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A Solvent-Stable Metalloprotease Produced by *Pseudomonas aeruginosa* A2 Grown on Shrimp Shell Waste and Its Application in Chitin Extraction

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Abstract A solvent-stable protease-producing bacterium was isolated and identified as *Pseudomonas aeruginosa* A2. The strain was found to produce high level of protease activity when grown in media containing only fresh shrimp waste (FSW) or shrimp waste powder (SWP), indicating that it can obtain its carbon, nitrogen, and salts requirements directly from shrimp waste. Maximum protease activities 17,000 and 12,000 U/mL were obtained with 80 g/L SWP and 135 g/L FSW, respectively. The optimum temperature and pH for protease activity were 60 °C and 8.0, respectively. The crude protease, at different enzyme/substrate (E/S) ratio, was tested for the deproteinization of shrimp waste to produce chitin. The crude enzyme of *P. aeruginosa* A2 was found to be effective in the deproteinization of shrimp waste. The protein removals after 3 h hydrolysis at 40 °C with an E/S ratio of 0.5 and 5 U/mg protein were about 56% and 85%, respectively. ^{13}C CP/MAS-NMR spectral analysis of the chitin prepared by treatment with the crude protease was carried out and was found to be similar to that of the commercial α-chitin. These results suggest that enzymatic deproteinization of shrimp waste by A2 protease could be applicable to the chitin production process.

Keywords Shrimp shell · Waste valorization · *Pseudomonas aeruginosa* · Protease · Enzymatic deproteinization · Chitin

Introduction

Chitin, an homopolymer of *N*-acetyl-D-glucosamine residues linked by ß-1,4 bonds, is the most abundant renewable natural resource after cellulose [1]. The main sources of raw material for the production of chitin are cuticles of various crustaceans, principally crabs and shrimps. Chitin in biomass is closely associated with proteins, inorganic compounds (such as calcium carbonate), lipids, and pigments. They all have to be quantitatively removed to achieve the high purity of chitins necessary for biological applications [2].

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Chitin is insoluble in alkali and in most organic solvent. Thus, due to its low reactivity, chitin is mainly used as raw material to produce chitin-derived products, such as chitosans, chito-oligosaccharides, and glucosamine. Chitin and its derivatives are biomolecules of a great potential, possessing versatile biological activities, demonstrating excellent biocompatibility, and complete biodegradability. Therefore, they have found extensive applications in pharmacy, medicine, agriculture, food and textile industries, cosmetics, and wastewater treatment [3–5].

To extract chitin from crustacean shells, chemicals processing for demineralization and deproteinization have been applied. Raw materials were first treated with dilute hydrochloric acid at room temperature to remove metal salts, particularly calcium carbonate, and then with strong bases to remove proteins [2]. However, the use of these chemicals may cause a partial deacetylation and depolymerisation of the chitin, resulting in final inconsistent physiological properties [6].

An alternative approach to these harsh chemical treatments is the use of proteolytic microorganisms [7–9] or proteolytic enzymes [7]. Bustos and Healy [10] demonstrated that chitin obtained by the deproteinization of shrimp shell waste with various proteolytic microorganism had higher molecular weights compared to chemically prepared shellfish chitin.

During a screening program on protease producing strains, *Pseudomonas aeruginosa* A2, producing high protease activity, was isolated. In this study, we describe the production and biochemical characterization of the extracellular solvent-stable protease produced by A2 strain. The use of the crude protease for shrimp waste deproteinization to produce chitin was also investigated.

Materials and Methods

Materials

Casein, ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), and α-chitin were purchased from Sigma Company (Sigma Co., St Louis, USA). Hydrochloric acid was from Panreac Quimica. Coomassie Brilliant Blue R-250 was from Bio-Rad Laboratories (Mexico). All other reagents were of analytical grade.

Methods

Screening and Identification of Protease-Producing Bacterium

A protease-producing bacterium A2 was isolated from marine water in Sfax city (Tunisia) by selective screening on skim milk agar plates containing (g/L): peptone 5, yeast extract 3, bacteriological agar 12, and skim milk 250 ml. The isolate was identified as *P. aeruginosa* according to the methods described in Bergey's Manual of Determinative Bacteriology and on the basis of the 16S rDNA sequence analysis. The 16S rDNA gene of the isolate was amplified using the upstream primer P1: 5′ CCGAATTCGTCGACAACA-GAGTTTGATCCTGGCTCAG3′ and the downstream primer P2: 5′CCCGGGATCCAAGCTT-AAGGAGGTGATCCAGCC3′ which generate a DNA fragment of approximately 1.5 kb. Amplification of DNA was carried out using the Techne TC-312 thermocycler under the following conditions: denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 1 min, 59 °C for 1 min, 72 °C for 2 min, and

final extension at 72 °C for 15 min. DNA nucleotide sequence was determined by the dideoxynucleotide chain termination method [11] using the ABI PRISM 3100 Avant (Applied Biosystem) sequencer according to the instructions of the manufacturer. Two independent PCR products were sequenced to confirm the fidelity of the sequence. Sequence comparison with the databases was performed using Basic Local Alignment Search Tool (BLAST) through the NCBI server [12].

Preparation of Fresh Shrimp Waste and Shrimp Waste Powder

The shrimp (*Metapeneaus monoceros*) shells were procured in fresh condition from a shrimp processing plant located at Sfax City, Tunisia. The fresh shrimp waste (FSW) and shrimp waste powder (SWP) were prepared in our laboratory. Briefly, to get FSW, shrimp waste were washed thoroughly with distilled water and then cooked 20 min at 100 °C (to eliminate the microbial flora present in the waste, and to inactivate all endogen enzyme activities present in the raw material), filtered, then minced and stored at −20 °C. To obtain SWP, FSW was dried, then minced again to obtain a fine powder, and stored in glass bottles at room temperature. The chemical composition (proteins, chitin, lipids, and ash) was determined.

Enzyme Production

P. aeruginosa A2 was cultivated in media composed only of SWP or FSW. Inocula were routinely grown in Luria-Bertani (LB) broth medium composed of (g/L): peptone 10.0, yeast extract 5.0, NaCl 5.0, and pH 7.0 [13]. Media were autoclaved at 121 °C for 20 min. For LB agar plates, media were solidified with 1.8% (*w/v*) agar [14].

Cultivations were performed on a rotary shaker (200 rpm) for 24 h at 37 °C, in 250 mL conical flasks with a working volume of 25 mL. The cultures were centrifuged and the supernatants were used for estimation of protease activity.

Zymography

Zymography was performed on sodium dodecyl sulfate (SDS)-PAGE according to the method of Garcia-Carreno et al. [15] with slight modification. After electrophoresis, the gel was immersed in 100 mM of Tris-HCl buffer (pH 8.0) containing 2.5% Triton X-100, with shaking for 1 h to remove SDS. Triton X-100 was removed by washing the gel three times with 100 mM Tris-HCl buffer (pH 8.0). The gel was then incubated with 1% (*w/v*) casein in 100 mM Tris-HCl buffer (pH 8.0) at 50 °C for 30 min. Finally, the gel was stained with Coomassie Brilliant Blue R-250 and destained with 5% ethanol −7.5% acetic acid. Clear zone on the blue background of the gel indicated the presence of protease activity.

Protease Assay

Protease activity was measured by the method described by Kembhavi et al. [16] using casein as a substrate. A 0.5-mL aliquot of the crude enzyme, suitably diluted, was mixed with 0.5 mL of Tris-HCl (pH 8.0) containing 1% (*w/v*) casein and incubated for 10 min at 60 °C. The reaction was stopped by the addition of 0.5 mL trichloroacetic acid 20% (*w/v*). The mixture was allowed to stand at room temperature for 15 min and then centrifuged at 10,000×*g* for 15 min to remove the precipitate. The acid-soluble material was estimated spectrophotometrically at 280 nm. A standard curve was generated using solutions of 0–

50 mg/L tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine per minute under the experimental conditions used.

Properties of the Enzyme

Effect of pH on Protease Activity and Stability The optimum pH of the protease was studied over a pH range of 5.0–12.0. For the measurement of pH stability, the enzyme was incubated for 1 h at 30 °C in different buffers and the residual protease activity was determined under standard assay conditions. The following buffer systems were used: 100 mM sodium acetate buffer for pH 5.0–6.0, Tris-HCl buffer for pH 7.0–8.0, glycine-NaOH buffer for pH 9.0–11.0, and Na_2HPO_4-NaOH buffer for pH 12.0.

Effect of Temperature on Protease Activity and Stability To investigate the effect of temperature, the activity was tested using casein as a substrate at the temperature range from 30 to 80 °C in 100 mM Tris-HCl buffer, pH 8.0. Thermal stability was examined by incubating the crude enzyme at different temperatures from 30 to 60 °C. Aliquots were withdrawn at desired time intervals to test the remaining activity at pH 8.0 and 60 °C. The non-preincubated enzyme was considered as control (100%).

Effects of Metal Ions and Enzyme Inhibitors on Protease Activity The influence of various metal ions, at a concentration of 5 mM, on enzyme activity (500 U/mL) was investigated by adding the monovalent (Na^+ and K^+) or divalent (Mg^{2+}, Hg^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Ba^{2+}, and Mn^{2+}) metal ions to the reaction mixture. The activity of the enzyme without any metallic ion was considered as 100%.

The effects of some enzyme inhibitors on protease activity (500 U/mL) were studied using PMSF, EDTA, and β-mercaptoethanol. The crude enzyme was preincubated with inhibitors for 30 min at 30 °C and then the remaining enzyme activities were measured at pH 8.0 and 60 °C, using casein as a substrate. The activity of the enzyme preincubated in the absence of inhibitors was taken as 100%.

Effect of Organic Solvents on Protease Stability The organic solvent stability of the enzyme was studied by incubating the crude enzyme with various organic solvents (50%, *v/v*): methanol, ethanol, N,N-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), diethyl ether, hexane, acetone, and acetonitrile at 30 °C with shaking (150 rpm). Aliquots were withdrawn at desired time intervals to test the remaining activity under standard assay conditions.

Enzymatic Deproteinization of Shrimp Waste

Shrimp shell waste (15 g) were mixed with water at a ratio of 1:3 (*w/v*), minced and then cooked for 20 min at 90 °C. The cooked sample was then homogenized in a Moulinex® blender for about 2 min. The pH of the mixture was adjusted to 8.0, and then the shrimp waste proteins were digested with A2 crude enzyme at 40 °C using different enzyme/substrate (E/S) ratio (unit of enzyme/mg of proteins) for various lengths of time. After, the reaction was stopped by heating the solution at 90 °C for 25 min to inactivate the enzyme. The shrimp waste protein hydrolysates were then centrifuged at 5,000×*g* for 20 min. The solid phase was washed and then pressed manually through four layers of gauze. The protein content was analyzed for calculation of the protein removal rate. The press cake was packed in a plastic bag and stored at −20 °C until further processing.

Deproteinization percentage (%DP) was calculated by the following equation as described by Rao et al. [17].

$$\%\mathrm{DP} = \frac{[(Po \times O) - (P_R \times R)] \times 100}{Po \times O}$$

where P_O and P_R are protein concentrations (%) before and after hydrolysis; while, O and R represent the mass (g) of original sample and hydrolyzed residue in dry weight basis, respectively.

Chemical Demineralization

Demineralization was carried out in dilute HCl solution. The solid fraction obtained after deproteinization of shrimp waste by A2 protease was treated with HCl (5%) in 1:10 (*w/v*) ratio at room temperature (25 °C) under constant stirring. The obtained chitin was washed to neutrality with deionized water.

Analysis

Moisture and ash were determined according to standard methods [18]. The pH value was measured using a pH meter (827 pH lab, Metrohm, swiss). Total nitrogen (TN) and chitin nitrogen (CN) were estimated by the Kjeldahl method [18]. Corrected protein was obtained by subtracting CN from TN and multiplying by 6.25, the Kjeldahl conversion factor for meat protein, assuming that protein has 16% nitrogen [19]. Calcium, sodium, potassium, and magnesium were analyzed for chitin using atomic absorption spectrometer.

For nuclear magnetic resonance (NMR) analysis, chitinous materials, obtained after deproteinization and demineralization of shrimp shell waste, were dried and then grind to obtain a fine powder. The quantity used for each NMR analysis was about 0.4 g. The NMR spectra of chitin samples were obtained by the ^{13}C cross-polarization, magic-angle-spinning (CP-MAS) spectroscopy method using a BRUKER-ASX300 instrument. NMR spectra were recorded at a ^{13}C frequency of 75.5 MHz (field of 7.04 T). CP-MAS sequence was used with the following parameters: the ^{13}C spin lattice relaxation time was 5 s, powdered samples were placed in an alumina rotor used for the double air-bearing type MAS system and spun as fast as 8 kHz. Contact time was 8 ms.

Results

Isolation and Screening of Protease-Producing Strain

Several bacteria strains producing extracellular proteases were screened from different marine waters. Samples collected were plated onto skim milk agar plates. Plates were incubated 24–48 h at 37 °C. Clear halos around colonies on skim milk agar gave an indication of protease-producing strains. Individual colonies were purified through repeated streaking on fresh agar plates. Among more than 20 strains, isolate A2, exhibiting a large and clear zone of hydrolysis on skim milk agar plates and able to grow in media containing only shrimp waste powder, was selected.

Morphological, physiological, and biochemical properties of the A2 strain were investigated by use of methods described in Bergey's Manual of Systematic Bacteriology

Table 1 Morphological and biochemical characteristics of the isolate A2

Characters	Result
Shape	Rod
Gram stain	–
Spore	–
Motility	+
Flagella	–
Production of pigment	+
H_2S production	–
Catalase	+
Oxidase	+
Citrate	+
Indole production	+
Arginine dihydrolase	+
Indole	–
Urease	–
Nitrate reduction	+
Nitrite reduction	–
Hydrolysis of gelatin	+
starch	+
Assimilation of glucose	+
lactose	+
arabinose	–
mannose	–
mannitol	+
NAG	–
maltose	–
gluconate	+
caprate	+
adipate	–
malate	–
citate	–
phenyl-acetate	–
Fermentation of glucose	–

(+) positive result, (−) negative result

[20]. API-20NE strip was used for biochemical characterization of the isolate strain. Table 1 summarized the morphological, physiological, and biochemical characteristics of A2 strain. The results showed a typical characteristic of *P. aeruginosa* strain. In addition, the external transcribed spacer region of the 16S rDNA (1.5 kb) was amplified and sequenced. The nucleotide sequence was analyzed with the GenBank database using BLAST program and the isolate was identified as *P. aeruginosa*.

Protease Production on FSW and SWP

The isolate *P. aeruginosa* A2 was first cultivated in a flask for protease production in media containing only fresh shrimp shell waste at different concentrations. As depicted in Fig. 1,

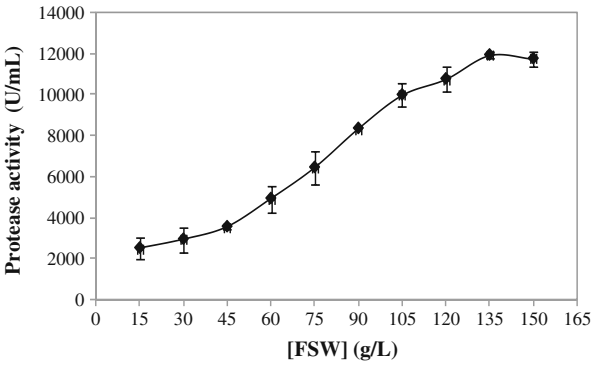

Fig. 1 Effect of fresh shrimp waste (FSW) concentrations on protease production by *P. aeruginosa* A2. Cultivations were performed for 24 h at 37 °C in media containing only FSW at different concentrations

A2 strain exhibited high level of protease activity. Protease production increased significantly with increasing concentration of FSW, and the highest level of protease activity (12,000 U/mL) was achieved at a concentration of 135 g/L (which correspond approximately to 45 g/L dry weight).

Protease production was also carried out in media containing only SWP as a complex growth substrate. The SWP contained high protein content (46–49%), relatively high ash and chitin contents (27–35%, 17–20%, respectively), and low lipid content (5–6.5%). As shown in Fig. 2, protease production increased with increasing SWP and reached a maximum value of 17,000 U/mL with 80 g/L. Beyond this concentration, protease activity decreased. These results indicated that the strain can obtain its carbon, nitrogen, and salts requirements directly from SWP.

The obtained results indicate that SWP is a suitable complex substrate for protease production by *P. aeruginosa* A2.

Biochemical Properties

Zymography is a sensitive and rapid assay method for analyzing protease activity. Zymography of the crude protease, using casein as a substrate, revealed only one clear band of casein hydrolysis, suggesting the presence of at least one protease activity (result not shown).

The pH activity profile of the crude enzyme, determined using different buffers of varying pH values, is depicted in Fig. 3a. The enzyme preparation was highly active

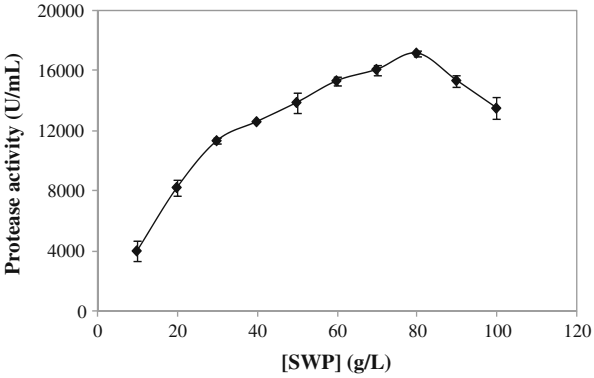

Fig. 2 Effect of shrimp waste powder (SWP) concentrations on protease production by *P. aeruginosa* A2. Cultivations were performed for 24 h at 37 °C in media containing only SWP at different concentrations

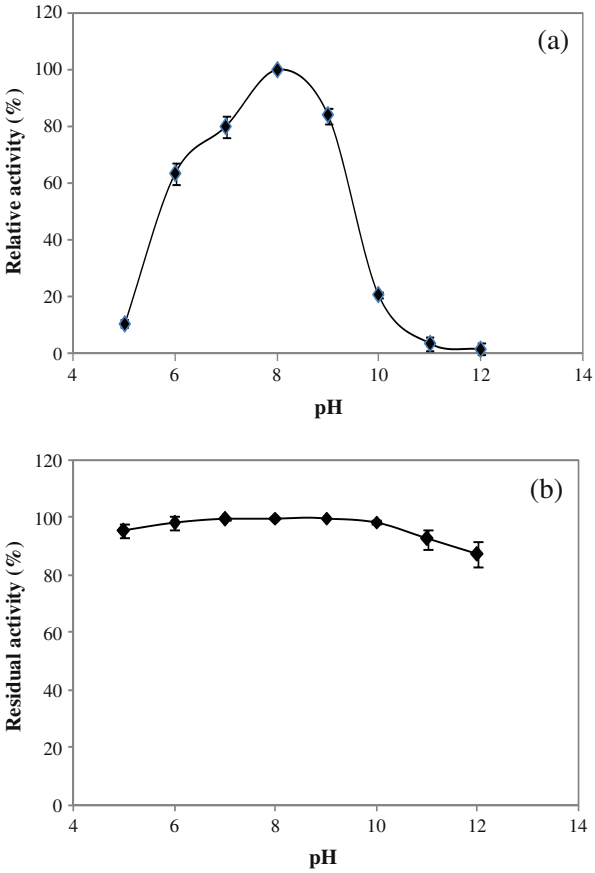

Fig. 3 Effect of pH on activity (**a**) and stability (**b**) of the A2 protease. The pH profile was determined in different buffers by varying pH values (5.0–12.0) at 60 °C. The maximum activity obtained at pH 8.0 was considered as 100% activity. The pH stability was determined by preincubating the enzyme in different buffers for 60 min at 30 °C and the residual activities were measured at pH 8.0 and 60 °C. The activity of the enzyme before preincubation was taken as 100%

between pH 6.0 and 9.0 with an optimum activity at pH 8.0. The relative activity at pH 9.0 was about 84%. The pH stability profile showed that the enzyme is stable in the pH range of 5.0–12.0 and retaining more than 85% of its initial activity after 1 h incubation at 30 °C (Fig. 3b).

The effect of temperature on the activity of A2 protease was examined at various temperatures. The crude protease was active between 30 and 80 °C, with an optimum at 60 °C (Fig. 4a). The relative activities at 55 °C and 65 °C were about 83% and 86%, respectively. Thermal stability of A2 protease was determined by incubating the enzyme for 7 h at different temperatures in 100 mM Tris-HCl buffer (pH 8.0) and the remaining activities were measured at appropriate times. As shown in Fig. 4b, A2 protease was stable and retained about 84%, 77%, and 59% of its initial activity after 7 h incubation at 30 °C, 40 °C, and 50 °C, respectively. At 60 °C, the half-life of the crude enzyme was approximately 2 h.

The effects of various metal ions, at a concentration of 5 mM, on the activity of the A2 protease were studied at pH 8.0 and 60 °C by the addition of the respective cations to the reaction mixture (Table 2). The enzyme activity was not affected by monovalent (K^+ and Na^+) ions. The Mg^{2+}, Co^{2+}, Ba^{2+}, and Ca^{2+} affect partially the protease activity with a relative activity between 87% and 96%. However, Mn^{2+}, Zn^{2+}, Cu^{2+}, and Hg^{2+} affect greatly the enzyme activity with more than 74% inhibition.

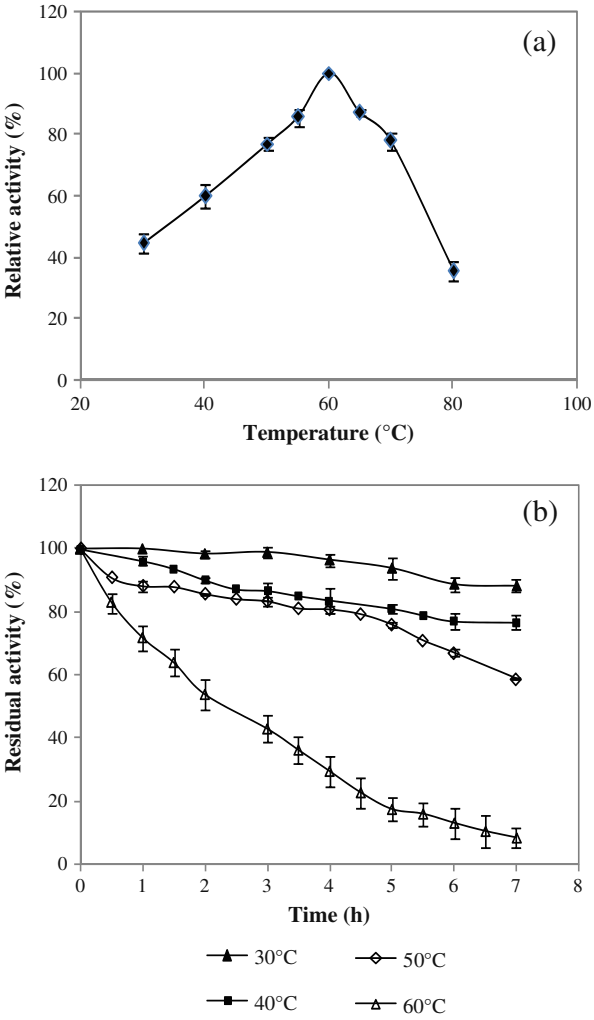

Fig. 4 Effect of temperature on activity (**a**) and stability (**b**) of the A2 protease. The temperature profile was determined by assaying protease activity at temperatures between 30 and 80 °C. The temperature stability was determined by preincubating the enzyme at temperatures from 30 to 60 °C for 7 h. The residual enzyme activities were measured under the standard conditions assay. The activity before preincubation was taken as 100%

The effects of various enzyme inhibitors, such as chelating agents and group-specific reagents, on the activity of the A2 protease were also investigated. PMSF, a serine enzyme inhibitor, slightly affect the activity of the enzyme. However, the enzyme was strongly inhibited by the metalloprotease inhibitor (EDTA; 10 mM), with 93.55% of its original activity being lost. This confirms that the protease from *P. aeruginosa* A2 strain belongs to the family of metalloproteases. *P. aeruginosa* A2 protease was not inhibited by the reducing agents β-mercaptoethanol, suggesting that the active form of the enzyme do not require disulfide bonds.

Stability in the Presence of Organic Solvents

The use of proteases has increased substantially in recent years as they catalyses the synthesis of peptide bonds as well as peptide–bond hydrolysis [21]. Peptide synthesis could be enhanced by the addition of organic solvents in the reaction mixture. Thus, to achieve a

Table 2 Effects of some metal ions and enzyme inhibitors on protease activity

Chemical/ion	Concentration (mM)	Activity (%)
None	–	100
EDTA	5	21.07
	10	6.45
PMSF	5	93.08
	10	68.55
β-mercaptoethanol	5	80.9
	10	67.13
K^+	5	105
Na^+	5	100.9
Mg^{2+}	5	96.1
Co^{2+}	5	96
Ba^{2+}	5	94.2
Ca^{2+}	5	87.4
Mn^{2+}	5	26.3
Zn^{2+}	5	7.9
Cu^{2+}	5	5.1
Hg^{2+}	5	3.4

The crude enzyme was preincubated with various inhibitors in 100 mM Tris-HCl buffer (pH 8.0) for 30 min at 30 °C and then the residual activities were determined at pH 8.0 and 60 °C. Effect of metal ions (5 mM) on protease activity was determined by incubating the enzyme for 10 min at 60 °C and pH 8.0. Enzyme activity measured in the absence of any additive was taken as 100%

high yield in protease-catalyzed peptide synthesis, protease stability in the presence of organic solvents is very important because enzymes are usually denatured or inactivated before completing the reaction.

Organic solvent stability of the A2 crude protease was evaluated by incubating the crude enzyme with 50% of various organic solvents at 30 °C with constant shaking. Half-lives of the crude protease activities are shown in Table 3. In the absence of organic solvent, the half-life of the A2 protease was about 28 days. In the presence of hexane, the half-life was about 31 days. Interestingly, the stability of the enzyme was highly enhanced by DMSO and diethyl ether; the half-lives of the crude enzyme in the presence of these solvents were more than 64 days. However, the stability of the A2 crude protease was highly affected by methanol, acetone, acetonitrile, and DMF.

Enzymatic Deproteinization of Shrimp Waste by A2 Protease

The A2 strain was found to grow well and produce proteases when it was cultivated in shell waste broth without any additional supplements. This indicates that the strain can deproteinize crustacean wastes to obtain its carbon and nitrogen requirements directly from protein hydrolysates. Therefore, the capacity of the crude protease in the deproteinization of shrimp waste was evaluated, since removing proteins from shrimp shell waste constitute a crucial step in the extraction procedure for obtaining chitin.

Many factors, such E/S ratio, temperature value, and hydrolysis time have been reported to influence the enzymatic deproteinization process. In the present study, different E/S ratios from 0.5 to 30 U/mg protein were first used to compare the shrimp waste

Table 3 Half-lives of the A2 protease in the presence of 50% (*v*/*v*) organic solvents

Organic solvent	Half-life (days)
None	28
Acetonitrile	0.5
Acetone	0.7
Ethanol	1.6
DMF	6
Methanol	7
Hexane	31
DMSO	64
Diethyl ether	65

Two milliliters of organic solvent was added to 2 ml of the cell-free supernatant and incubated at 30 °C, 150 rpm. The remaining proteolytic activities were measured regularly

deproteinization efficiency. Deproteinization was conducted for 3 h at 40 °C. As shown in Fig. 5, the deproteinization rate with an E/S ratio of 0.5 U/mg proteins was 56%. The percentage of protein removal increased with increasing E/S ratio and reached about 85% with an E/S of 5 U/mg protein, and no significant increase in the deproteinization was obtained when higher ratios were used.

Deproteinization with an E/S ratio of 5 U/mg protein was also followed during different times (1–7 h). The optimal deproteinization rate (85%) was obtained after 3 h of hydrolysis (Table 4).

The effect of temperature on the enzymatic deproteinization of shrimp waste was also investigated (Table 5). Optimal temperature was found to be 40 °C, with deproteinization rate of 85%.

Chemical Demineralization

Different contact time of hydrochloric acid 5% were tried to remove ash. This treatment converts the insoluble calcium carbonate into soluble calcium chloride that can subsequently be removed by washing. Mineral content (calcium, sodium, potassium, and magnesium) in the chitin prepared from the shrimp shells is shown in Table 6. From this table, it is clear that calcium content is higher than other minerals in chitin. Furthermore, increasing acid contact time showed improvement in demineralization efficiency. After a contact time of 6 h, mild acid treatment reduced the mineral content to permissible limits in the chitin.

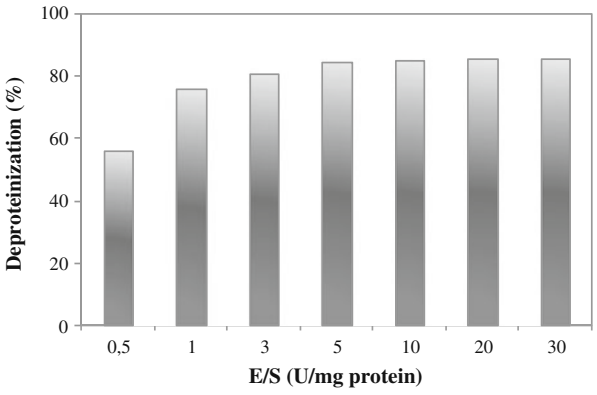

Fig. 5 Effects of the E/S ratio on the deproteinization of shrimp shell wastes. Protein hydrolysis was performed for 3 h at 40 °C

Table 4 Effect of hydrolysis time on the deproteinization of shrimp shell waste

Time (h)	1	2	3	5	7
Deproteinization (%)	60	79	85	85	85

Protein hydrolysis was performed at 40 °C and with an E/S ratio of 5

^{13}C CP/MAS-NMR Spectroscopic Analysis

NMR is one of the most powerful tools in the study of polysaccharide composition and sequential structure. NMR is a nondestructive method resulting in retained structure and conformation of the polysaccharide, making it possible to monitor reactions and other structural and physical properties under different solvent conditions.

Solid-state ^{13}C CP/MAS-NMR is known to be very sensitive to changes in the local structure. ^{13}C CP/MAS-NMR spectrum of the chitin sample, prepared by enzymatic deproteinization is shown in Fig. 6. NMR analysis of the shrimp waste chitin gave similar peak pattern to that of commercial chitin. There are eight signals for the eight carbon atoms of chitin. The C1-C6 carbons of N-acetylglucosamine monomeric unit are observed between 50 and 110 ppm, indicating high structural homogenity. The carbonyl group is around 173 ppm, while the methyl group of the acetyl group produced a peak at around 23 ppm. The ^{13}C signals for C3 (73.88) and C5 (75.86) in the spectrum were clearly separated into two signals. The eight signals appeared at chemical shifts given in Table 7.

Discussion

The expansion of biotechnology has created an increasing demand for new and low-cost medium. In most instances, in the production of industrial enzymes, up to 30–40% of the production cost is accounted by the growth substrate [22]. The use of cost-effective growth medium for the production of proteases can significantly reduce the cost of enzyme production. In the present study, *P. aeruginosa* was found to grow and produce appreciable level of protease in media containing only SWP as microbial growth substrate. In fact, 12,000 U/mL protease activity was obtained at 135 g/L concentration of FSW, corresponding to 45 g/L dry weight; while maximum protease activity (17,000 U/mL) was obtained when culture was conducted with 80 g/L of SWP. This suggests that the strain can obtain its carbon, nitrogen, and salts requirements directly from SWP. Therefore, the use of SWP, a cheap and readily available byproduct, could result in a substantial reduction in the cost of the enzyme production by *P. aeruginosa* A2.

The level of protease activity obtained by A2 strain in medium containing only SWP was largely higher than that of *P. aeruginosa* K-187 (21.2 U/mL). The highest protease activity produced by K-187 strain was carried out in medium containing 5% (*w/v*) shrimp

Table 5 Effects of temperature on the deproteinization of shrimp shell waste

Temperature (°C)	40	50	60
Deproteinization (%)	85	84	78

Protein hydrolysis was performed for 3 h with an E/S ratio=5

Table 6 Mineral content of the demineralized chitin

Mineral (μg/g) time (h)	Ca^{2+}	Na^{+}	Mg^{2+}	K^{+}
0	134,500	5,800	1,200	700
1	569	437	42	210
3	233	320	266	392
6	101	98	19	11
18	101	90	9	6
Commercial chitin	3900	810	<10	<10

and crab shell powder, 1% lactose, 0.5% NH_4NO_3, 0.1% K_2HPO_4, 0.5% $MgSO_4$, and 0.5% $FeSO_4$ $7H_2O$ [23]. The level of A2 protease activity was also higher than that of *P. aeruginosa* (1,160–1,175 U/mL) obtained on another solid waste discharged from leather manufacturing industries called animal fleshing [24].

Proteolytic activity of the crude protease was first studied by casein zymography. The crude preparation revealed the presence of a single band of protease activity, suggesting the presence of at least one protease in the crude enzyme.

Most proteases from *Pseudomonas* exhibited optimum pH values ranging from 7.0 to 9.0 [25–27]. Maximal proteolytic activity of A2 protease toward casein was observed at pH 8.0. The A2 protease was stable between pH 5.0 and 12.0 when incubated for 60 min. This stability was higher than those of *P. aeruginosa* MN7 and *P. aeruginosa* PseA proteases which were stable in the pH range from 6.0 to 11.0 [26] and 6.0 to 9.0 [25], respectively. The optimum temperature of A2 protease was 60 °C. This optimum was

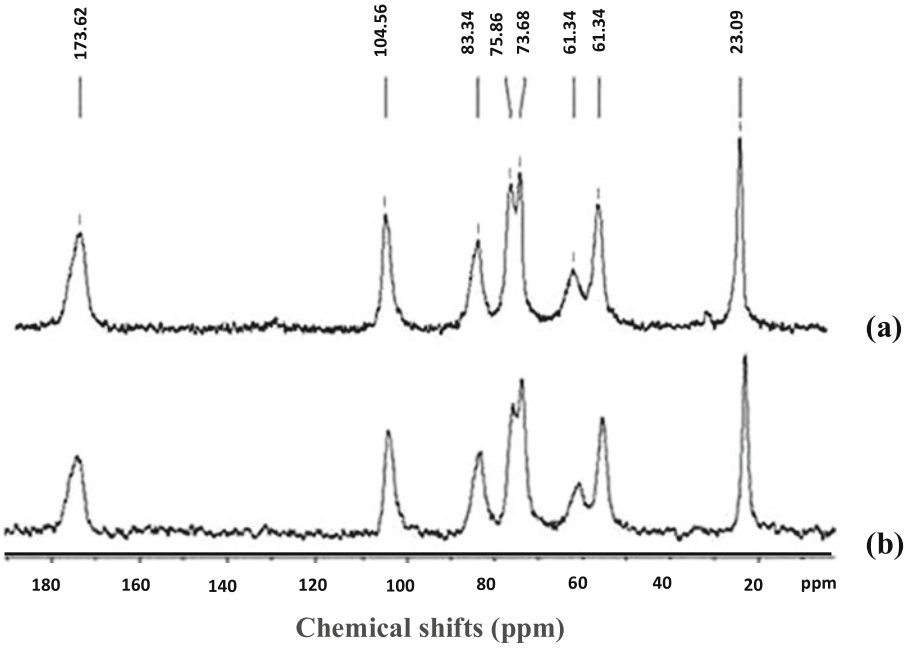

Fig. 6 ^{13}CP/MAS-NMR solid-state spectra of shrimp shell wastes chitin and commercial α-chitin. **a** Chitin extracted from shrimp shells treated with A2 crude protease, **b** commercial chitin

Table 7 Chemical shifts of chitins obtained by ^{13}CP/MAS-NMR

^{13}C signal assignment	A (ppm)	B (ppm)
C1	104.56	104.33
C2	55.69	55.42
C3	73.68	73.71
C4	83.34	83.14
C5	75.86	75.73
C6	61.34	60.8
CH3	23.09	23.02
C = O	173.62	174.09

A: chitin obtained by enzymatic deproteinization, B: commercial α-chitin

similar to the values reported earlier for proteases from other *Pseudomonas* strains [25, 26], and higher than that of PST-01 protease which had optimum temperature at 55 °C [27]. A2 protease was slightly affected by serine protease inhibitor, but metalloprotease inhibitor EDTA was found to be inhibitory (93.55% inhibition at 10 mM), suggesting that the enzyme is a metalloprotease.

A2 crude protease was stable in various organic solvents, especially DMSO and diethyl ether. From a biotechnological perspective, there are many advantages of employing enzymes in organic media, such as high regioselectivity and stereoselectivity, minimal side-chain protection requirements. However, the major drawback of using enzymes in organic solvents is their significantly reduced activity compared to that in buffer media. If enzymes were naturally stable in organic solvents and exhibited high activity, they would be more useful for synthetic reactions. Since most of the enzymes including proteases are not stable in organic solvents, several contemporary techniques such as site-directed mutagenesis, immobilization, chemical modification, and directed evolution have been used to obtain organic solvent stable proteases. However, exploring naturally organic solvent stable proteases shall mitigate some of the limitations encountered by above-mentioned modified proteases.

The application of microorganisms or proteolytic enzymes for deproteinization of marine crustacean wastes is a current research trend in conversion of wastes into useful biomass. It is a simple and environment-friendly alternative to chemical methods employed in the preparation of chitin. In this study, the A2 culture protease was used for the deproteinization of shrimp shell waste. After optimization of E/S ratio, hydrolysis time and temperature, the maximum deproteinization rate was 85% with an E/S ratio of 5 U/mg protein after 3 h of hydrolysis at 40 °C. The deproteinization activity of A2 crude protease was better than many proteases reported in previous studies. In fact, the percentage of protein removal from natural shrimp waste was only 78% after a 7-day incubation at 37 °C with the culture supernatant from *P. aeruginosa* K-187 [23]. Bustos and Healy [10] reached no more than 64% deproteinization by using purified *P. maltophilia* protease, and a maximum value of 82% was achieved after 6 days of fermentation. With *Bacillus* sp. TKU004 which produce a solvent-stable metalloprotease, the percentage of protein removal reached only 73% after 3 days of incubation [28]. On the other hand, Manni et al. [29] investigated the utilization of an oxidant and organic solvent-stable metalloprotease produced by *Bacillus cereus* SV1 on the deproteinization of shrimp waste. The protein removal with an E/S ratio of 20 U/mg protein was 88.8%.

The fact that deproteinization cannot reach 100% may be explained by the non-accessibility of enzymes to some proteins protected by chitin. In fact, the proteins in the inner layer of shrimp shell waste are protected by the outer layer chitin from the attack by proteases, thus no further proteolysis could occur [28]. In addition, some portions of peptides are suggested to be linked covalently to a small number of the C-2 amino groups of chitins.

Although the deproteinization percentage is lower than that of chemical treatment, enzymatic deproteinization avoids many problems of chemical treatments such as heavy metal residues, overhydrolysis, and breakdown of chitins, etc. However, retaining some protein in the chitin preparation might provide some uses [28]. For instance, Chui et al. [30] reported the demineralization of shrimp shell waste from *Penaeus marginatus* by dilute hydrochloric acid in small columns to yield shrimp chitin, without removal of proteins. When chitin is to be used as a biosorbent for removal and recovery of heavy metals (such as Cu^{2+}, Cr^{3+}), retaining protein in the shrimp shells enables the prepared chitin (protein-containing shrimp chitin) to possess enhanced metal chelating properties comparable to chitosan [28].

One of the factors determining the good quality of chitin is the low mineral content [31]. To produce chitin of standard quality, ash content of the deproteinized residue has to be removed by mild acid treatment. Calcium is the most abundant mineral in the shrimp shell waste. This is due to the fact that the main constituent of minerals in shrimp shell is calcium carbonate [32]. After 6 h treatment in 5% acid, the mineral content was reduced to permissible limits in the chitin. Sini et al. [9] confirm that the optimum concentration of acid used for demineralization depends on the ash content of the raw material.

NMR analysis of the shrimp waste chitin, obtained after enzymatic deproteinization and chemical demineralization, gave similar peaks pattern to that of commercial chitin. The C-1–C-6 carbons of the main N-acetylglucosamine are found between 50 and 100 ppm, the carbonyl peak (acetyl group) is around 170 ppm, and the methyl peak of the acetyl group is around 20 ppm. The separation in the C-1–C-6 and methyl peaks was less distinct for fungal chitin, although the essential peaks used for chitin identification are essentially present.

The ^{13}C signals for C3 (73.68) and C5 (75.86) in the spectrum were clearly separated into two signals, similarly to the α-chitin reported by Cárdenas et al. [33] and Focher et al. [34]. In contrast, for the β-chitin from *Illex argentinus* squid pens, the C3 to C5 merge into single resonance centered at 75.0 ppm [35].

Conclusion

This work describes the characterization of the crude protease highly produced by *P. aeruginosa* A2 in media containing only shrimp waste powder. The highest level of protease activity (17,000 U/mL) was obtained with 80 g/L SWP. Since SWP is cheaply obtained, its use as a growth substrate instead of commercial substrates may reduce considerably the cost of the enzyme production.

The optimum pH and temperature of the proteolytic activity were 8.0 and 60 °C, respectively. The crude enzyme was found to deproteinize shrimp waste. The protein removal after 3 h at 40 °C with an E/S ratio of 5 U/mg protein was about 85%.

The obtained results demonstrated that the crude of *P. aeruginosa* A2 could be used effectively in the deproteinization of shrimp shell waste to produce chitin. Furthermore, the biological deproteinization may overcome the disadvantages of chemical deproteinization.

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