

Chitosanase Production by *Paenibacillus ehimensis* and its Application for Chitosan Hydrolysis

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ABSTRACT

The chitosanase production by *Paenibacillus ehimensis* was studied in submerged cultures and the chitosan hydrolysis was evaluated by using these enzymes without purification. The bacterium produced inducible enzymes after 12 h of growth in a culture medium containing 0.2% (w/v) of soluble chitosan as carbon source. The enzyme production was strongly repressed by the presence of glucose. The production started as soon as the available sugars finished in the culture medium. The maximum level of chitosanase activity was 500 U.L⁻¹ at 36°C after 36 h incubation. The crude enzyme was optimally active at pH 6.0 and 55°C and in these conditions, the enzyme presented good stability (6 days). The enzyme without purification was used to hydrolyze the chitosan which resulted chitooligosaccharides between 20 and 30 min of reaction.

Key words: Chitosanase, *Paenibacillus ehimensis*, chitosanase production, crude enzymes and TLC

INTRODUCTION

Chitosan, a D-glucosamine polymer, is a totally or partially deacetylated derivative of chitin. Chitin can be extracted from shrimp or crab shells and is second most abundant polysaccharides in the nature, with an annual production of 10¹⁰ to 10¹¹ tons (Kumar, 2000; Kurita, 2001). The accumulation of large amounts of crustaceans processing discard has become a major concern in the seafood processing industry. This waste has been a challenge for shellfish-production. Therefore, the production of high added-value products, such as, chitin, chitosan and their derivatives along with their application in different fields are of utmost interest (Shahidi et al. 1999).

Chitosan and its derivatives showed functional properties making them useful in many fields including, food, cosmetics, medicine and pharmaceuticals (Shahidi et al. 1999; Kim and Rajapakse, 2005). However, its poor solubility makes the chitosan difficult to be used in food and biomedical applications. On the other hand, chitosan oligosaccharides (COS) are readily soluble in water due to their shorter chain lengths and free amino groups in the D-glucosamine units (Li et al. 2005). COS, composed of 2 to 10 units of D-glucosamines, are easily absorbed in the intestine and quickly get into the blood flow. COS also present a systemic biological effects in the organism, such as antitumor, prebiotic and

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antimicrobial activities (Kim and Rajapakse, 2005).

Conversion of chitosan into COS can be done either by acid or enzyme hydrolysis. Chemical hydrolysis is carried out at high temperatures under highly acidic conditions, resulting in a large amount of glucosamine (chitosan monomer), due to the difficulties in the formation process control. Therefore, this method produces low yields of pentamers and hexamers. Enzymatic hydrolysis has some advantages in producing COS. Chitosanases can catalyze the hydrolysis under mild conditions (Kuo et al. 2004; Ming et al. 2006). Chitosanases have been recognized as enzymes that attack chitosan but not chitin and defined as the enzyme performing endohydrolysis of β -1,4-linkages between D-glucosamine residues in a partly acetylated chitosan (Su et al. 2006). Chitosanase has been found in a variety of microorganisms, including various bacterial species (Kurakake et al. 2000; Choi et al. 2004; Kim et al. 2004; Su et al. 2006; Sun et al. 2007; Zhu et al. 2007; Gao et al. 2008; Wang and Yeh, 2008; Wang et al. 2008a; Wang et al. 2008b) and fungi (Chen et al. 2005; Ike et al. 2007).

Although, microbial chitosanases shows excellent performances in COS production, they are expensive to be utilized in large-scale industrial applications, because enzyme hydrolysis requires multi-steps, particularly, enzyme preparation and purification.

In an attempt to obtain a chitosanase which could be used without purification for chitosan oligomers production, a bacterium isolated from soil rich in chitin was employed in this work. The induction and the production of chitosanolytic enzymes from *Paenibacillus ehimensis* were studied. The chitoooligosaccharides obtained using these crude enzymes and the enzymes stability were also evaluated.

MATERIALS AND METHODS

Microorganism and maintenance of culture

The bacterial strain used in this study was the *Paenibacillus ehimensis* NRRL B-23118, previously isolated from a soil sample collected in Japan (Kuroshima et al. 1998). The strain was activated in nutrient broth for 48 h at 36°C. For the maintenance, the supernatant was removed by centrifugation at 2500 rpm for 15 min at 20°C and

1.5 mL of 10% (v/v) glycerol solution was added on the bacterial cells and they were stored at -20 and -196°C.

Materials

Chitosan was purchased from Sigma-Aldrich Co. (St. Louis, Mo.). Other reagents were of analytical grade.

Preparation of soluble chitosan

Soluble chitosan was prepared as follows: 10 g of powder chitosan was suspended and dissolved through stirring in 1 L of HCl solution (0.1 M). Insoluble materials were removed by filtration through sintered glass filter and the pH of the solution was adjusted to 7.0 by wise drop addition of 10 N NaOH for enzyme production and 6.0 for oligosaccharides production (Choi et al. 2004).

Enzyme production in submerged cultures

For the production of chitosanase, the culture medium consisted ($\text{g}\cdot\text{L}^{-1}$): peptone 6.0, yeast extract 6.0, glucose 1.0, K_2HPO_4 1.0, $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ 0.5 and soluble chitosan 2.0. The initial pH was adjusted to 7.0 with NaOH and the medium was sterilized by autoclaving at 121°C for 15 min. The above medium (45 mL in 250 mL Erlenmeyer flasks) was inoculated with 5 mL of a pre-culture in exponential growing phase as previously defined (Pagnoncelli et al. 2007) and incubated on an orbital shaker at 36°C and 120 rpm. Triplicate flasks were withdrawn at regular intervals and analysed for reducing sugars, enzymatic activity and pH. Then the contents were centrifuged at 12.000 rpm for 15 min and the cell-free supernatant was used for analysis.

Enzyme assay

Chitosanase activity was assessed in triplicate by measuring the reducing sugars produced from chitosan hydrolysis. Five hundred microliters of the supernatant (crude enzymes) was mixed with 500 μL of soluble chitosan (pH 6.0), and the reaction mixture was incubated for 30 min at 55°C. To stop the reaction, 2.5 mL of dinitrosalicylic acid was added and the reducing sugars were measured immediately by using DNS method (Miller, 1959) with D-glucosamine as the standard. One unit of chitosanase was defined as the amount of enzyme that released 1 μmol of D-glucosamine per minute under the conditions described above.

Effect of temperature on chitosanase activity

To determine the optimum temperature, the supernatant and the soluble chitosan were incubated at 30-85°C for 30 min in acetate buffer (pH 6.0). The reducing sugars were measured immediately by using DNS method.

Effect of pH on chitosanase activity

For the measurement of optimum pH, the supernatant was incubated with soluble chitosan at 3.0 and 9.0 at 55°C for 30 min. The reducing sugars were measured immediately by DNS method.

Stability on chitosanase activity

The thermal stability of the enzymes was investigated after incubating the supernatant for six days at 55°C in absence of the soluble chitosan (substrate) at pH 6.0, 7.5 and 9.0. Aliquots were sampled and the remaining activity was assayed at the optimum pH and temperature as previously determined.

Analysis of hydrolytic product of soluble chitosan

The reaction mixture, consisting of 2 mL of 1% (w/v) of soluble chitosan and 2 mL of supernatant (crude enzymes), was incubated at 55°C for 9 h. The reaction was stopped by boiling for 10 min, followed by centrifugation at 12.000 rpm for 15 min and filtered. The COS mixture was analyzed by TLC. The reaction mixture (30 µL) was spotted

on silica gel 60 plates (Merck) and developed in a solvent system composed of n-butanol: 30% ammonia water (2:1, v/v) (Choi et al. 2004). COS standard (obtained from Seikagaku Co) were run in parallel to the COS mixture. After solvent development, plate was dried by hot air and immersed in a saturated silver nitrate solution (0.7 g/ 200 mL acetone). Plate was dried by hot air again and COS were detected by spraying the plate with 0.5 N sodium hydroxide/ethanol solution then heating at 80°C for 20 min (Hsiao et al. 2008).

RESULTS AND DISCUSSION

Enzyme production

Figure 1 shows the time-course profile of chitosanase production by *P. ehimensis* in a basal medium containing soluble chitosan. Chitosanase production started after the total glucose consumption by the microorganism, reaching a maximum at 36 h (500 U.L⁻¹). While the culture medium had glucose as carbon source, the enzyme production induction was not seen and it started when the glucose levels dropped to 0.2 g.L⁻¹. Thus, the chitosanase produced by *P. ehimensis* was inducible like most of reported chitosanases. These enzymes might be synthesized in the presence of either their substrate in the surrounding medium or compounds structurally related to them, a behaviour common in all the inducible enzymes (Suto and Tomita, 2001; Costa et al. 2008).

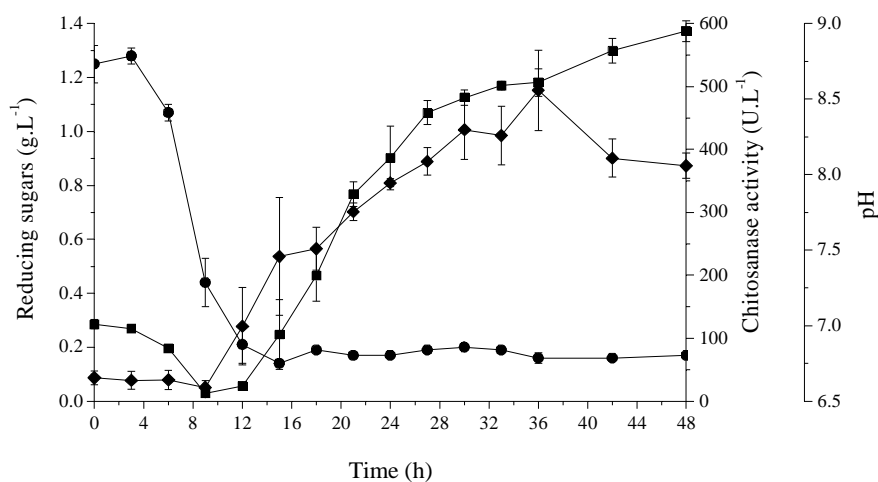


Figure 1 - Chitosanase production, reducing sugar consumption and pH as a function of cultivation time of *Paenibacillus ehimensis* grown at 36°C on soluble chitosan in shake flasks. (■) pH, (●) reducing sugar and (◆) chitosanase activity.

The pH of the medium initially dropped as glucose consumption started, but as soon as the enzyme production was initiated, the pH started to rise. This indicated that some organic nitrogen was being consumed with consequently ammonia formation. It was observed that during the enzyme production in the absence of simple sugars such as amylase, chitinase and chitosanase, the microorganisms needed to use the nitrogen sources for energy. The subsequent decrease in the enzyme activity after 36 h could probably be due to the inactivation of the enzymes by other constituent such as proteases (Sumantha et al. 2006). Kinetics studies of chitosanase and chitinase production by *Sphingomonas* sp. CJ-5 and *Pseudomonas* sp. TKU 015 and chitosanase production by *Microbacterium* sp. OU01 and *Serratia marcescens* TKU011 have shown the same behavior (Sun et al. 2007; Zhu et al. 2007; Wang et al. 2008a; Wang et al. 2008b). This way the chitosanase activity obtained by these microorganisms should not be compared to the activity obtained using the enzymes produced by *P. ehimensis* because the reaction conditions were different, like the substrate added in the reaction and the time of the reaction.

Chitosanase produced by *Pseudomonas* sp. TKU 015 after 72 h had a chitosanase activity of 25 $\text{mU}\cdot\text{mL}^{-1}$, and after 30 minutes in contact with a 1% chitosan solution, the relation between substrate and enzyme was 2:1 (Wang et al. 2008a). Best results were obtained using chitosanase from *Sphingomonas* sp. CJ-5, 0.9 $\text{U}\cdot\text{mL}^{-1}$ after 30 minutes, in the same conditions using the enzymes produced by *P. ehimensis*, but the enzyme production reaching a maximum only after 96 h (Zhu et al. 2007). A 72 h old culture of *Serratia marcescens* TKU011 presented a chitosanase activity around 0.025 $\text{U}\cdot\text{mL}^{-1}$ which was after 30

minutes of a enzyme / substrate (1:5) reaction. The highest level of chitosanase activity in the *Bacillus cereus* D-11 culture after cultivation for 72 h was 4.85 $\text{U}\cdot\text{mL}^{-1}$, but this activity was investigated after 30 minutes of enzyme / substrate (1:45) reaction (Gao et al. 2008). Chitosanase produced by *Microbacterium* sp. OU01 after 96 h had a maximum chitosanase activity of 118 $\text{U}\cdot\text{mL}^{-1}$, after 15 minutes in contact with a 1% chitosan solution, the relation between enzyme and substrate was 1:10 (Sun et al., 2007). The activity of the chitosanase produced by *P. ehimensis* was evaluated after 30 minutes and the relation between the substrate and enzyme was 1:1, and probably, with increasing the substrate the enzyme activity will also increase. In this work, the enzyme production conditions were satisfactory, since the most important was the time of oligosaccharide formation and the use of one enzyme without purification as well.

Enzyme activity optimal conditions

Chitosanases produced by others *Bacillus* sp. strains showed optimum activities at pH values between 4.0 and 8.0, the optimum temperatures of activity have been reported to be in the ranges 30-70°C (Kurakake et al. 2000; Aktuganov et al. 2003; Jo et al. 2003; Choi et al. 2004; Kim et al. 2004; Su et al. 2006; Gao et al. 2008; Wang and Yeh, 2008). The effect of pH and temperature on the catalytic activity of chitosanase from *P. ehimensis* was studied by using soluble chitosan as substrate changing the pH and temperature during the assay.

The enzyme activity was assayed at temperatures ranging from 30 to 85°C at a constant pH of 6.0. Chitosanase presented constant activity from 50 to 75°C (Fig. 2). For practical purposes, further assays were carried out at 55°C.

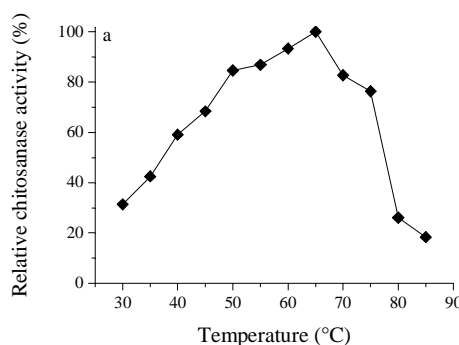


Figure 2 - Effect of temperature on chitosanase activity produced by *Paenibacillus ehimensis*.

The effect of pH on the catalytic activity was studied by using chitosan as a substrate under the standard assay conditions. The experimental domain was set for pH between 3.0 and 9.0. The enzyme had an optimum pH at pH 6.0 (Fig. 3).

Enzyme stability

To determine the thermal stability of chitosanase

produced by *P. ehimensis*, the residual activity was measured after incubation of the supernatant at various pH for 6 days at 55°C in the absence of substrate (soluble chitosan) and the residual activity was measured along the time (Fig. 4).

The chitosanase produced was stable at pH 6.0, lost around 5% activity at pH 7.5 and 60% at pH 9.0.

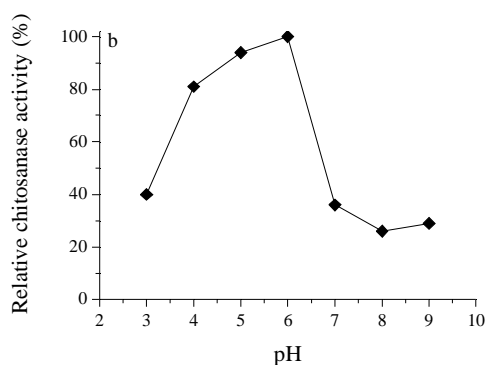


Figure 3 - Effect of pH on chitosanase activity produced by *Paenibacillus ehimensis*.

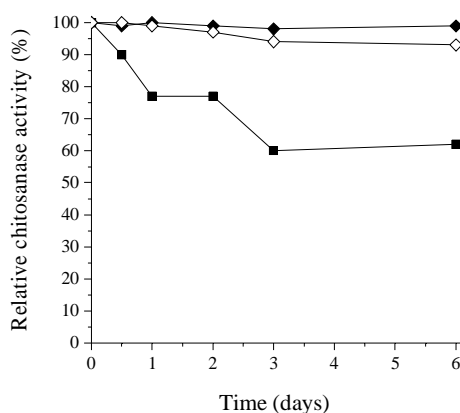


Figure 4 - Effect of temperature on the stability of chitosanase at pH 6.0 (◆), 7.5 (◇) and 9.0 (■).

Enzymes exposed to extremes pH and temperatures lose activity. *Microbacterium* sp OU01 produced two chitosanases, which lost the activity easily. One was optimally active at 50°C and the half-life was 30 min, the other enzyme presented optimal temperature of 60°C and after 20 min half activity was detected (Sun et al. 2006). After purification, chitosanase produced by *Serratia marcescens* TKU011 presented the optimum temperature of 60°C, but was rapidly inactivated retaining only 60% of its initial activity

after 60 min at this temperature (Wang et al. 2008b). Chitosanase purified from the culture of *Pseudomonas* sp TKU011 presented 40% of activity loss when exposed for 30 min at 50°C (Wang et al. 2008a). Purified chitosanases produced by *B. cereus* S1 exposed for 30 min at 50°C lost 35% of enzyme activity. Enzyme exposition for the same time at 60°C resulted in complete enzyme denaturation (Kurakake et al. 2000). Purified chitosanases produced by *Bacillus* sp. KCTC 0377BP showed maximum activity at

60°C, but in 5 min exposure at this temperature, the activity was half reduced (Choi et al. 2004). Purified enzymes produced by *B. subtilis* showed optimal activity at 37°C and after 60 min, half activity was lost (Wang and Yeh, 2008). Purified enzyme from *Bacillus* sp. MET 1299 showed optimal activity at 60°C and after 60 min, 80% of activity was lost (Kim et al. 2004). The crude chitosanases produced by *P. ehimensis* showed higher stability at optimal activity condition compared to purified chitosanases produced by other microorganisms. The high stability of the enzyme is important for industrial applications of the crude enzymes, which could reduce the process costs.

Analysis of chitosanase hydrolytic products of soluble chitosan

The products obtained by enzymatic hydrolysis of soluble chitosan using the crude chitosanases from

P. ehimensis (supernatant) were analyzed by TLC (Fig. 5). The synthesis was carried out at pH 6.0 and 55°C (optimum activity conditions) containing 1% (w/v) of soluble chitosan. Chitosan was hydrolyzed to (GlcN)₂ and (GlcN)₆ at the initial stage of the reaction. After incubation for 2 h, the amounts of (GlcN)₂ and (GlcN)₃ in the hydrolysates increased.

COS was obtained after 20 min of reaction and their maximum concentration (visualized by the spot intensity on TLC plate) was obtained between 20 and 30 min of reaction. The crude chitosanase enzyme complex from *P. ehimensis* produced COS with different sizes, suggesting that the mode of action of the enzyme was endo-type (Chen et al. 2005). The crude enzymes resulted in COS profile comparable to that obtained using purified chitosanases produced by other microorganisms (Kim et al. 2004; Ike et al. 2007).

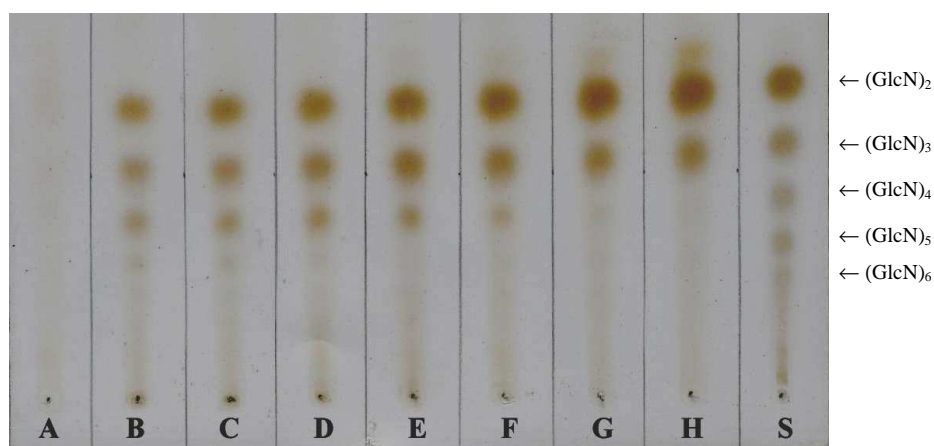


Figure 5 - TLC profiles of chitooligosaccharides produced in the hydrolysis of soluble chitosan by crude chitosanase from *Paenibacillus ehimensis*. Lane A-H, present hydrolysates obtained after enzyme reaction for 10, 20, 30 min, 1, 2, 3, 6 and 9 h; Lane S, COS standard.

CONCLUSIONS

In this work, the inductive chitosanase effect of chitosan on *P. ehimensis* B- 23118 was observed. The chitosanase obtained by this microorganism showed maximum activity at pH 6.0 at 50-70°C. Thus, 55°C was chosen as the optimum temperature for further assays. Crude enzyme showed good stability at optimum activity

conditions being suitable for direct utilization without purification.

The crude chitosanases hydrolyzed soluble chitosan into biofunctional oligomers (COS). The use of crude enzymes instead purified ones, are of industrial interest because enzyme purification steps are expensive steps in industrial enzyme production. Crude chitosanase from *P. ehimensis* B-23118 showed good industrial potential.

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RESUMO

A produção de quitosanases pelo *Paenibacillus ehimensis* foi estudada em culturas submersas e a hidrólise da quitosana foi realizada utilizando essas enzimas sem purificação. As enzimas foram obtidas após 12 horas de crescimento desta bactéria em meio de cultivo contendo 0,2% (p/v) de quitosana solúvel como fonte de carbono. A produção das enzimas foi fortemente reprimida na presença de glicose, sendo obtida após o consumo total dos açúcares disponibilizados no referido meio de cultivo. A máxima atividade quitosanolítica foi obtida após 36 horas de cultivo a 36°C, atingindo valores de 500 U.L⁻¹. As enzimas utilizadas no extrato bruto apresentaram melhores atividades quando submetidas a condições de pH e temperatura de 6,0 e 55°C, respectivamente, e nessas condições permaneceram estáveis durante 6 dias. Essas enzimas sem serem submetidas aos processos de purificação foram utilizadas para hidrolisar a quitosana, obtendo os quito-oligosacarídeos entre 20 e 30 minutos de reação.

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