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Production and purification of a protease, a chitosanase, and chitin oligosaccharides by *Bacillus cereus* TKU022 fermentation

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ABSTRACT

A protease- and chitosanase-producing strain was isolated and identified as *Bacillus cereus* TKU022. The protease and chitosanase were both produced using 1.5% (w/v) shrimp head powder (SHP) as the sole carbon/nitrogen source, and these enzymes were purified from the culture supernatant. The molecular masses of the TKU022 protease and chitosanase determined using SDS–PAGE were approximately 45 and 44 kDa, respectively. The high stability of the TKU022 protease toward surfactants, an optimal pH of 10 and an optimal temperature of 50–60 °C suggest that this high-alkaline protease has potential applications for various industrial processes. Concomitant with the production of the TKU022 chitosanase, *N*-acetyl chitooligosaccharides were also observed in the culture supernatant, including $(GlcNAc)_2$, $(GlcNAc)_4$, $(GlcNAc)_5$, and $(GlcNAc)_6$ at concentrations of 201.5, 12.4, 0.5, and 0.3 μg/mL, respectively, as determined using an HPLC analysis. The chitin oligosaccharides products were also characterized using a MALDI-TOF mass spectrometer. A combination of the HPLC and MALDI-TOF MS results showed that the chitin oligosaccharides of the TKU022 culture supernatant comprise oligomers with degree of polymerization (DP) from 2 to 6. Using this method, the production of a protease, a chitosanase, and chitin oligosaccharides may be useful for various industrial and biological applications.

1. Introduction

Proteases constitute one of the most important groups of industrial enzymes. Among the various proteases, bacterial proteases are the most significant compared with proteases of animal, plant, or fungal origin, and bacteria of the genus *Bacillus* produce most of the commercial proteases used today[1] because the alkaline proteases of *Bacillus* spp. exhibit high proteolytic activity and stability under alkaline conditions.[2] The characterization of alkaline proteases is interesting for bioengineering and biotechnological applications. The major application of these proteases is in the detergent industry because the pH of laundry detergents is generally in the range of 9.0–12.0. In addition, proteases have applications in leather processing, food processing, and the production of protein hydrolysates.[1]

Chitosanases have been found in abundance in a variety of bacteria.[3–5] Most of the chitosanase-producing strains use colloidal chitosan or chitosan as a major carbon source.[4,5] However, preparation of chitin/chitosan involves the demineralization and deproteinization of shellfish waste using strong acids or bases.[6] The utilization of shrimp head wastes not only solves environmental problems but also decreases the production cost of microbial chitosanases. Among these published protease- and/or chitosanase-producing strains, few have been found to utilize marine wastes as a carbon/nitrogen source. The production of inexpensive proteases and chitosanases is an important element in the process.

Recent studies on chitosan/chitin have attracted interest for converting these species into oligosaccharides because the oligosaccharides not only are water-soluble but also possess versatile functional properties such as antitumor activity and antimicrobial activity.[6–8] Traditionally, chitin oligosaccharides were processed using chemical methods in industries. Many problems exist in these chemical processes, such as the production of a large amount of short-chain oligosaccharides, low yields of oligosaccharides, the high cost of separation, and environmental pollution. Alternatively, with its advantages in environmental compatibility, low cost, and reproducibility, enzyme hydrolysis has become more popular in recent years.[8] However, in our work, the chitin oligomers were produced directly by *B. cereus* TKU022 fermentation. With this method, the production of chitin oligomers will omit the procedure for purifying enzymes and help decrease the cost of chitin oligomer production more effectively. In summary, in the present study, shrimp head wastes can be utilized for the production of enzymes (both protease and chitosanase) and *N*-acetyl chitooligosaccharides (GlcNAc-oligomers) by *B. cereus* TKU022 fermentation. The

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above technologies facilitate the potential use of this process in industrial applications and functional foods.

2. Results and discussions

2.1. Isolation and identification of a protease/chitosanase-producing strain

The microorganisms were isolated from soils using the procedure described above. Among more than 100 strains, isolated in the laboratory and screened for protease and chitosanase activity, TKU022 strain was selected. The TKU022 strain that showed the highest protease and chitosanase activity was isolated, maintained on nutrient agar, and used throughout the study.

TKU022 is a gram-positive and endospore-forming bacillus that contains catalase but not oxidase and that grows in both aerobic and anaerobic environments. According to the result of a 16S rDNA partial base sequence (approximately 1.5 kbp) analysis, strain TKU022 is most closely related to *Bacillus cereus* and *Bacillus thuringiensis*. According to the API identification, TKU022 was most closely related to *B. cereus*, with a 98.2% similarity. Therefore, the isolate was identified as *B. cereus.*

2.2. Production of extracellular protease and chitosanase by *B. cereus* TKU022

In our preliminary experiments, the protease, chitosanase, and chitinase activities were measured in the culture supernatant of strain TKU022 during various cultivation times. The culture supernatants exhibited protease and chitosanase activities, but the results were negative for the chitinase assay in the supernatants. To further investigate the production of protease and chitosanase by strain TKU022 during 6 days of cultivation in the production medium, the 50 mL of basal medium containing 1.5% SHP was the most suitable for the production of protease and chitosanase at 37 °C (data not shown). After a 6-h lag phase, exponential growth was observed for 3 days, and stationary phase was achieved after 3 days. Interestingly, strain TKU022 secreted extracellular chitosanase at the beginning of the exponential phase and achieved maximal production of up to 0.6 U/mL at this phase (2 days; Fig. 1). Thereafter, the chitosanase activity decreased remarkably with the appearance of protease activity (Fig. 1). This secretion pattern of the protease is similar to those reported in previous studies in which the majority of proteases were not produced or were only produced at a low rate in the exponential growth phase and normally reached the maximal production in the stationary phase.[2,11] Therefore, the protease production was closely related to the cell growth. This result indicated that the production of protease is cell growth dependent, and *B. cereus* TKU022 is a promising protease producer.

To investigate the reason why the chitosanase activity decreased with the first appearance of protease, *B. cereus* TKU022 was incubated under the optimized conditions for the production of protease and chitosanase. *B. cereus* TKU022 was incubated for 2 days (for chitosanase production) and for 5 days (for protease production). Using the culture supernatants of *B. cereus* TKU022, we assayed the enzyme activities of protease and chitosanase. Equal volumes of protease and chitosanase preparations were mixed and reacted at 37 °C for 0, 10, 20, 30, and 60 min. The results are shown in Fig. 2. After 60 min of reaction, the chitosanase in the absence of protease retained 96% of its original activity, but the chitosanase in the presence of protease retained only 38% of its original activity. It is conjectured that the reason for the obvious decrease in chitosanase activity might be that the chitosanase was hydrolyzed by the protease and therefore lost its activity. Similar to this study, *B. subtilis* IMR-NK1 is also a protease- and chitosanase-producing strain.[3] Because the recovery yield was lower for the purified chitosanase, the authors of this previous study conjectured that the hydrolysis by the protease caused the decrease in the activity of the chitosanase.

Comparison of productivity by activity was often seen in review papers concerning enzymes. The maximum values of TKU022 protease activity (0.5 U/mL) and chitosanase activity (0.6 U/mL) were higher than those of some other reports in the same reaction system and the same definition of enzyme activity, such as *Bacillus* sp. TKU004 protease (0.067 U/mL),[12] *Bacillus* sp. TKU004 chitosanase (0.16 U/mL),[13] and *B. subtilis* TKU007 chitosanase (0.03 U/mL).[14] However, it is indeed very difficult to make comparison because the conditions used for assaying were usually different in the literature. In addition, for comparison to be meaningful, the reaction system and definition of unit for enzyme activity need to be the same. We could not make comparison of enzyme activity of our

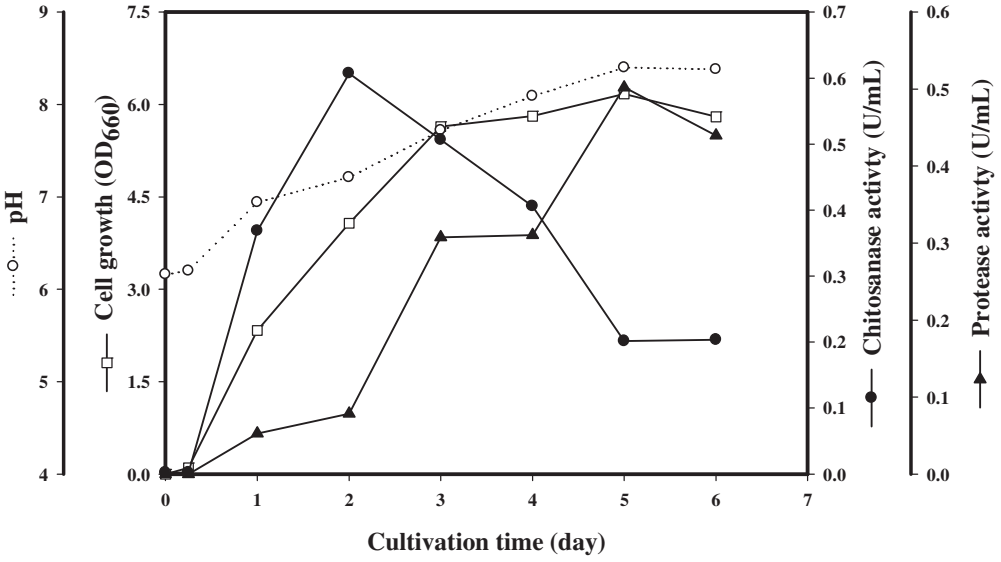

Figure 1. Time courses of the protease and chitosanase production in a culture of *B. cereus* TKU022 on shrimp head-containing media: (▲) protease activity (U/mL); (●) chitosanase activity (U/mL); (□) cell growth; (○) pH.