

Full Length Research Paper

Evaluation of proteases produced by *Erwinia chrysanthemi* for the deproteinization of crustacean waste in a chitin production process

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This study evaluated the potential use of proteases produced by *Erwinia chrysanthemi* for the extraction of pure chitin from crustacean wastes using standard methods. The crude bacterial protease had activity of 22.4 U/ml. Protein removal by the commercial protease were similar in both the mineralized and demineralized wastes and amounted to about 75 and 80%, respectively, in 16 h. A similar trend was observed with higher protein removal efficiency (95%) for the crude protease from both the mineralized and demineralized wastes. Also, treatment of the powder with 5% NaOH resulted in the removal of protein in the demineralized and mineralized wastes decreased by about 96 and 87.6%, respectively, in same period from an initial concentration of 0.3 mg/ml. It was concluded that proteases produced by *E. chrysanthemi* could be used to transform waste from sea-food processing industries into products of commercial value.

Key words: *Erwinia chrysanthemi*, extra cellular, proteases, chitin, value-added products.

INTRODUCTION

Crustacean shells are major wastes of seafood processing industries and they tend to cause unpleasant odors or environmental pollution if not adequately processed. However, since crustacean wastes are rich in such valuable substances such as protein, astacene, chitin and calcium, they could be used to produce high value-added materials if recycled. Chitin, chitosan and their oligosaccharides have so many useful functions and applications in agriculture, medicine, food industry, chemical engineering and environmental protection (Perberdy, 1999).

Chitin is a high molecular weight linear polymer of N-acetyl-D-glucosamine (N-acetyl- 2 -amino- 2 -deoxy- D-glucopyranose) units linked by β -D-glucopyranose bonds. It is the most prominent structural polysaccharide in the exoskeleton of insects, crustaceans and invertebrates in general. The shells of crabs and lobsters are common sources of chitin and hence it is the major source of surface pollution in coastal areas (Simpson et al., 1994).

In many respects, chitin plays an analogous role to that played by collagen in higher animals and by cellulose in plants. Even though chitin is widely distributed in nature, it is never found in its pure form (Simpson et al., 1994). In its natural state, chitin is tightly associated with proteins, lipids, pigments and calcium deposits (Simpson et al., 1994) and needs to be purified before it is of any commercial use. Currently, the purification of chitin consists of two main steps, demineralization with dilute acid or chelating agent and deproteinization with dilute alkali or proteolytic enzyme (No et al., 1989). However, prolonged alkaline treatment under severe conditions causes depolymerization and deacetylation (Chaussard and Domard, 2004), which in the case of the later is not a disadvantage as chitin is usually converted to chitosan (Simpson et al., 1994; Kumar, 2000). Depolymerization however, will affect the viscosity of the final product and hence should be avoided. The growing interest in finding alternative methods for chitin extraction, which can maximize shell waste utilization and at the same time being environmentally friendly has brought about the use of bacterial proteolytic enzymes for protein removal from chitin rich fractions (Lagarreta et al., 1996).

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The use of proteolytic enzymes for the deproteinization of crustacean wastes is a current trend in the conversion of shell fish wastes into useful biomass (Aytekin and Elibol, 2009; Manni et al., 2009). It is a simple and inexpensive alternative to chemical methods for the deproteinization of crustacean wastes in chitin preparation (Aytekin and Elibol, 2009; Manni et al., 2009). Proteases are degradative enzymes that catalyse the total hydrolysis of proteins and execute a large variety of other functions. Also, proteases have a long history of application in the food and detergent industries (Bhaskar et al., 2007; Ma et al., 2007; Wang et al., 2008; Wang et al., 2009). Their application in the leather industry for dehairing and bating of hides to substitute currently used toxic chemicals developed over a decade ago has conferred added biotechnological importance (Rao and Deshpande, 1998).

Since proteases are physiologically necessary for living organisms, they are found in a wide diversity of sources such as plants, animals and microorganisms. Microorganisms represent an excellent source of enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation. Microbial proteases account for approximately 40% of the total worldwide enzyme sales (Godfray and West, 1996) and are preferred to plant and animal proteases since they possess almost all the characteristics desired for biotechnological applications (Rao et al., 1998). In this study, the potentials of proteases produced by *Erwinia chrysanthemi* for the deproteinization of crustacean wastes in chitin production were assessed.

MATERIALS AND METHODS

Processing and treatment of crustacean wastes

Crustacean wastes in the form of lobster heads (*Palinurus* sp.) were obtained from Lusitania fishing company in Port Elizabeth, South Africa. The heads of lobsters were defrosted in sunlight and the inner parts removed and discarded. The shells were sun-dried for a week and thereafter grounded into a powder using a grinder (Relsch Muhle, AGE Mbsh 10 VDE 0660) and stored in tightly sealed 25 l buckets.

The calcium present in the crustacean waste was removed using the modified No and Meyer (1997) acid-based demineralization approach as follows: The shellfish powder was mixed with 1.5 N HCl (1:2 ratio, w/v or sufficient enough to cover the sample) and the solution stirred continuously for 2 h. The acid was decanted from the test slurry and the chitinaceous material was washed with water until the effluent was neutral. The pH was determined and the sample further treated with HCl, washed again with water and dried.

The assay for calcium was carried out as described by Fatoki and Mathabatha (2001). Shellfish powder (0.5 g) was weighed into a 100 ml beaker and concentrated nitric acid (HNO₃) (5 ml) was added. The mixture was gently heated to 70°C and held at that temperature for 30 min. After cooling, 2 ml of perchloric acid (HClO₄), 5 ml of concentrated nitric acid (HNO₃) and 5 ml of hydrofluoric acid (HF) were added and the mixture heated to dryness. The walls of the beaker were rinsed with double distilled water (5 ml) and the solution further heated until white dense fumes developed. The beaker was allowed to cool and concentrated HNO₃ (10 ml) was

added to dissolve the salts. The solution was transferred to a 50 ml standard flask, made up to the mark with double distilled water and the amount of calcium was then analyzed using the Thermo ICE 3300 AA Spectrometer (Essex, United Kingdom). The digestion was done in triplicate.

Erwinia chrysanthemi strain and protease production

A Lac⁺, slimy *E. chrysanthemi* mutant (ECS) (Gray et al., 1984) was used in this study. The stock culture was maintained on nutrient agar slant at 5°C and sub-cultured weekly. The high-calcium *Xanthomonas campestris* (HXC) medium described by Muyima et al. (2001) was adjusted to pH 7 before sterilizing at 101 kPa (121°C) for 20 min, allowed to cool and used for the determination of protease activity. The medium contained 1% sodium polypectate (NaPP) as carbon source. A loop of *E. chrysanthemi* cells from the stock culture was used to inoculate 100 ml HXC broth medium. The cell culture was incubated (30°C, 180 rpm, 12 h), centrifuged (27,200 × g, 10 min, 4°C) and the supernatant used for the determination of protease activity.

Protease assay

Protease activity was assayed as described by Muyima et al. (2001). The assay mixture contained the following ingredients: azocasein (1.5 % w/v, 0.25 ml), 0.1 M imidazole-HCl buffer (pH 6.2; 0.25 ml). The mixture was maintained at 37°C for 10 min before adding cell-free extract (0.5 ml) and incubated for a further 30 min. The reaction was stopped by adding 1.5 M HClO₄ (0.5 ml). A blank was prepared by adding HClO₄ prior to the addition of cell-free extract. The reaction mixture was centrifuged on a microfuge (MiniStar Plus, Hangzhou Allsheng Instruments Co, Ltd., China) at 9000 rpm for 2 min. The supernatant (0.5 ml) was mixed with 1 M NaOH (0.5 ml) and the absorbance was determined at 440 nm wavelength in a Milton Roy Spectronic 1201 spectrophotometer (Milton Roy, New York, USA). One enzyme unit is defined as the amount that catalyses the hydrolysis of 1 µg of azocasein (1% w/v in 0.1 M NaOH). After determining the absorbance at 440 nm, a factor of 29.411 was used to convert net A₄₄₀ to U/ml. Net A₄₄₀ was obtained by subtracting the absorbance at 440 nm of the blank tube from that of the assay tube.

Estimation of protein content

Protein concentration in the processed shells was determined using the method described by Stoscheck (1990). Bovine serum albumin (BSA) was used as a standard protein. Absorbance was determined spectrophotometrically at 260 and 280 nm (Milton Roy, New York, USA) and converted to protein concentration using the following equation:

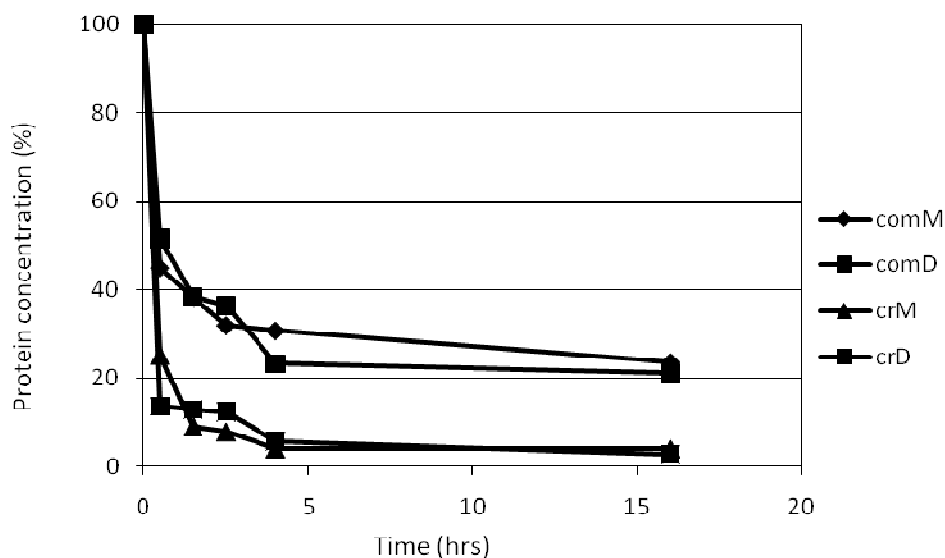
$$\text{Protein (mg/ml)} = 1.55 A_{280} - 0.76 A_{260}$$

Deproteinization

Chemical deproteinization using NaOH (Synowiecki and Al-Khateeb, 2000; Wang et al., 2008; Aytekin and Elibol, 2009) was performed in parallel with enzymatic deproteinization for comparison purposes. Two different concentrations of NaOH (5 and 10% w/v) were used. Three replicates of mineralized and demineralized powder (10 g each) were weighed into 250 ml flasks and NaOH (30 ml) was added to immerse the powder. The shell samples were incubated at 37°C for varied times up to 16 h with continual shaking. Samples were washed with a pre-determined volume of tap water and dried.

Table 1. Concentration of calcium in crustacean shell powder before and after treatment with HCl.

Treatment	Shellfish powder (g) \pm SD	Calcium concentration (mg/ml) \pm SD
Before	25 \pm 00	103.6 \pm 0.721
After	14.95 \pm 0.139	0.534 \pm 0.172

**Figure 1.** Deproteinization of crustacean shell powder by proteases. M = mineralized sample, D = Demineralized sample, com = Commercial protease, cr = Crude *E. chrysanthemi* protease).

Protein concentration was determined before and after the deproteinization process.

For enzyme deproteinization, demineralized powder (10 g) was weighed into 250 ml flasks. An appropriate volume of cell-free extract was added to degrade the protein in the powder. The samples were incubated at 37°C for varied times up to 16 h with continual shaking. A commercial protease was used as a control to compare with *E. chrysanthemi* produced proteases. Samples were washed with a pre-determined volume of tap water and dried. Protein concentration was determined before and after the deproteinization process.

RESULTS AND DISCUSSION

The demineralization process resulted in the reduction of the initial shell waste powder from 25 g to about 15 g (Table 1). Simultaneously, the concentration of calcium also decreased from 103.6 to 0.534 mg/ml after treatment with 1.5 N HCl (Table 1), a drop of over 99%. The decrease in the initial amount of powder could be attributed to the removal of HCl by washing with water. The initial protein concentration before deproteinization with the enzyme was 0.421 mg/ml and *E. chrysanthemi*-produced protease activity was measured at 22.4 U/ml, which was slightly higher than the 20.2 U/ml reported by Yang et al. (2000) for *Bacillus subtilis*.

Protein removal by the commercial protease were similar in both the mineralized and demineralized wastes and amounted to about 75 and 80% respectively in 16 h. A similar trend was observed though with a higher protein removal efficiency (95%) for the crude protease from both the mineralized and demineralized wastes (Figure 1).

Dow et al. (1998) showed that the proteases produced by *X. campestris* were able to degrade various hydroxyproline-rich glycoproteins that were thought to be the building blocks of plant cell walls. These proteins have also been reported to play a role in immobilizing bacterial cells after infection (Taggart et al., 2005; Baoyu et al., 2007). Wang and Chio (1997) used *Pseudomonas aeruginosa K-187* cells to diminish the protein content of shrimp and crab shells. Also, Gagne and Simpson (1993) investigated the utilization of chymotrypsin and papain for the deproteinization of shrimp waste and reported reduced residual protein levels after using either of the enzymes.

Both the mineralized and demineralized wastes powders were also deproteinized using two different concentrations of NaOH (Figure 2). When the powder was treated with 5% NaOH, protein concentration in the demineralized and mineralized wastes decreased by about 96 and 87.6%, respectively, in 16 h from an initial concen-

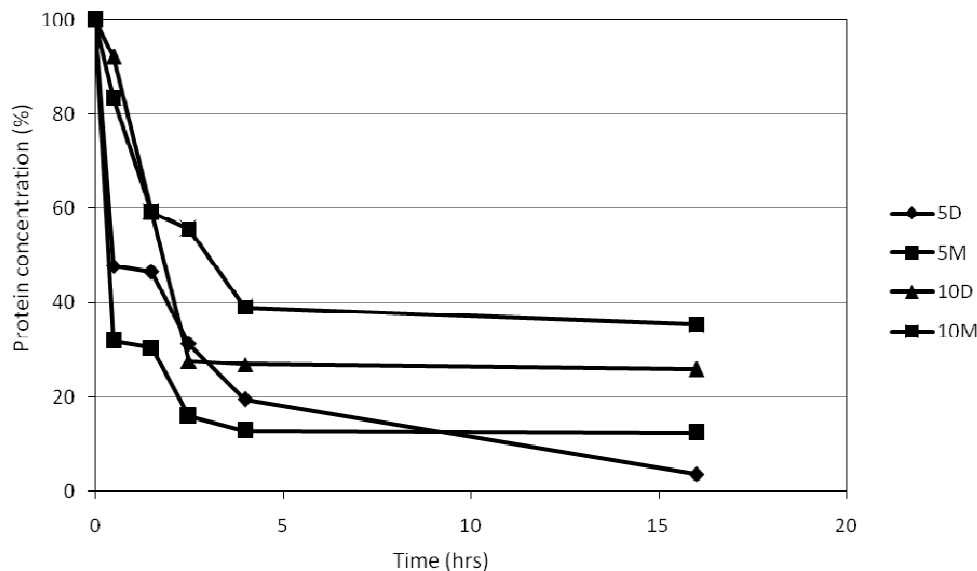


Figure 2. Reduction of protein concentration at different NaOH concentrations. M = Mineralized; D = Demineralized.

tration of 0.3 mg/ml. Also, at a 10% NaOH concentration, protein concentration in the demineralized and mineralized waste decreased by about 74 and 64.6%, respectively, over the same period (Figure 2). Regardless of the type of treatment used, effective deproteinization seems to be accomplished over a longer incubation period of reaction.

Most studies carried out on *E. chrysanthemi* proteases have concentrated on the isolation and characterization of the enzyme with little examination of its role in plant pathogenicity and the present study suggests that high performing *E. chrysanthemi* strain could find applications in the biotransformation of agricultural waste as has been corroborated by Muyima et al. (2001). In addition, compared to the chemical approach, the use of microorganisms promises to be less costly and more environmentally friendly.

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