Carbohydrate Research 344 (2009) 979–984

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Utilization of squid pen for the efficient production of chitosanase and antioxidants through prolonged autoclave treatment

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ARTICLE INFO

Article history:
Received 10 December 2008
Received in revised form 10 March 2009
Accepted 12 March 2009
Available online 17 March 2009

Keywords:
Chitosanase
Antioxidant
Squid pen wastes
Bacillus sp.
Autoclave treatment

ABSTRACT

We have developed a culture system for efficient production of chitosanase by *Bacillus* sp. TKU004. TKU004 was cultivated by using squid pen powder as the sole carbon/nitrogen source. The effects of autoclave treatments of the medium on the production of chitosanase were investigated. Autoclave treatment of squid pen powder for 45 min remarkably promoted enzyme productivity. When the culture medium containing an initial squid pen powder concentration of 3% was autoclaved for 45 min, the chitosanase activity was optimal and reached 0.14–0.16 U/mL. In addition, extracellular surfactant-stable chitosanase was purified from the TKU004 culture supernatant. The antioxidant activity of TKU004 culture supernatant was determined through the scavenging ability of DPPH, with 70% per mL. With this method, we have shown that marine wastes can be utilized efficiently through prolonged autoclave treatments to generate a high value-added product, and have revealed its hidden potential in the production of functional foods.

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1. Introduction

Chitosan, a D-glucosamine polymer, is a totally or partially deacetylated derivative of chitin. It is usually obtained by the artificial deacetylation of chitin in the presence of alkali.[1] Recent studies on chitin and chitosan have attracted interest for converting them to oligosaccharides because these derivatives are not only water soluble but also possess versatile functional properties such as antitumor activity and antimicrobial activity.[1–3] Traditionally, chitosan oligosaccharides are processed by chemical methods in industries. There are many problems with the existing chemical processes, such as a large amount of short-chain oligosaccharides produced, low yields of oligosaccharides, high cost of separation, and environmental pollution. Consequently, chitosanase hydrolysis has become more and more popular in recent years given its advantages with regard to environmental compatibility, low cost, and reproducibility.[4,5]

Bioconversion of chitinous materials has been proposed as a waste treatment alternative for the disposal of marine wastes. To further enhance the utilization of chitin-containing marine waste, we have recently investigated the bioconversion of shellfish chitin wastes for the production of proteases and/or chitinases.[1,6–8] To obtain additional potentially useful proteases and/or chitinases, we were successful in isolating a number of bacteria that produced extracellular proteases and/or chitinases by using squid pen as the sole carbon/nitrogen source. Among these was the bacterium *Bacillus* sp. strain TKU004, which produced a solvent-stable protease in the culture supernatant maximally on the fourth day.[6] A chitosanase activity, found on the second day, disappeared with the subsequent appearance of the protease activity.

Chitosanases have been found in abundance in a variety of bacteria, including *Bacillus* spp.[9,10] Almost all of the chitosanase-producing strains , such as *Bacillus cereus* D-11[10] and *Aspergillus* sp. CJ22-326,[11] use colloidal chitosan or chitosan as a major carbon source. However, preparation of chitin/chitosan involves demineralization and deproteinization of shellfish waste with the use of strong acids or bases.[1,8] The utilization of squid pen waste not only solves environmental problems, but also decreases the production cost of microbial chitosanases. Besides, the chitin in squid pen is of the β form, which has better degradation than α form chitin found in shrimp and crab shells. The production of inexpensive chitosanase is an important element in the process. Among the published chitosanase-producing strains, a few microorganisms have been found to utilize marine wastes as carbon/nitrogen sources and produce chitosanase and proteases. Because chitosanase disappears with the pursuant appearance of protease, in the present paper, we attempted to optimize the culture conditions of *Bacillus* sp. TKU004 for maximal chitosanase production by using squid pen powder as a cheap source of carbon/nitrogen. The medium based on squid pen is reported to be effective for producing larger amounts of chitosanase than other media[12,13] and be cost effective in the industrial production of chitosanase using *Bacillus* sp.

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doi:10.1016/j.carres.2009.03.011

TKU004. Therefore, the effects of various factors in the preparation of media based on squid pen and in fermentation on the chitosanase productivity by *Bacillus* sp. TKU004 were investigated to optimize chitosanase production. In addition, the chitosanase isolated from *Bacillus* sp. TKU004 was also purified, characterized, and compared with chitosanases isolated from other bacterial sources.

2. Materials and methods

2.1. Materials

The squid pen powder (SPP) used in these experiments was prepared as described earlier.[6] Squid pens were purchased from Shin-Ma Frozen Food Co. (I-Lan, Taiwan). For the preparation of the SPP, the squid pens were washed thoroughly with tap water and then dried. The dried materials obtained were milled to powders for use as the carbon/nitrogen source for chitosanase production. DEAE–Sepharose CL-6B and Sephacryl S-100 were purchased from GE Healthcare UK Ltd (Little Chalfont, Buckinghamshire, England). Weak-base anion-exchanger Macro-prep DEAE was obtained from Bio-Rad (Hercules, CA, USA). All other reagents used were of the highest grade available.

2.2. Preparation of culture medium and enzyme production

Bacillus sp. TKU004 was cultivated in SPP media prepared with autoclave treatment (121 °C) for 15–60 min. Media were composed of 1–3% SPP, 0.1% K_2HPO_4, and 0.05% $MgSO_4{\cdot}7H_2O$ (pH 7). Various volumes of the resultant media were aerobically cultured at 30 °C for 1–5 days on a rotary shaker (150 rpm) in 250 mL Erlenmeyer flasks. After centrifugation (12,000*g*, 4 °C, for 20 min), the supernatants were collected for measurement of chitosanase activity and reducing sugar concentration.

2.3. Purification of the chitosanase

2.3.1. Production of chitosanase

For the production of chitosanase, *Bacillus* sp. TKU004 was grown in 100 mL of liquid medium in an Erlenmeyer flask (250 mL) containing 3% SPP, 0.1% K_2HPO_4, and 0.05% $MgSO_4{\cdot}7H_2O$ (pH 7). One milliliter of the seed culture was transferred into 100 mL of the same medium and was grown in an orbital shaking incubator for 2 days at 30 °C and at pH 7 (the pH was 7.5 after autoclaving). After incubation, the culture broth was centrifuged (4 °C and 12,000*g* for 20 min), and the supernatant was used for further purification by chromatography.

2.3.2. DEAE–Sepharose CL-6B chromatography

To the culture supernatant (1000 mL), ammonium sulfate was added (608 g/L). The resultant mixture was kept at 4 °C overnight and the precipitate formed was collected by centrifugation at 4 °C for 20 min at 12,000*g*. The precipitate was then dissolved in a small amount of 50 mM sodium phosphate buffer (pH 7), and was dialyzed against the buffer. The resultant dialyzate (50 mL) was loaded onto a DEAE–Sepharose CL-6B column (5 cm x 30 cm) equilibrated with 50 mM sodium phosphate buffer (pH 7). The chitosanase was eluted with a linear gradient of 0–1 M NaCl in the same buffer. The adsorbed chitosanase fractions were combined and concentrated by ammonium sulfate precipitation. The resultant precipitate was collected by centrifugation and dissolved in 5 mL of 50 mM sodium phosphate buffer (pH 7).

2.3.3. Macro-prep DEAE chromatography

The obtained enzyme solution was then chromatographed on a column of Macro-prep DEAE (12.6 mm × 40 mm), which had been equilibrated with 50 mM sodium phosphate buffer (pH 7). As shown in Figure 1, the chitosanase was eluted with a linear gradient of 0–1 M NaCl in the same buffer. The sample showing higher chitosanase activity (Fig. 1) was combined and concentrated by ammonium sulfate precipitation. The resultant precipitate was collected by centrifugation and dissolved in 50 mM sodium phosphate buffer (pH 7).

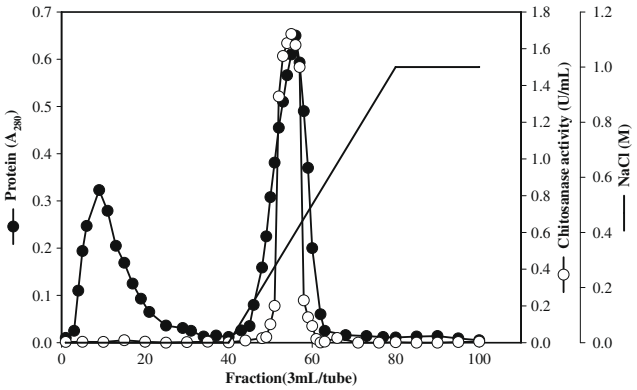

Figure 1. Elution profile of TKU004 chitosanase on Macro-prep DEAE: (●) absorbance at 280 nm; (○) chitosanase activity (U/mL).

2.3.4. Sephacryl S-100 chromatography

The resultant enzyme solution was loaded onto a Sephacryl S-100 gel filtration column (2.5 cm × 120 cm), which had been equilibrated with 50 mM sodium phosphate buffer (pH 7), and was then eluted with the same buffer. One sample exhibiting chitosanase activity was obtained, combined, and used as a purified preparation.

2.4. Protein determination

Protein content was determined by the method of Bradford using Bio-Rad dye reagent concentrate and bovine serum albumin as the standard. After column chromatography, the protein concentration was estimated by measuring the absorbance at 280 nm.[6]

2.5. Measurement of enzyme activity

Chitosanase activity of the enzyme was measured by incubating 0.2 mL of the enzyme solution with 1 mL of 0.3% (w/v) water-soluble chitosan (Kiotec Co., Hsinchu, Taiwan; with 95% deacetylation) in 50 mM phosphate buffer, pH 7, at 37 °C for 30 min. The reaction was stopped by heating it at 100 °C for 15 min. The amount of reducing sugar produced was measured by the method of Imoto and Yagishita with glucosamine as a reference compound. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of reducing sugar per minute.[12]

2.6. Determination of molecular mass

The molecular mass of the purified chitosanase was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli. The standard proteins used for calibration were phosphorylase b (molecular mass, 97.4 kDa), albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). Before electrophoresis, proteins were exposed overnight to 10 mM phosphate buffer (pH 7) containing β-mercaptoethanol. The gels were stained with Coomassie Brilliant Blue

R-250 in methanol–acetic acid–water (5:1:5, v/v), and were decolorized in 7% acetic acid. The molecular mass of the TKU004 chitosanase in the native form was determined by a gel filtration method. The sample and standard proteins were applied to a Sephacryl S-100 column (2.5 cm x 120 cm, Amersham Pharmacia) equilibrated with 50 mM phosphate buffer (pH 7). Bovine serum albumin (molecular mass, 67 kDa), *Bacillus* sp. α-amylase (50 kDa), and hen egg white lysozyme (14 kDa) were used as molecular mass markers.[12]

2.7. Mass spectrometry and protein identification

Bands of interest on SDS–PAGE gel were excised and in-gel digested with trypsin. The identification of TKU004 chitosanase was done by using liquid chromatography–tandem mass spectrometry (LC–MS/MS) by the Mission Biotech, Taiwan. Fragment spectra were searched against the NCBI non-redundant protein database. Database searches were carried out using the MASCOT search engine.

2.8. Scavenging ability on 1,1-diphenyl-2-picrylhydrazyl radicals

TKU004 culture supernatant (150 μL) was mixed with 37.5 μL of methanolic solution containing 0.75 mM DPPH (Sigma Chemical Co., St. Louis, MO) radicals. The mixture was shaken vigorously and was left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against a blank.[14] The scavenging ability was calculated as follows: Scavenging ability (%) = [(ΔA_{517} of control − ΔA_{517} of sample)/ΔA_{517} of control] × 100.

3. Results and discussion

3.1. Culture conditions and enzyme production

In our preliminary experiments, we found that 100 mL of basal medium (0.1% K_2HPO_4 and 0.05% $MgSO_4{\cdot}7H_2O$, pH 7) containing 2% SPP was better for the production of chitosanase by strain TKU004 at 30 °C. To study the effect of cultivation volume on the production of chitosanase, we found that 100 mL of medium was more suitable for chitosanase production than 25 mL, 50 mL, 150 mL, and 200 mL of medium. To study the effect of carbon/nitrogen sources on the production of chitosanase, growth was carried out in 100 mL of basal medium (0.1% K_2HPO_4 and 0.05% $MgSO_4{\cdot}7H_2O$, pH 7) containing additional carbon/nitrogen sources of 1–3% (w/v) SSP or SPP, respectively. The result showed that 3% SPP was more suitable as an inducer for chitosanase production than others (data not shown). Therefore, in the following experiments, 100 mL of basal medium (0.1% K_2HPO_4 and 0.05% $MgSO_4{\cdot}7H_2O$, pH 7) containing additional carbon/nitrogen source of 3% (w/v) SPP was used.

3.1.1. The effects of various heat treatments of SPP medium on the production of chitosanase

Because SPP is an insoluble material, various heat treatments were tested to efficiently extract the nutrients from the SP or to change the structure of SP. Figure 2 shows the effect of autoclave time length of media on the production of chitosanase. Larger amounts of chitosanase were produced by fermentation with the medium autoclaved for 45 or 60 min than with that autoclaved for 15 min and 30 min. In addition, the production of chitosanase by batch fermentation using the media with and without insoluble materials was investigated. To remove insoluble materials in the medium after autoclave treatments for 45 min, the medium was centrifuged at 12,000*g* for 20 min using sterilized tubes and was used for fermentation. The productivity of chitosanase was only slightly reduced when the fermentation was done using the medium without insoluble materials (data not shown). Therefore, *Bacillus* sp. TKU004 mainly metabolizes solubilized nutrients. These results indicate that the nutrients contained in SPP are degraded and extracted into the medium by autoclaving for a sufficiently long time. Microorganisms metabolize these nutrients more efficiently. The chitosanase activities reached up to approximately 0.14–0.16 U/mL when the media prepared with 45–60 min autoclave treatments were used. Because this value is much higher than those reported previously (0.024 U/mL)[12] and (0.03 U/mL),[13] this medium preparation procedure is very effective for chitosanase production by *Bacillus* sp.. Therefore, in the following experiments, autoclave treatment for 45 min was used to prepare the media.

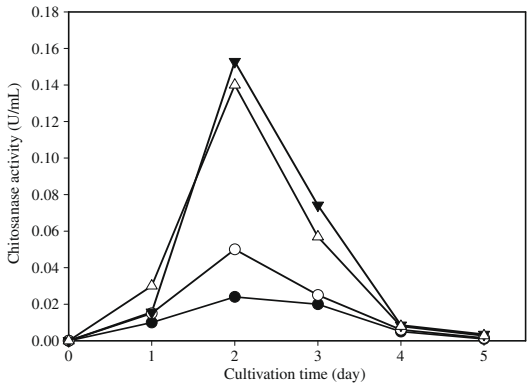

Figure 2. Effect of autoclave time length of the media on the production of chitosanase by batch fermentation. SPP (3%) media were autoclaved for 15 min (●), 30 min (○), 45 min (▼), and 60 min (△).

3.1.2. The effect of SPP concentration on the cell growth and production of chitosanase

Figure 3A and B shows the effect of SPP concentration on cell growth and production of chitosanase, respectively. In all cases, both the cell concentration and enzyme activity reached the maximum after 2 days of fermentation and decreased gradually after that. This result indicates that production of chitosanase is cell growth dependent. Although the maximum cell concentration increased with increasing initial SPP concentration, production of chitosanase was the highest when 3% SPP was used. This is probably because excessive extracted nutrients reduce enzyme productivity of the cells.

3.1.3. The production of reducing sugar in liquid phase fermentation

Figure 4 shows the time course of the reducing sugar and remaining SPP content in the culture medium during the fermentation. Because the medium is based on SPP, the reducing sugars were mainly chitooligosaccharides. The reducing sugar content increased during initial 3 days of fermentation and reached constant values thereafter. In addition, the amount of SPP in the culture media decreased with an increase in reducing sugar content. Comparing the differences in culture time for optimal production, it was found that the highest reducing sugar content (on the third day) appeared later than the optimal enzyme production (on the second day). These results indicate that reducing sugars might be produced from SPP by microorganism enzymatic hydrolysis and be present in the culture supernatant.

Considering that the accuracy of chitosanase activity assay might be affected by reducing sugars, the chitosanase activity of the culture supernatant after dialysis was also analyzed for comparison. There were no differences between the chitosanase

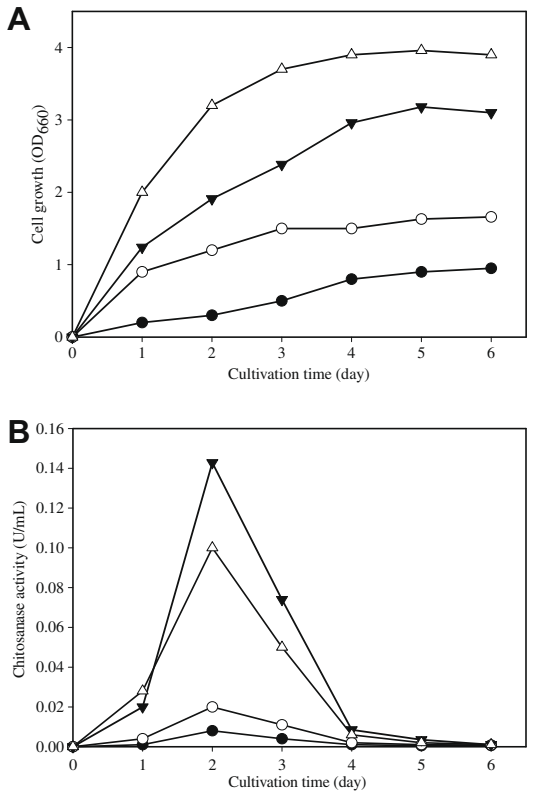

Figure 3. Effect of SPP concentration on cell growth (A) and production of chitosanase (B) by batch fermentation. The media containing 1% (●), 2% (○), 3% (▼), and 4% (△) SPP were autoclaved for 45 min.

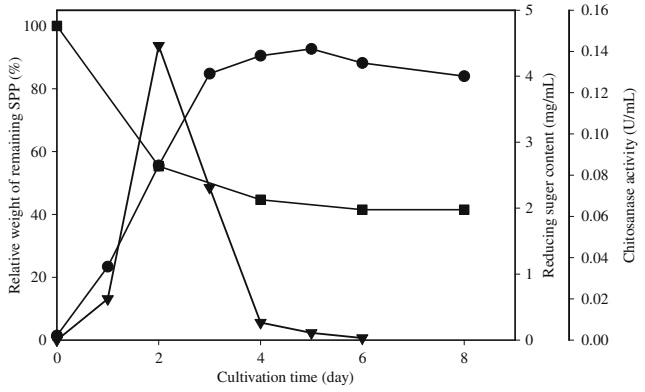

Figure 4. Time courses of the reducing sugar (●), remaining SPP (■) content, and chitosanase activity (▼) in the culture medium during batch fermentation. The medium containing 3% SPP was autoclaved for 45 min.

activities of non-dialyzed and dialyzed samples. Furthermore, along with the chitosanase, TKU004 protease was produced on the fourth day in the same medium.[6] While investigating the variation in chitosanase activity, the maximum chitosanase activity appeared on the second day, and then gradually decreased with the appearance of protease activity and reducing sugars. Chitosanase activity was almost undetectable on the fourth day.

Conjecturing from above results, the reducing sugars in the culture supernatant might have originated from the hydrolyzates (chitooligosaccharides) obtained from hydrolysis of squid pen chitin by chitosanase. Presumably, there are two reasons for the almost complete disappearance of the chitosanase activity after 4 days. First, chitosanase was inactivated due to decomposition by the protease produced afterward. Second, chitosanase still existed but was inhibited by chitosanase inhibitor produced afterward, and consequently, chitosanase activity of the culture supernatant could not be detected. However, the second reason was less likely as chitosanase activity was not found in the previous purification procedures of protease.[6] Therefore, it was inferred that the disappearance of the chitosanase activity should be related to the appearance of protease afterward and the resulting proteolysis of the chitosanase.

3.2. Isolation and purification

The purification of the TKU004 chitosanase from the culture supernatant (500 mL) is described in the materials and methods section (Section 2). As shown in Table 1, the purification steps were combined to give an overall purification of about 527-fold. The overall activity yield of the purified chitosanase was 20%, with a specific activity of 2800 mU/mg. The final amount of TKU004 chitosanase obtained was 10 mg. After DEAE–Sepharose CL-6B chromatography, one protein sample containing the chitosanase activity was eluted from the DEAE–Sepharose CL-6B column, and another sample without enzyme activity was washed from the column. By further purification, the purified chitosanase was obtained. The purified chitosanase was also confirmed to be homogeneous by SDS–PAGE (Fig. 5). The molecular weights of the chitosanase were determined by SDS–PAGE and gel filtration and were approximately 29 and 25 kDa, respectively.

The molecular mass of TKU004 chitosanase (29 kDa) was obviously different from those of most of the other *Bacillus* chitosanases, such as those of *Bacillus subtilis* IMR-NK1 (41 kDa),[9] *B. cereus* S1 (45 kDa),[15] *Bacillus ehimensis* EAG1 (31 kDa),[16] *Bacillus megaterium* P1 (43, 39.5, 22 kDa),[17] *Bacillus* sp. KCTC0377BP (45 kDa),[18] *Bacillus* sp. MET1299 (52 kDa),[19] *Bacillus* sp. 739 (46 kDa),[20] *Bacillus* sp. P16 (45 kDa),[21] and *Bacillus* sp. 7-M (41 kDa).[22] The chitosanases of *B. subtilis* GM9804 (27 kDa),[23] *B. subtilis* KH-1 (28 kDa),[24] and *Bacillis* sp. DAU101 (27 kDa)[5] were the only *Bacillus* chitosanases that had the molecular mass similar to that of *Bacillus* sp. TKU004 chitosanase.

3.3. Effect of pH and temperature on the enzyme activity

The effect of pH on the catalytic activity was studied by using soluble chitosan as a substrate under the standard assay conditions. The pH activity profile of the chitosanase had maximum values at pH 7. The pH stability profile of the chitosanase activity was determined by the measurement of the residual activity at pH 7 after incubation at various pH values at 37 °C for 60 min. The chitosanase activity was stable at pH 4–7 (data not shown).

The effect of temperature on the activity of chitosanase was studied with soluble chitosan as a substrate. The optimum temper-

Table 1
Purification of chitosanase from *Bacillus* sp. TKU004

Step	Total protein (mg)	Total activity (U)	Specific activity (mU/mg)	Purification fold	Yield (%)
Culture supernatant	25960	138	5.316	1	100
$(NH_4)_2SO_4$ ppt	3460	93	26.879	5.056	67
DEAE–Sepharose	1354	86	63.516	11.948	62
Macro-prep DEAE	395	54	136.709	25.717	39
Sephacryl S-100	10	28	2800.000	526.712	20

Bacillus sp. TKU004 was grown in 100 mL of liquid medium in an Erlenmeyer flask (250 mL) containing 3% squid pen powder, 0.1% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$ in a shaking incubator for 2 days at 30 °C.

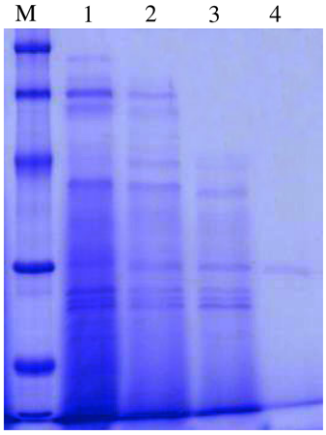

Figure 5. SDS–PAGE analysis of the purified chitosanase produced by strain TKU004. Lanes: M, molecular markers (97.4, 66.2, 45, 29, 20.1, 14.4 kDa); (1) concentrated culture supernatant; (2) purified by DEAE–Sepharose CL-6B chromatography; (3) purified by Macro-prep DEAE chromatography; and 4, the purified chitosanase.

ature for TKU004 chitosanase was 30–50 °C. To examine the heat stability of TKU004 chitosanase, the enzyme solution in 50 mM phosphate buffer (pH 7) was allowed to stand for 60 min at various temperatures, and then the residual activity was measured. TKU004 chitosanase maintained its initial activity from 25 to 40 °C but was completely inactivated at 80 °C (data not shown).

3.4. Substrate specificity

The activities of the TKU004 chitosanase with various substrates are summarized in Table 2. The enzyme showed activities slightly toward colloidal chitin, chitin, and chitosan with 82–95% deacetylation, but no activity toward chitosan with the other different degrees of deacetylation.

3.5. Effects of various chemicals on the enzyme activity

To characterize further the *Bacillus* sp. TKU004 chitosanase, we next examined the effects of some known enzyme inhibitors and divalent metals on its activities. The effects of various chemicals on the enzyme activity were investigated by preincubating the enzyme with chemicals in 50 mM phosphate buffer (pH 7) for 30 min at 37 °C and then measuring the residual chitosanase activity by using soluble chitosan as substrate. The results showed that TKU004 chitosanase was inhibited by 5 mM Cu^{2+} and Fe^{2+} (Table 3). Similar results were also found for the chitosanase of *Bacillus* sp. DAU101[5,25] and *B. subtilis* TKU007.[13]

Table 2
Substrate specificity of TKU004 chitosanase

Substrate	Relative activity (%)
Water-soluble chitosan	100
Chitosan (95% DD)	10
Chitosan (85% DD)	5
Chitosan (82% DD)	2
Chitosan (80% DD)	0
Chitosan (73% DD)	0
Chitosan (60% DD)	0
Colloidal chitin	6
Chitin (α-type)	0
Chitin (β-type)	2

Table 3
Effects of various chemicals and surfactants on chitosanase activity of TKU004

Chemicals	Concentration (mM)	Relative activity (%)
None	0	100
PMSF	5	97
EDTA	5	90
Mg^{2+}	5	86
Cu^{2+}	5	59
Fe2+	5	23
Ca^{2+}	5	90
Zn^{2+}	5	82
Mn^{2+}	5	83
Ba^{2+}	5	85
SDS	0.5/1/2	100/97/95
Tween 20	0.5/1/2 (%)	99/95/94
Tween 40	0.5/1/2 (%)	99/98/96
Triton X-100	0.5/1/2 (%)	97/94/93

Purified enzyme was preincubated with the various reagents at 37 °C for 30 min and residual chitosanase activity was determined as described in the text. One hundred percent was assigned to the activity in absence of reagents.

3.6. Effect of various surfactants on the enzyme activity

Enzymes are usually inactivated by the addition of surfactants to the reaction solution. The effect of different surfactants (2%, w/v) on stability of the purified TKU004 chitosanase was also studied. The purified chitosanase was incubated with surfactants (0.5–2%, w/v) at 37 °C for 30 min and the remaining enzymatic activity was determined under normal assaying conditions. The chitosanase activity of the sample without any surfactants (control) was taken as 100% (0.15 U/mL). It was found that , even in the presence of 2% Tween 20, Tween 40, Triton X-100 (non-ionic surfactant), and 2 mM SDS (anionic surfactant), the TKU004 chitosanase retained 94%, 96%, 93%, and 95% of its original activity, respectively (Table 3).

3.7. Identification and N-terminal sequence of TKU004 chitosanase

To identify the protein having chitosanase activity, which appeared as a prominent 29 kDa band on SDS–PAGE gel, the band was excised and analyzed after tryptic digestion. The band from the SDS–PAGE gel was subjected to electrospray tandem mass spectrometry analysis. The fragment spectra were subjected to the NCBI non-redundant protein database search. The spectra matched three tryptic peptides that were identical to chitosanase from *B. subtilis* subsp. *subtilis* str. 168 (GenBank accession number gi16079742) with 23% sequence coverage. The sequence of this chitosanase gave the calculated nominal mass of 31477 Da, similar to the experimental values obtained with the purified TKU004 chitosanase. The identification of TKU004 chitosanase was carried out by the Mission Biotech, Taiwan.

The first 10N-terminal amino acid residues of the purified chitosanase were identified as MKISLKKKAG. The N-terminal amino acid sequence of TKU004 chitosanase was analyzed using a BLAST search against GenBank. The first 10N-terminal amino acids sequence showed 100% identity to the first 10 amino acids in the sequence of *Bacillus* sp. DAU101 chitosanase.[5] *Bacillus amyloliquefaciens* chitosanase,[26] and *B. amyloliquefaciens* FZB42 plant growth promoter.[27] However, only the first through fourth and seventh amino acids of TKU004 chitosanase (M, K, I, S, and K, respectively) are the same as N-terminal amino acid residues of *B. subtilis* 168 chitosanase. Comparison of TKU004 chitosanase with *Bacillus* sp. DAU101 chitosanase, *B. amyloliquefaciens* chitosanase, *B. subtilis* 168 chitosanase, and *B. amyloliquefaciens* FZB42 plant growth promoter, showed that they all presented the AVMYDTVIQHGDGDDPDSFYALIK peptide sequence, and belong to glycosyl hydrolase family 46 chitosanase

domain. Considering the differences in the first 10 N-terminal amino acids sequence, the differential function among these *Bacillus* chitosanases, namely whether they act as a plant growth promoter or exhibit chitosanase activity, may well be correlated to the presence or absence of this sequence of 10 N-terminal amino acid residues.

3.8. Antioxidant activity of culture supernatant from SPP fermented by strain TKU004

It has been reported that chitin, chitosan, and peptide have antioxidative[28–31] and anticarcinogenic[3,8] properties. TKU004 protease had been purified and characterized in our previous experiments.[6] To increase the utilization of these chitin/protein-containing marine wastes, we incubated *Bacillus* sp. TKU004 for 1–4 days with various concentrations of squid pen powder under the optimal culture conditions described above (100 mL, 30 °C) and analyzed the antioxidant activity and enzyme activity of the culture supernatants. The antioxidant activity assayed was the scavenging ability on DPPH. After heating the medium that contained SPP in an autoclave (121 °C for 45 min), antioxidant activities (30–35% per mL) were found in the supernatants. However, as shown in Figure 6, it was found that the antioxidant activities increased significantly and reached approximately 70% after fermentation by TKU004. The optimal antioxidant activity was found in the TKU004 culture supernatant (3% SPP) incubated for 3 days (Fig. 6). Comparing the differences in culture time for optimal production of different materials (Fig. 6), it was found that the optimal antioxidant activities of TKU004 appeared later than optimal enzyme production.

The chitooligosaccharides in the supernatant were recovered by the method previously described[8] and the antioxidant activity was measured. It was found that the antioxidant activity of the chitooligosaccharides in the supernatant was approximately 65% (data not shown). These results demonstrated that antioxidative oligosaccharides might have been hydrolyzed by enzymes present in the culture supernatant. This is consistent with the previous observation of the increase in the reducing sugar content. The third day culture supernatant showed both high reducing sugar content and high antioxidant activity. The antioxidant materials may contain oligosaccharides (and a little oligopeptides) that are electron donors and are able to react with free radicals to terminate the radical chain reaction. TKU004 culture supernatants displayed much more antioxidant activity than the supernatants only after being heated in an autoclave (121 °C for 45 min). It is assumed that even though the autoclave treatment degrades SPP and produces some of the antioxidant materials, most of the antioxidant materials are produced by strain TKU004.

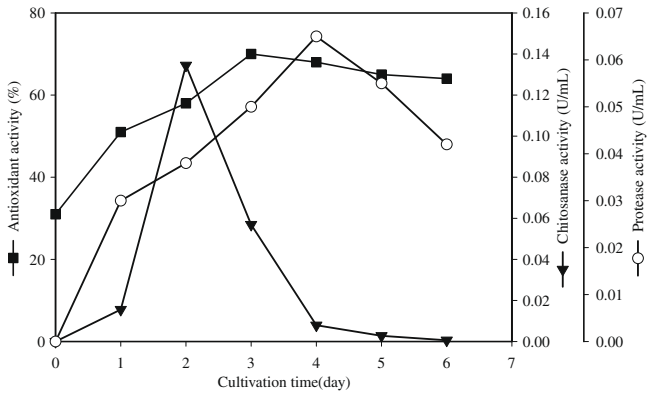

Figure 6. Time courses of enzymes and antioxidants in a culture of *Bacillus* sp. TKU004 in squid pen-containing medium: (▼) chitosanase activity (U/mL); (○) protease activity (U/mL); (■) antioxidant activity (%).

4. Conclusions

This research used squid pen powder as the sole carbon/nitrogen source to produce chitosanase. This is different from most other chitosanase-producing strains, which require chitosan as the carbon/nitrogen source. In this study, we have succeeded in developing an efficient production procedure of chitosanase by *Bacillus* sp. TKU004 using a cheap medium based on squid pen. Although the production is significantly improved by the optimization of cultivation conditions, further studies on the improvement of the productivity of *Bacillus* sp. strains by mutation or genetic engineering approaches are important to develop the industrial production of chitosanase. In addition, we have also purified and characterized the chitosanase, and found that the culture supernatant had antioxidant activity.

Acknowledgment

This work was supported in part by a Grant from the National Science Council, Taiwan (NSC96-2313-B-032-002-MY3).

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