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Isolation, Purification and Characterization of Chitosanase from *Bacillus subtilis* CH1

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Bacillus subtilis strain with highly active chitosanase was isolated from the intestine of *Sebastiscus marmoratus* (scorpion fish). It was named as *Bacillus subtilis* CH1 by morphological, biochemical and 16S rRNA gene analysis. The optimal conditions for chitosanase production were investigated. The optimum carbon and nitrogen sources for *Bacillus subtilis* CH1 were 2% starch and 1% yeast extract respectively. Unlike other chitosanases, the expression of this chitosanase was not induced or slightly induced with chitosan. The chitosanase secreted into the medium were concentrated with ammonium sulfate precipitation and purified by gel permeation chromatography. The molecular weight of purified chitosanase was 30 kDa. The optimum pH and temperature of purified chitosanase were 5.5 and 60°C respectively. The purified chitosanase was continuously thermostable at 40°C and showed stable activity between pH 6.0 and 8.0. Chitosanase activity of *Bacillus subtilis* CH1 under optimum condition was 4.1 units/ml.

Keywords: Chitosanase, *Bacillus subtilis*, Bacteria screening, Identification, Protein purification

Introduction

Chitin is mainly compound of shell of crustaceans and the shells are produced 1,000 billion tons per annum in worldwide and most of which are being disposed into the sea, creating a potential environmental problem (Allan et al., 1978). Chitosan was first identified as a minor component of cell walls of *Phycomyces blakesleeanus* (Kreger, 1954) and can be produced from chitin through the chemical N-deacetylation. Chitosans are high molecular weight linear polysaccharides consisting of 1,4-ß-linked D-glucosamine residues and are hydrolyzed by chitosanase.

Chitosan and its partially degraded oligosaccharides are becoming important because of their potential usefulness and novel application in the field of functional foods, medical aids, pharmaceuticals, and agricultural agents (Li et al., 1992). In addition, chitosanases may find important industrial applications in the utilization of the enormous chitosan substrates as it is reported that chito-oligosaccharides has antibacterial activity (Jeon et al., 2001; Savard et al., 2002; Tokoro et al., 1989), antifungal activity (Kendra et al., 1989), antitumor activity (Suzuki et al., 1986; Tokoro et al., 1988b) and immuno-enchancing effects (Tokoro et al., 1988a).

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Three main classes of chitosanases are identified according to their substrate specifities. The first class is the chitosanases that degrade chitosan upon recognizing a GlcNAc-GlcN bond, those that recognize both the GlcNAc-GlcN and the GlcNGlcN bond, and those that are specific to the GlcNGlcN bond only. The final group of chitosanases has been purified from *Bacillus* sp. PI-7S (Seino et al., 1991) and *Bacillus* sp. No. 7-M (Izume et al., 1992).

Chitosanase activity has been detected in a variety of prokaryotes (Palletier and Sygusch, 1990), fungi (Fenton and Eveleigh, 1981), plants (Ouakfaoui and Asselin, 1992), and viruses (Yamada et al., 1997). Among these, bacterial chitosanases awaits a break through as it could be especially useful in obtaining large amounts of chitosan oligomers and the enzyme can be easily prepared. Most bacteria and fungi producing chitosanases are known to secrete chitosanase extracellularly (Boucher et al., 1992; Pelletier and Sygusch, 1990; Sakai et al., 1991; Yamasaki et al., 1992), which mostly induced by chitosan as the carbon source. Intracellular chitosanase are found in plants and zygomycete fungi.

In this study, our aim was to isolate a micro-organism from the intestine of *Sebastiscus marmoratus* that produces high active chitosanase, optimize the chitosanase producing conditions and purify and characterize the enzyme in order to obtain the maximum use in the industry.

Materials and Methods

Screening of chitosanase producing bacteria

In order to isolate chitosanase producing bacterial strains, samples from intestine of *Sebastiscus mamoratus* were used and chitosan (Sigma co., USA) was used as the only carbon source in minimal medium containing $(NH_4)_2HPO_4$ 0.5%, K_2HPO_4 0.21%, KH_2PO_4 0.09%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4$ 0.001%, $ZnSO_4$ 0.0001%, $MnCl_2$ 0.0001%, agar 1.2%. Isolated bacteria were incubated in Luria Bertani (LB) broth for 16 hours at 30°C and at 200 rpm. The cultured bacteria were centrifuged at 15,000 rpm for 1 min and the supernatant was tested for chitosanase activity. Bacterial strains with high chitosanase activity were pooled.

Identification of bacteria

The morphological characteristics of the selected bacteria were observed under the scanning electron microscope (SEM) (Hitachi S-2460N, Japan). The bacteria was incubated on LB agar plate for SEM and grown at 30°C for 12 hrs. One bacterial colony was cut (0.5×0.5 cm) from LB agar plate and treated with 2.5% glutaraldehyde solution for 2 hrs. The colony was washed two times with 1X PBS for 5 min and dehydrated in 40, 50, 60, 70, 80, 90 and 100% ethanol for 1 hr in each solution. The colony was moved into mixing solution (isoamyl acetate : ethanol = 1 : 3, 1 : 1, 3 : 1) for 1 hr and kept in 100% isoamyl acetate for more than 1 hr. A photograph of the colony was taken by the SEM after treatment and drying with CO_2 and coating with gold.

The biochemical characteristics of the bacteria were tested by the method described in Mac Faddin (1995).

To identify 16S rRNA gene from the bacteria, chromosomal DNA was extracted with QIAamp DNA Mini Kit (Qiagen Inc., Germany). 16S rRNA gene was amplified using polymerase chain reaction (PCR) with the primer 5'-CCAGACTCCTACGGGAGGCAGCA-3' as the sense primer and 5'-TTGACGTCRTCCCCACCTTCCTC-3' as the antisense primer. These primers were designed by comparing 16S rRNA sequence of other chitosanase producing bacteria from NCBI data. The PCR mixture included 0.2 μg of template, 20 pmole of each primer, 10 mM of dNTP mixture, 5 μl of 10X *pfu* DNA polymerase buffer (Mg^{2+} included) and 3 units of *pfu* DNA polymerase (Bioneer co., Korea) in 50 reaction volume. The initial denaturation step was 5 min at 94°C, 30 cycles of amplification were carried out with the following cycles: 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min. Finally, extension step was carried out for 10 min at 72°C. The PCR product was identified on 1% agarose gel and purified with Gel Purification Kit (Bioneer co., Korea). The purified PCR product was cloned into pBlueScript SK (-) vector and transformed into *Escherichia coli* DH5α. Plasmid extraction was carried out with plasmid extraction kit (Bioneer co., Korea). Sequencing of 16S rRNA gene was conducted by Macrogen (Korea). Sequencing result was compared with published 16S rRNA sequences using NCBI Blast search.

Chitosanase assay

Chitosanase activity was assayed using soluble chitosan as the substrate. Soluble chitosan (1%) was prepared by adding 1 ml of acetic acid into 80 ml of distilled water followed by adding 1 g of chitosan. The solution was mixed and pH was adjusted to 5.5 with 1 N NaOH. Finally, the volume was made up to 100 ml with distilled water. Cultured supernatant was used as the enzyme solution and added into 1% soluble chitosan. Reaction was carried out at 50°C for 10 min according to the method invented by Rondle and Morgan (1995).

Optimization of the conditions for chitosanase production

Starch, soluble chitosan, chitosan powder, chitin powder, mannitol, lactose, galactose, glycerol, fructose, maltose, glucose and sucrose were used in order to identify the optimal carbon source. 0.5% of each component in minimal medium containing 0.3% yeast extract as the nitrogen source was used in this experiment. After determining the optimal carbon source, optimal concentration was determined.

Yeast extract, pepton, trypton, $NaNO_3$ and $(NH_4)_2SO_4$ were used in order to identify the optimal nitrogen source. After determining the optimal nitrogen source, optimal concentration was also determined.

Each culture was grown at 30°C for 10 hrs with shaking at 200 rpm. After incubation, culture was centrifuged at 15,000 rpm for 1 min and then the supernatant was used for chitosanase assay.

To identify the activity at optimal condition, the strain was cultured at 30°C for 28 hrs with optimal carbon and nitrogen sources and activity was checked every 4 hrs with the supernatant. Also, to compare the activity of chitosanase from culture grown in LB broth, the same method was followed.

Purification of chitosanase

Chitosanase producing bacteria was incubated in 1 L of LB broth at 30°C for 20 hrs and supernatant was collected by cen-

trifugation (4000 rpm for 10 min at 4°C). Crude proteins was precipitated by adding ammonium sulfate to the final concentration of 90% (w/v) and proteins were harvested by centrifugation (15,000 rpm for 30 min at 4°C). The pellet was dissolved in 1X PBS and dialyzed in order to desalt crude protein using dialysis membrane (MWCO:12-14,000). After desalting, the solution was added into sephadex-G 100 column for gel permeation chromatography and 1 ml fractions were collected. Each fraction was assayed for activity and SDS-polyacrylamide gel electrophoresis was performed to detect the purified chitosanase.

Characterization of chitosanase

Soluble chitosan (1%) was mixed with purified chitosanase and kept at a range of different temperatures from 30 to 80 degrees with 5°C intervals for 10 min. To test the thermostability, enzyme samples were subjected to 40, 50, 60, 70 and 80°C for 10, 20, 30 and 60 min. Chitosanase activity was also measured at different pH (3.5~7.5). Different buffers used to test the pH stability were 0.1 M acetate buffer (pH 3.5~5.5), 0.2 M phosphate buffer (pH 6.5~7.5) and glycine-NaOH buffer (pH 8.5~10.5). The pH stability of the chitosanase was determined by measuring the residual activity at 40°C for 1 hr.

Results

Screening and identification of chitosanase producing bacteria strain

Bacterial strain that produces chitosanase was found from intestine of *Sebastiscus marmoratus*. The strain showed high chitosanase activity with chitosan. The strain was identified by morphological, biochemical, physiological and genetic methods. Size of the strain was observed as 1.5~3.0 μm under SEM (Fig. 1). Taxonomic characteristics are shown in Table 1. 16S rRNA sequence of the strain was 99% similar to that of *B. subtilis*. With these results, the strain was called as *Bacillus subtilis* CH1.

Table 1. Morphological and biochemical characteristics of *B. subtilis* CH1

	Characteristics result
Size	0.4-0.6 μm width × 1.5-3.0 μm length
Gram stain	+
Shape of cell	rod
Spore	+
Oxidase	-
Catalase	+
MR	-
VP	-
indole	-
O/F	O
TSI	K^W/A
Motility	+

Optimal condition of *B. subtilis* CH1 for chitosanases production

Effect and the concentration of the carbon source on chitosanase production are shown in Fig. 2 and 3. The highest chitosanase activity was observed for *B. subtilis* CH1 when 2% starch was used. Effect and the concentration of nitrogen source on chitosanase production is shown in Fig. 4 and 5. The highest chitosanase activity was observed when 1% yeast

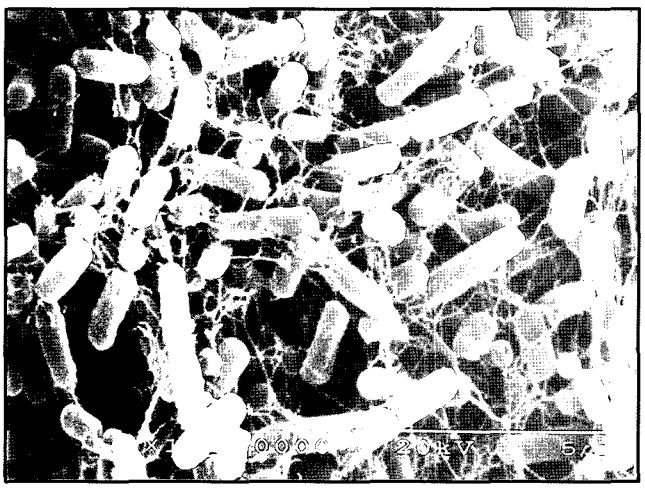

Fig. 1. Scanning electron micrograph (SEM) of *B. subtilis* CH1.

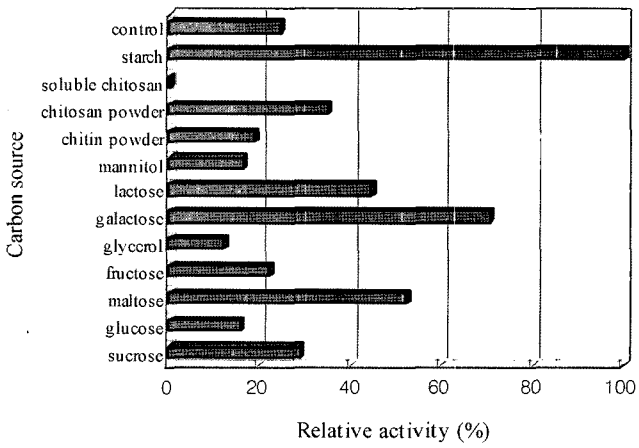

Fig. 2. Effect of carbon sources on the chitosanase production of *B. subtilis* CH1. Experiment was carried out at 30°C for 10 hrs. The basal medium alone was used in the control.

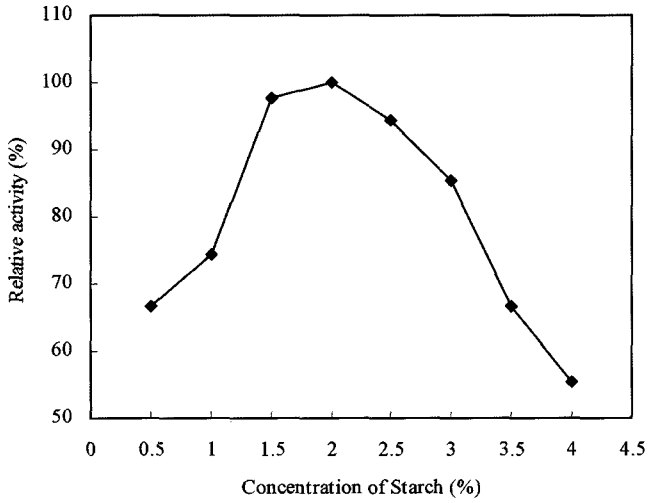

Fig. 3. Effect of starch concentration on the chitosanase production of *B. subtilis* CH1. Experiment was carried out at 30°C for 10 hrs.

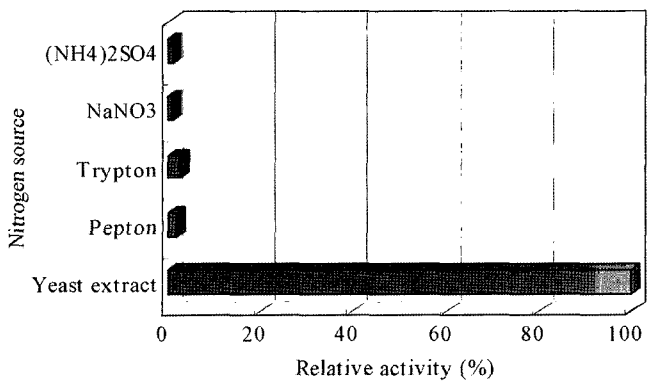

Fig. 4. Effect of nitrogen source on the chitosanase production of *B. subtilis* CH1. Experiment was carried out at 30°C for 10 hrs.

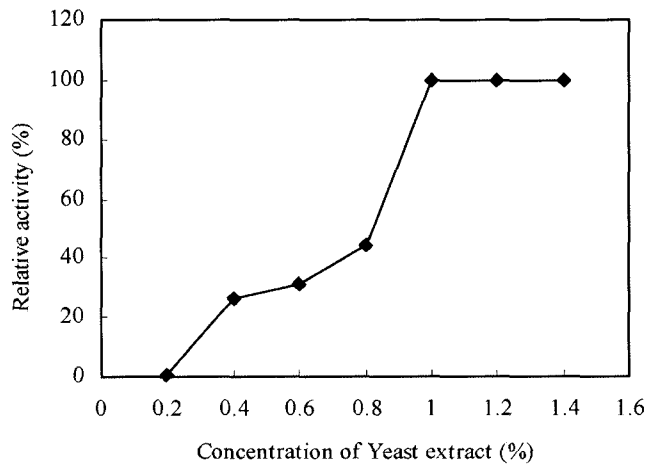

Fig. 5. Effect of yeast extract concentration on the chitosanase production of *B. subtilis* CH1. Experiment was carried out at 30°C for 10 hrs.

extract was used. Thus, 2% starch and 1% yeast extract were used to provide optimal culture conditions and the activity acquired under these conditions was 4.1 units/ml after 24 hrs

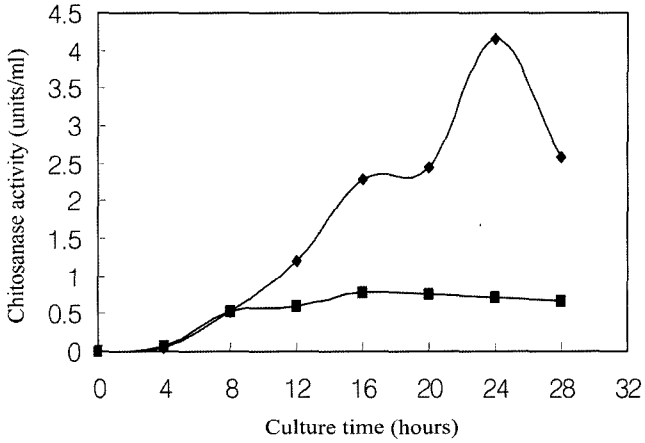

Fig. 6. Activity of chitosanase following different time period under LB broth (■) and optimum condition (◆). Experiment was carried out at 30°C for 28 hrs.

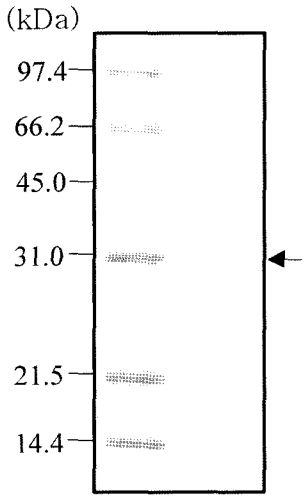

Fig. 7. Gel electrophoresis analysis of purified chitosanase from *B. subtilis* CH1 by gel permeation chromatography. lane 1 : protein marker, lane 2 : purified chitosanase from *B. subtilis* CH1.

at 30°C (Fig. 6). The highest activity obtained for chitosanase grown with LB broth was 0.78 units/ml.

Purification of chitosanase

Crude protein solution was obtained by salting out the enzyme solution with ammonium sulfate. Chitosanase was purified by gel permeation chromatography using sphadex-G 100 column. The molecular weight of the purified chitosanase when analysed with SDS-PAGE showed around 30 kDa (Fig. 7).

Characterization of purified enzyme

Optimal temperature and pH of purified chitosanase was 60°C (Fig. 8) and 5.5 (Fig. 9) respectively. Thermal stability

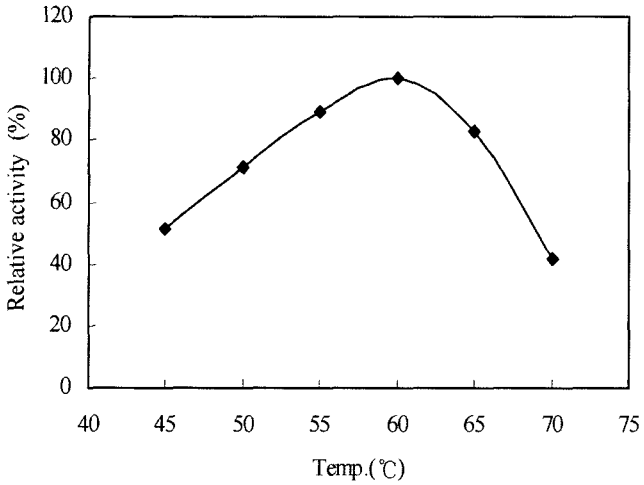

Fig. 8. Effect of temperature on the activity of chitosanase obtained from *B. subtilis* CH1.

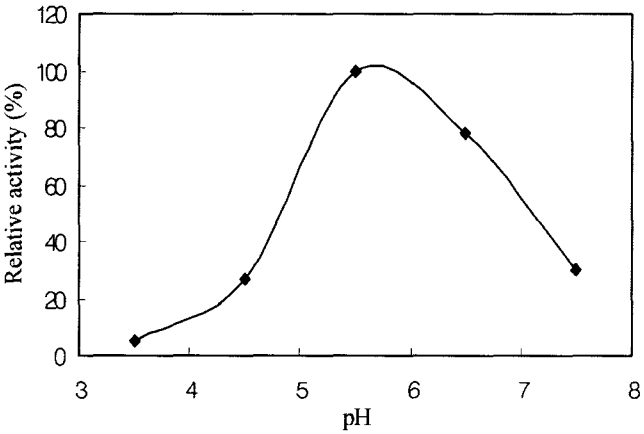

Fig. 9. Effect of pH on the activity of chitosanase obtained from *B. subtilis* CH1.

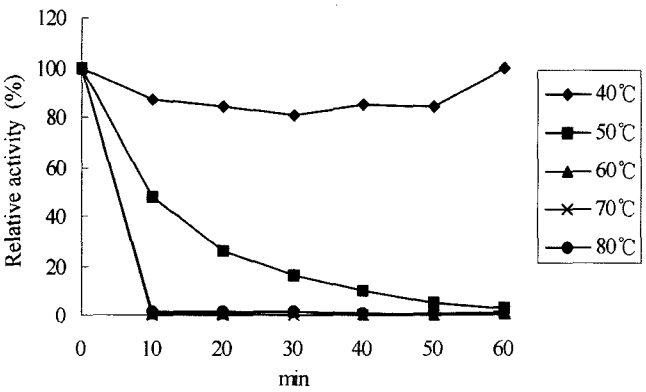

Fig. 10. Thermal stability of chitosanase obtained from *B. subtilis* CH1.

of chitosanase at different incubation temperatures showed that chitosanase was stable upto 40°C and the stability was gradually decreased when the temperature was increased to 50°C but rapidly decreased at 60°C (Fig. 10). Chitosanase was

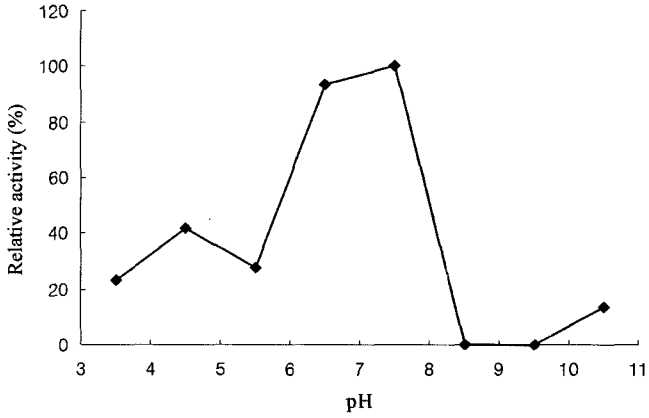

Fig. 11. pH stability of chitosanase obtained from *B. subtilis* CH1.

stable between pH 6.0 and 8.0 (Fig. 11).

Discussion

A bacterial strain that secretes chitosanase extracellularly from intestine of *S. marmoratus* was isolated. The chitosanase secreted by the strain had high activity towards chitosan. The selected strain was gram positive rod bacteria and the size was 1.5-3.0 μm in length. The colony form was round in shape and the mucus was secreted in large quantity (data not shown). The strain was similar to *B. subtilis* according to morphological, biochemical and 16S rRNA sequence analysis. Therefore, we conclude that this strain belongs to *B. subtilis* and we named it as *B. subtilis* CH1. Growth rate of the strain and the chitosanase activity was directly proportional (date not shown).

Optimal carbon source was starch and showed high activity at 2%. Most of the other studies have reported that bacteria need chitosan as inducer for production of chitosanase (Price and Storck, 1975; Pelletier and Sygusch, 1990; Seino et al., 1991; Tominaga and Tsujisaka, 1975; Yoshihara et al., 1990). However, chitosanase production of *B. subtilis* CH1 was slightly induced or not induced by chitosan, rather induced highly by another carbon sources. Glucosamine was also tried as the carbon source and the activity observed was also high compared to other carbon sources (data is not shown). However, glucosamine can not be used in industry due to economical reasons.

Optimal nitrogen source for chitosanase production was observed to be yeast extract, on the other hand, activities observed for other nitrogen sources were significantly low. Optimal concentration of yeast extract for chitosanase activ-

ity was shown to be at 1%. However, more than 1% could not increase the activity but maintained at a same level. The highest chitosanase activity under optimal condition showed 4.1 units/ml at 24 hrs. The activity observed for chitosanase under optimal medium was 5 folds higher compared to that of chitosanase incubated in LB broth.

Purification of chitosanase was performed by applying concentrated crude protein directly into sephadex-G 100 column. Each fraction was tested for chitosanase activity and subjected to SDS-PAGE to find out purified chitosanase. The molecular weight of purified chitosanase was about 30 kDa. The size of the chitosanase from *B. subtilis* CH1 is similar to the size of chitosanases from *Myxobacter* AL-1 (Hedges and Wolfe, 1974), *Bacillus* R4 (Tominaga and Tsujisaka, 1975) and *Penicillium islandicum* (Fenton and Eveleigh, 1981).

Optimal temperature of the purified chitosanase was 60°C, however it was not stable at this temperature. It was more stable at 40°C continuously. In the other reported papers, optimal temperatures of chitosanase from *Bacillus circulans* (Davis and Eveleigh, 1984) and *Bacillus subtilis* 168 (Rivas et al., 2000) were 60°C. Chitosanase of *Bacillus subtilis* 168 (Rivas et al., 2000) was observed to be stable at 37°C but the stability rapidly decreased above 50°C. Optimal pH of purified chitosanase was 5.5 and the activity was stable between pH 6.0 and 8.0. Optimal pH of most of the reported chitosanases vary between 4.0 to 8.0 (Somashekar and Joseph, 1996).

The high active chitosanase produced by the *B. subtilis* CH1 could be useful in chito-oligosaccharide industries provided the optimal culture media and the enzyme conditions. we are planning to conduct future studies in analysing the gene responsible for chitosanase and overexpress it in a suitable recombinant expression system.

Acknowledgement

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