, p.37-43.

Hirano, S., 1996. Chitin biotechnology applications. *Biotechnol. Annu. Rev.*, **2**:237-258.

Kang, S.C., Sanggyu Park, S., Lee, D.G., 1998. Isolation and characterization of a chitinase cDNA from the entomopathogenic fungus, *Metarhizium anisopliae*. *FEMS Microbiol. Lett.*, **165**(2):267-271. [doi:10.1111/j.1574-6968.1998.tb13156.x]

Kawase, T., Saito, A., Sato, T., Kanai, R., Fujii, T., Nikaidou, N., Miyashita, K., Watanabe, T., 2004. Distribution and phylogenetic analysis of family 19 chitinases in *Actinobacteria*. *Appl. Environ. Microbiol.*, **70**(2):1135-1144. [doi:10.1128/AEM.70.2.1135-1144.2004]

Kawase, T., Yokokawa, S., Saito, A., Fujii, T., Nikaidou, N., Miyashita, K., Watanabe, T., 2006. Comparison of enzymatic and antifungal properties between family 18 and 19 chitinases from *Streptomyces coelicolor* A3(2). *Biosci. Biotechnol. Biochem.*, **70**(4):988-998. [doi:10.1271/bbb.70.988]

Laemmli, U.K., 1970. Cleavage of structural proteins duing the assembly of the head of bacteriophage T4. *Nature*, **227**(5259):680-685. [doi:10.1038/227680a0]

Lan, X., Zhang, X., Hu, J., Shimosaka, M., 2006. Cloning, expression and characterization of a chitinase from the chitinolytic bacterium *Aeromonas hydrophila* strain SUWA-9. *Biosci. Biotechnol. Biochem.*, **70**(10): 2437-2442. [doi:10.1271/bbb.60169]

McCreath, K.J., Specht, C.A., Robbins, P.W., 1995. Molecular cloning and characterization of chitinase genes from *Candida albicans*. *Proc. Natl. Acad. Sci. USA*, **92**(7): 2544-2548. [doi:10.1073/pnas.92.7.2544]

Mendonsa, E.S., Vartak, P.H., Rao, J.U., Deshpande, M.V., 1996. An enzyme from *Myrothecium verrucaria* that degrades insect cuticles for biocontrol of *Aedes aegypti* mosquito. *Biotechnol. Lett.*, **18**(4):373-376. [doi:10.1007/BF00143454]

Morimoto, K.S., Karita, T., Kirchman, D.L., 1993. Role of chitinase proteins in the specific attachment of the marine bacterium *Vibrio harveyi* to chitin. *Appl. Environ. Microbiol.*, **59**(2):373-379.

Morimoto, K.S., Karita, T., Kimura, T., Sakka, K., Ohmiya, K., 1997. Cloning, sequencing, and expression of the gene encoding *Clostridium paraprtrificum* chitinase chiB and analysis of the functions of novel cadherin-like domains and a chitin-binding domain. *J. Bacteriol.*, **179**(23): 7306-7314.

Park, J.K., Okamoto, T., Yamasaki, Y., Tanaka, K., Nakagawa, T., Kawamukai, M., Matsuda, H., 1997. Molecular cloning, nucleotide sequencing, and regulation of chiA gene encoding one chitinase from *Enterobacter* sp. G-1. *J. Ferment. Bioeng.*, **84**(6):493-501. [doi:10.1016/S0922-338X(97)81901-0]

Pishko, E.J., Kirkland, T.N., Cole, G.T., 1995. Isolation and characterization of two chitinase-encoding genes (cts1, cts2) from the fungus *Coccidioides immittis*. *Gene*, **167**(1-2):173-177. [doi:10.1016/0378-1119(95)00654-0]

Rivas, L.A., Parro, V., Moreno-Paz, M., Mellado, R., 2000. The *Bacillus subtilis* 168 *csn* gene encodes a chitosanas with similar properties to a *Streptomyces* enzyme. *Microbiology*, **146**:2929-2936.

Saito, J., Kito, A., Higuchi, Y., Nagata, Y., Ando, A., Miki, K., 1999. Crystal structure of chitosanase from *Bacillus circulans* MH-K1 at 1.6-A resolution and its substrate recognition mechanism. *J. Biol. Chem.*, **274**(43): 30818-30825. [doi:10.1074/jbc.274.43.30818]

Sakka, K., Kusaka, R., Kawano, A., Karita, S., Sukhumavasi, J., Kimura, T., Ohmiya, K., 1998. Cloning and sequencing of the gene encoding chitinase ChiA from *Xanthomonas* sp. strain AK and some properties of ChiA. *J. Ferment. Bioeng.*, **86**(6):527-533. [doi:10.1016/S0922-338X(99)80001-4]

Sandorf, P.A., 1989. Chitosan: Commercial Uses and Potential Applications. *In*: Chitin and Chitosan, Elsevier, London, p.51-69.

Somashekar, D., Joseph, R., 1996. Chitosanase-properities and applications: a review. *Bioresource Technology*, **55**(1): 35-45. [doi:10.1016/0960-8524(95)00144-1]

Sun, Y., Liu, W., Han, B., Zhang, J., Liu, B., 2006. Purification and characterization of two types of chitosanase from a *Microbacterium* sp. *Biotechnol. lett.*, **28**(17):1393-1399. [doi:10.1007/s10529-006-9101-z]

Techkarnjanaruk, S., Goodman, A.E., 1999. Multiple genes involved in chitin degradation from the marine bacterium *Pseudoalteromonas* sp. strain S91. *Microbiology*, **145**(4): 925-934.

Tsujibo, H., Orikoshi, H., Tanno, H., Fujimoto, K., Miyamoto, K., Imada, C., Okami, Y., Inamori, Y., 1993. Cloning, sequence, and expression of a chitinase gene from a marine bacterium, *Alteromonas* sp. strain O-7. *J. Bacteriol.*, **175**(1):176-181.

Watanabe, T., Kanai, R., Kawase, T., Tanabe, T., Mitsutomi, M., Sakuda, M., Miyashita, K., 1999. Family 19 chitinases of *Streptomyces* species: characterization and distribution. *Microbiology*, **145**(12):3353-3363.

Xia, G., Jin, C., Zhou, J., Yang, S., Jin, C., 2001. A novel chitinase having a unique mode of action from *Aspergillus fumigatus* YJ-407. *Eur. J. Biochem.*, **268**(14):

4079-4085. [doi:10.1046/j.1432-1327.2001.02323.x]

Yabuki, M., Uchiyama, A., Suzuki, K., Ando, A., Fujii, T., 1988. Purification and properties of chitosanase from *Bacillus circulans* MH-K1. *J. Gen. Appl. Microbiol.*, **34**:255-270.

Yamasaki, Y., Ohto, Y., Morita, K., Nakagawa, T., Kawamukai, M., Matsuda, H., 1992. Isolation, identification, and effect of oxygen supply on cultivation of chitin and chitosan degrading bacterium. *Biosci. Biotech. Biochem.*, **56**(8):1325-1326.

Yanai, K., Takaya, N., Kojima, N., Horiuchi, H., Ohta, A., Takagi, M., 1992. Purification of two chitinase from *Rhizopus oligosporus* and isolation and sequencing of the encoding genes. *J. Bacteriol.*, **174**(22):7398-7406.

Zhu, X.F., Wu, X.Y., Dai, Y., 2003. Fermentation conditions and properties of a chitosanase from *Acinetobacter* sp. C-17. *Biosci. Biotechnol. Biochem.*, **67**(2):284-290. [doi:10.1271/bbb.67.284]