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Identification and Production of Constitutive Chitosanase from *Bacillus* sp. HW-002

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A chitosanase-producing bacteria was isolated on chitosan agar plate from soil samples. The strain was spore-forming gram positive bacteria, catalase positive, and rod shape. The strain was identified as *Bacillus cereus*. The strain did not need an inducer for the synthesis of chitosanase. Chitosanase from *Bacillus* sp. HW-002 was constitutive enzyme. The optimal medium for the production of the enzyme was composed of 0.5% sucrose and 1.5% yeast extract-tryptone (1 : 1 w/w) mixture at pH 6.5. After *Bacillus* sp. HW-002 was cultivated at 32°C for 32 h, maximal productivity was gained to be about 27,200 U/l. Chitosanase from *Bacillus* sp. HW-002 was a mixed growth-linked metabolite.

Chitosan is a deacetylated deriverative of chitin, which is a linear polysaccharide consisting of β-1,4-N-acetylglucosamine. Chitin is a plentiful biomass which is widely distributed in nature as the skeletal structure of crustaceans, insects, mushrooms, and the cell wall of fungi. Conservative estimates of the amount of crustacean shells produced worldwide fall in the range of 1.5×10^5 metric tons *per annum* (3). Most of crustacean shells are being deposited into the sea and creating a potential environmental problem. Transformation of this unused biomass could be advantageous, since chitin and chitosan derivatives have numerous applications (15). Chitosan can be obtained by chemically deacetylation of chitin that has been extracted from abundant natural sources (15). Chitin is insoluble but chitosan is soluble in water, therefore enzymatic hydrolysis of chitosan is more effective than enzymatic hydrolysis of chitin.

Low molecular weight chitooligosaccharides have received attention, because of their interesting biological properties, including their inhibitory effects on the growth of fungi (8, 9) and bacteria (2, 22), host associated antitumor activity (19, 21), activation of immune response (18, 20), and their ability to elicit phytoalexin production in higher plants (6, 7). Chitosanase-producing microorganisms have been found in many bacteria, actinomycetes and fungi (5, 12, 14, 16, 23, 26). In previous reports (5, 12, 14, 16, 23, 26), the microorganisms needed chitosan as an inducer for the production of chitosanases. However, if chitosanase-producing microorganism which do not need chitosan as an inducer is screened, it has technical advantages at the fermentation because chitosan has a viscosity in the broth and react with other organic compounds of medium for heat sterilization. Chitosanase can be used for the production of chitooligosaccharide because enzymatic hydrolysis has advantages over chemical depolymerization (3). The advantages are production of selectively depolymerized chitooligosaccharide due to enzymatic specificity, mild reaction, and low pollutant production etc.

In order to find useful enzyme for production of selectively depolymerized chitooligosaccharide, microorganisms which were able to use chitosan as a sole carbon source were screened from soil samples. Bacteria was screened for the large production of extracellular chitosanase, and the bacteria produced constitutively chitosanase. Therefore, this strain was investigated into the taxonomical properties, lipid composition for the classification, and cultural conditions for the production of chitosanase.

MATERIALS AND METHODS

Isolation and Screening of Chitosanase-producing Microorganisms

The microorganisms were isolated from soil samples on chitosan agar plates as the sole carbon source. Isolated bacteria and actinomycetes were incubated in chitosan broth with shaking at 30°C for 4-6 days. The chi-

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tosan broth consisted of 0.5% chitosan, 0.1% yeast extract, 0.1% K_2HPO_4 and 0.05% $MgSO_4 \cdot 7H_2O$ at pH 6.5. When chitosan was used as a carbon source, it was autoclaved separately from other constituents after it was dissolved in 0.2 N acetic acid and the pH of the solution was adjusted to pH 6.5 with 1.0 N sodium acetate. The culture was centrifuged at 10,000×g and the chitosanase activity of the supernatant was examined. Bacteria which actively produced extracellular chitosanase were screened and the bacteria were used in the experiments for the production of chitosanase. During the experiments, the strain was maintained on tryptic soy broth agar (TSBA; Difco) at 4°C and transferred at 2 weeks.

Enzyme Assay and Measurement of Cell Growth

Chitosanase activity was examined by measuring the amount of reducing sugars released from the enzyme reaction using the dinitrosalicylic acid (DNS) method (4). The substrate was 0.3% chitosan dissolved in 50 mM sodium acetate buffer (pH 5.6). In 490 μl substrate solution, 10 μl enzyme solution was added and the reaction mixture was incubated in a water bath at 40°C for 10 min. The reaction was terminated by addition of 500 μl 1 N KOH, and then the mixture was centrifuged at 5,000×g for 5 min. The supernatant (0.5 ml) was transferred to a 5 ml tube and added to a 0.5 ml DNS solution. The mixture was boiled for 15 min, and then cooling with tap water. The optical density was measured by spectrophotometer (Shimadzu UV-240) at 540 nm. One unit (U) of activity was defined as the amount of enzyme which liberated 1 μmole of reducing sugar per min, and glucosamine was used as the standard. Cell growth was gauged as a measure of turbidity at 660 nm by spectrophotometer.

Taxonomical Tests of Bacteria

Taxonomical studies were carried out according to biochemical tests for the identification of medical bacteria (10). The morphological and sporulated characteristics were investigated using a scanning electron microscope or light microscope. The strain was classified according to Bergey's Manual of Systematic Bacteriology (13). Major fatty acid composition was analyzed by gas liquid chromatography (GLC) with a fused silica capillary column (25 m×0.2 mm). The bacteria was cultivated on TSBA at 30°C for 24 h and then 40-50 mg cells were harvested. The cells were treated by NaOH and HCl in aqueous methanol, and extracted with hexane/ether. The analysis conditions with GLC were at 170-270°C column temperature(elevated at 5°C/min), 250°C injector temperature and 300°C detector temperature. The carrier gas was nitrogen. The result was analyzed with a microbial identification system (MIS, Hewlett-Packard 5890A) (1, 11).

Cultural Conditions for the Production of Chitosanase

To determine the conditions for the production of chitosanase, the basal medium used through out this experiments was 0.5% starch, 0.75% tryptone and 0.75% yeast extract at pH 6.5. The chitosanase-producing bacteria were cultured in 300 ml Erlenmeyer flasks containing 50 ml medium. The effects of pH and temperature on the production of chitosanase were investigated with basal media from pH 3.0 to 10.0 and from 26 to 38°C, respectively. For the investigation into the effects of carbon and nitrogen sources, the media were supplemented with 0.5% carbon source and 1.5% nitrogen source. The best carbon and nitrogen concentrations were shown to be between 0.25 and 2.0% for the optimal production of chitosanase. The media were adjusted to pH 6.5 and were autoclaved at 121°C for 15 min. A loop of bacteria was transferred to the medium, and then incubated with shaking(180 strokes, width 6 cm) at 32°C for 36-56 h. The cultures were centrifuged at 10,000×g for 10 min and then the supernatant was used for chitosanase assay.

RESULTS AND DISCUSSION

Isolation and Screening of Chitosanase-producing Microorganism

About 50 strains of bacteria and actinomycetes which produced clear zone around the colonies were isolated from soil samples. The isolated strains were cultured on broth containing chitosan, and then the chitosanase activity of the supernatants were measured. The strain of *Bacillus* sp. HW-002 had the best chitosanolytic activity and the bacteria was chosen for the production of chitosanase.

Identification of the Isolated Bacteria

The strain used in this work was gram positive, rod shape, endospore-forming bacteria, catalase positive, and motility positive, but did not produce paracrystal. Utilization of glucose was positive but utilizations of manitol, arabinose and xylose were negative. The strain hydrolyzed starch, gelatin and casein, and grew at pH 6.8, pH 5.7 and 7% NaCl in nutrient broth. These results are shown in Fig. 1 and Table 1. The strain was similar to *Bacillus cereus* for most of the traits tested but differed in its nitrate reduction ability. The major fatty acids were 15 : 0 iso (34.57%), 13 : 0 iso (13.17%) and 17 : 0 iso (10.09%) etc., as shown in Fig. 2 and Table 2. According to IMS, this strain was very similar to the *B. cereus* group. The strain was identified as *B. cereus* or relative strain, according to the results. Therefore, the strain used in this work was tentatively designated as *Bacillus.* sp. HW-002.

B. megaterium P-1 (12), *B. circulans* MH-K1 (24), *Bacillus.* sp. R-4 (23), and *Bacillus.* sp. PI-7S (16) have been reported as chitosanase-producing microorganisms but *B. cereus* has not been reported upon until now.

Cultural Conditions for the Production of Chitosanase

Bacillus sp. HW-002 was incubated in basal medium at pH 3.0-10.0 for 48 h and the chitosanase activity was examined by DNS method (4). The chitosanase was produced at pH 6.0-8.0 and the maximum yield of the chitosanase was achieved at initial pH of 6.5 as shown in Fig. 3.

The *Bacillus* sp. HW-002 was incubated on basal medium at 26-38°C for 48 h. Maximum productivity of 24 U/ml was obtained at 32°C, but the maximum cell growth was at 34°C as shown in Fig. 4.

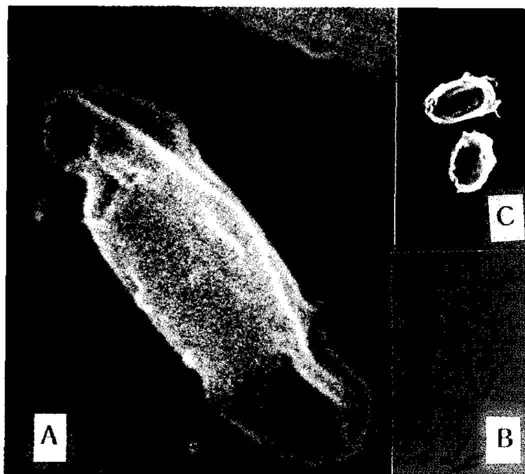

Fig. 1. Scanning electron microscopic photogram and light microscopic photogram.

(A) Vegitative cell (25,000×). (B) Cell chains (1,000×), *Bacillus*. sp. HW-002 was cultivated on TSBA at 30°C for 18 h. (C) Spore (10,000×), after the culture plate was incubated for 3 days.

Table 1. Morphological, biological and physiological characteristics of *Bacillus* sp. HW-002.

Characteristics	Result	Characteristics	Results
Width of rod (μm)	1.0-1.3	Hydrolysis of gelatin	+
Length of rod (μm)	3-5	casein	+
Spore shape	Ellipsoidal	starch	+
Spore position	Central	Utilization of citrate	+
Motility	+	Nitrate reduction	+
Parcrystal	-	Growth at pH	
Catalase	+	6.8 nutrient broth	+
Anaerobic growth	+	5.7 〃	+
V-P test	+	Growth in NaCl 7%	+
MR test	-	10%	-
Acid from		Growth at 10°C	-
D-glucose	+	30°C	+
L-arabinose	-	40°C	+
D-xylose	-	50°C	-
D-manitol	-		
Gas from glucose	-		

+, Positive reaction; -, Negative reaction.

Bacillus sp. HW-002 was cultivated in a basal medium containing 0.5% of various carbon sources at 32°C

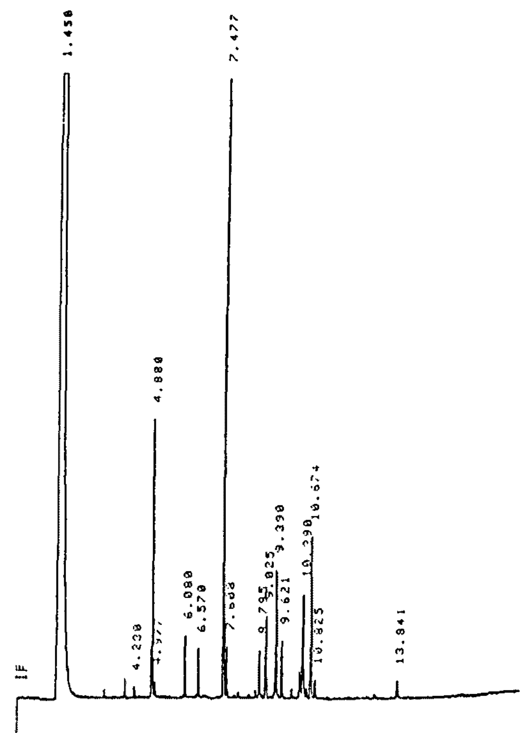

Fig. 2. Fatty acid gas-liquid chromatogram of *Bacillus* sp. HW-002.

The strain was cultivated on TSBA at 30°C for 1 day, 40-50 mg cells was harvested, methylated in NaOH aqueous methanol at 100°C for 20 min and extracted with hexane/ether. The analysis conditions of GLC were below; fused silica capllary column (25 m×0.2 mm), column temperature (170-270°C), injector temperature (250°C) and detector temperature (300°C).

Table 2. The composition of major fatty acids of *Bacillus* sp. HW-002.

RT	Name	%
3.912	12 : 0 ISO	0.90
4.890	13 : 0 ISO	13.17
4.985	13 : 0 ANTEISO	0.89
6.090	14 : 0 ISO	3.50
6.580	14 : 0	2.91
7.487	15 : 0 ISO	34.57
7.618	15 : 0 ANTEISO	2.68
8.805	12 : 0 ALDE?	3.03
9.035	16 : 0 ISO	5.24
9.401	15 : 0 ISO 2OH/16 : 1 w7t	8.16
9.630	16 : 0	3.72
9.279	ISO 17 : 1 wl0c	2.16
10.400	ISO 17 : 1 w5c	7.60
10.684	17 : 0 ISO	10.09
10.639	17 : 0 ANTEISO	1.41

for 56 hr. A cultural medium of 0.5 ml was sampled and the chitosanase activity was tested every 8 hr during the cultivation course.

Chitosanase was produced in various carbon sources but production time was affected by the carbon source. Chitosanase was rapidly produced at exponential growth in the medium with sucrose but chitosanase production was delayed with glucose, fructose or starch. The two peaks of chitosanase activity were observed in the basal medium containing glucosamine or *N*-acetylglucosamine, and the production of chitosanase was relatively poor with glucosamine, *N*-acetylglucosamine or chitosan (Fig. 5). Sucrose was a good carbon source, as illustrated from the high yield and rapid enzyme production. The effect of sucrose concentration on chitosanase production is shown in Fig. 6. Sucrose 0.5% was the best concentration for the production of chitosanase but higher concentrations of sucrose showed catabolic repression on enzyme synthesis. Other sugars also showed catabolic repression. As was previously demonstrated (5, 12, 14, 16, 23, 26), the majority of microorganisms produced chitosanase by induction, but the strain used in this work produced the enzyme in the absence of chitosan as an inducer. Therefore, chitosanase from *Bacillus* sp. HW-002

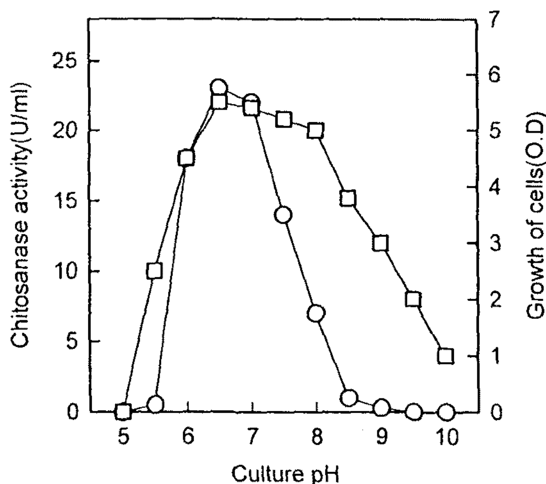

Fig. 3. Effect of pH on the production of chitosanase.
Bacillus sp. HW-002 was cultivated with shaking (180 rpm, width 6 cm) at 32°C for 48 h. The medium was composed of 0.5% starch, 0.75% yeast extract, 0.75% tryptone and initial pH 6.5 was adjusted with 0.1 N NaOH or 0.1 N HCl. The medium was autoclaved at 110°C for 10 min. Productivity of chitosanase (○), growth of cells (□).

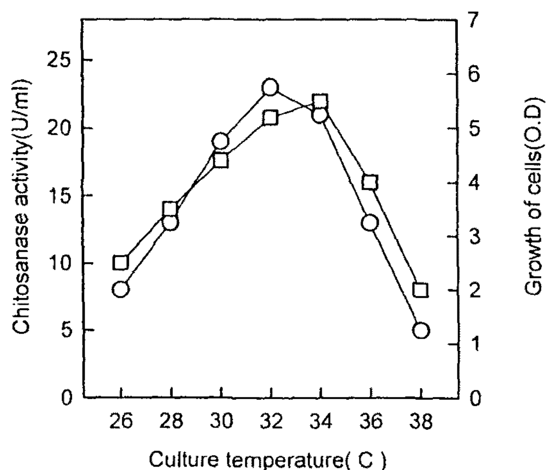

Fig. 4. Effect of temperature on the production of chitosanase.
Bacillus sp. HW-002 was cultivated with shaking (180 rpm, width 6 cm) at 26-38°C for 48 h. The medium was composed of 0.5% starch, 0.75% yeast extract, 0.75% tryptone and pH 6.5. Productivity of chitosanase (○), growth of cells (□).

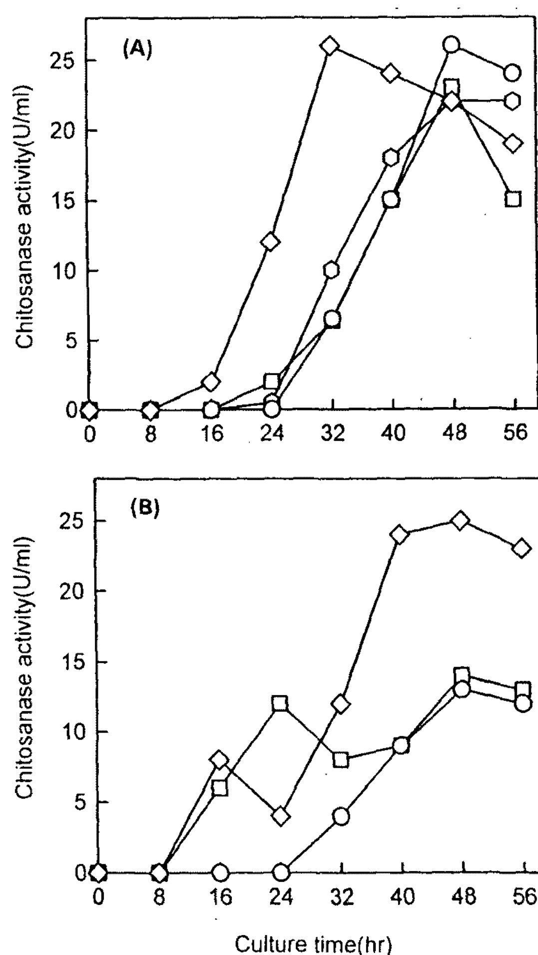

Fig. 5. Effect of carbon source on the prodction of chitosanase.
Bacillus sp. HW-002 was cultivated with shaking (180 rpm, width 6 cm) at 32°C for 56 h. The chitosanase activity was examined per 8 h on the cultivation course. The medium was composed of 0.5% various carbon source, 0.75% yeast extract, 0.75% tryptone and pH 6.5. (A); glucose (□), fructose (O), starch (○), sucrose (◇). (B); chtosan (○), *N*-acetylglucosamine (◇), glucosamine (□).

was an constitutive enzyme. The cultural characteristics were similar to *Acinetobacter* sp. CHB 101 (17), for which higher productivity was obtained in the M9-glucose medium than in the M9-chitosan medium. Chitosanase from *Pseudomonas* sp. (26) was produced both in media containing chitosan and media without chitosan, but the chitosanase productivity was increased about 3 fold with the addition of chitosan. In contrast with *Pseudomonas* sp., the chitosanase productivity of *Bacillus* sp. HW-002 decreased with the addition of chitosan.

Bacillus sp HW-002 was cultivated in 0.5% sucrose and 1.5% various nitrogen sources at 32°C for 36 h. The effects of the nitrogen sources on the production of chitosanase are shown in Table 3. Good yields were achieved in the yeast extract, tryptone, and yeast extract-other nitrogen source mixture. Yeast extract and yeast extract-tryptone (1 : 1 w/w) mixture were the best nitrogen sources with maximum productivities of about 26 U/ml (Table 3). The chitosanase activity decreased very rapidly when yeast extract was used as a single nitrogen source. Therefore, yeast extract-tryptone mixture was selected as the nitrogen source for the production of chitosanase. Chitosanase productivity increased with a 1.5% nitrogen source, but decreased in higher concentrations (Fig. 6). For the production of chitosanase, *Bacillus* sp. HW-002 required a greater source of nitrogen than a carbon source.

The composition of chitosanase production medium was determined on 0.5% sucrose, 0.75% yeast extract and 0.75% tryptone at pH 6.7. For the preparation of seed culture, a loop of bacteria was inoculated into a 1 l Erlenmeyer flask containing 100 ml medium and was incubated with shaking (180 rpm) at 32°C for 2 days. The chitosanase production medium was autoclaved at 121°C for 10 min, was cooled and inoculated with an approximately 5% seed culture. Working conditions were 2 l working volume, 300 rpm agitation and 1.5 vvm air supply in a 3 l jar fermenter with 0.01% silicon oil added as an antifoam. The 1 ml medium was sampled every 4 h during the cultivation course and was examined for chitosanase activity.

Fig. 7 shows the time course on the production of chitosanase in the chitosanase production medium. The chitosanase was secreted into the broth after 12 h. There-

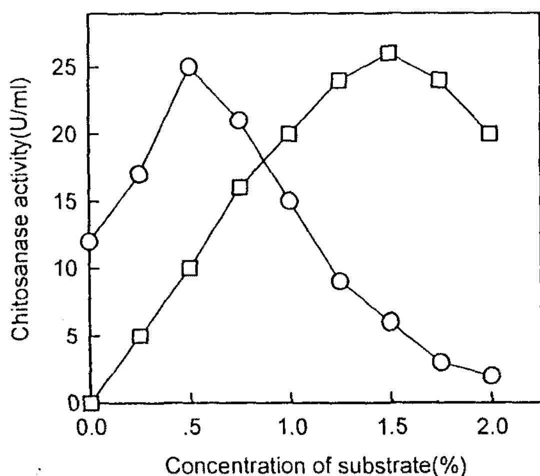

Fig. 6. Effect of sucrose and yeast extract-peptone mixture concentration on the production of chitosanase.
Bacillus sp. HW-002 was cultivated with shaking (180 rpm, width 6 cm) at 32°C for 48 h. The medium for effect of sucrose concentration was composed of 2% sucrose, 0.75% yeast extract, 0.75% tryptone and pH 6.5. The medium for effect of yeast extract-peptone mixture concentration was composed of 0.5% sucrose, 0-2% yeast extractpeptone mixture and pH 6.5. Effect of sucrose concentration on the productivity of chitosanase (○), effect of yeast extract-peptone mixture concentration on the productivity of chitosanase (□).

Table 3. The effect of nitrogen source on the production of chitosanase.

Nitrogen source	Activity (U/ml)	Cell growth (OD)
Peptone	6.0	4.6
Yeast extract	26.0	6.3
Cassamino acid	12.0	4.8
Albumin	4.8	4.2
Tryptone	19.0	4.0
Soy bean meal	7.9	-
Yeast extract-Cassamino acid (1:1)	22.7	5.1
Yeast extract-Tryptone (1:1)	25.5	5.0
Yeast extract-Albumin (1:1)	20.9	4.7
Yeast extract-Peptone (1:1)	21.0	4.1

Bacillus sp. HW-002 was cultivated with shaking (180 rpm, width 6 cm) at 32°C for 32 h. The medium was composed of 0.5% sucrose, 1.5% various nitrogen source and pH 6.5.

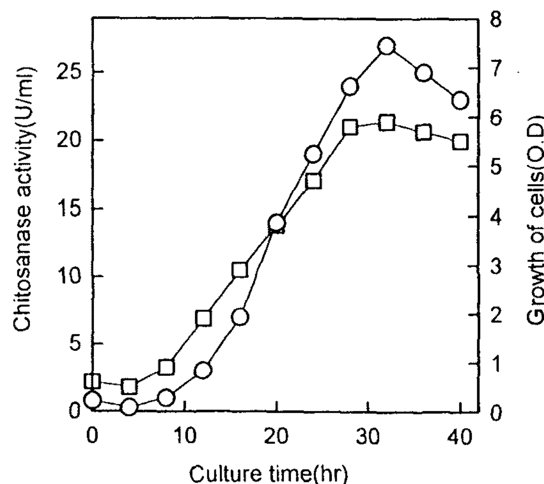

Fig. 7. Time course on the production of chitosanase.
Working conditions were 2 l working volumem, 300 rpm agitation, 1.5 vvm air supply, at 32°C in 3 l jar fermenter. The medium was composed of 0.5% sucrose, 0.75% yeast extract, 0.75% peptone, 0.01% silicone oil and pH 6.5. productivity of chitosanase (○), growth of cells (□).

after, the chitosanase activity increased linearly and reached a maximal activity of 27,200 U/l after 32 h. Maximal growth of cells was reached after 28 h. It was noted that chitosanase was secreted during the exponential growth phase and initial stationary phase, thus the chitosanase from *Bacillus* sp. HW-002 was a mixed growth-linked metabolite. This result was similar to that of chitosanase synthesis from *Pseudomonas* sp. (26) for which synthesis is associated with growth, but the chitosanase from *Aceinetobacter* sp. CHB 101 (17) was non-growth associated metabolite. Chitosanase from the strain used in this work was produced very rapidly with high yields of chitosanase, which was comparable with other microbes (5, 12, 14, 16, 23, 26). Since optimization of cultural conditions produced high yields of chitosanase in the absence of the substrate chitosan, and the production of chitosanase was very fast, we concluded that *Bacillus* sp. HW-002 has great potential for the production of chitosanase for industrial use.

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