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An Oxidant- and Solvent-Stable Protease Produced by *Bacillus cereus* SV1: Application in the Deproteinization of Shrimp Wastes and as a Laundry Detergent Additive

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Abstract The current increase in amount of shrimp wastes produced by the shrimp industry has led to the need in finding new methods for shrimp wastes disposal. In this study, an extracellular organic solvent- and oxidant-stable metalloprotease was produced by *Bacillus cereus* SV1. Maximum protease activity (5,900 U/mL) was obtained when the strain was grown in medium containing 40 g/L shrimp wastes powder as a sole carbon source. The optimum pH, optimum temperature, pH stability, and thermal stability of the crude enzyme preparation were pH 8.0, 60 °C, pH 6–9.5, and <55 °C, respectively. The crude protease was extremely stable toward several organic solvents. No loss of activity was observed even after 60 days of incubation at 30 °C in the presence of 50% (*v*/*v*) dimethyl sulfoxide and ethyl ether; the enzyme retained more than 70% of its original activity in the presence of ethanol and *N*,*N*-dimethylformamide. The protease showed high stability toward anionic (SDS) and non-ionic (Tween 80, Tween 20, and Triton X-100) surfactants. Interestingly, the activity of the enzyme was significantly enhanced by oxidizing agents. In addition, the enzyme showed excellent compatibility with some commercial liquid detergents. The protease of *B. cereus* SV1, produced under the optimal culture conditions, was tested for shrimp waste deproteinization in the preparation of chitin. The protein removal with a ratio *E*/*S* of 20 was about 88%. The novelties of the SV1 protease include its high stability to organic solvents and surfactants. These unique properties make it an ideal choice for application in detergent formulations and enzymatic peptide synthesis. In

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addition, the enzyme may find potential applications in the deproteinization of shrimp wastes to produce chitin.

Keywords Shrimp wastes · *Bacillus cereus* SV1 · Enzymatic deproteinization · Organic solvent-stable protease · Oxidant stable

Introduction

Shrimp by-products have been identified as an animal protein source of a great potential and also as an important source of chitin and asthaxanthin [1]. Only 65% of the shrimp is edible. The remainder is discarded as inedible waste (cephalothorax and exoskeleton).

Over the years, techniques have been developed for the recovery and exploitation of these by-products in valuable biopolymers such as chitin and chitosan [2–4]. These biomolecules are widely used in the food industry, pharmacy, textiles, chemical industries, etc. [5]. Conventionally, to extract chitin from crustacean shells, chemical processing for demineralization and deproteinization has been applied by treatment with strong acids and bases to remove calcium carbonate and proteins, respectively [6]. However, the use of these chemicals may cause a partial deacetylation of the chitin and hydrolysis of the polymer resulting in final inconsistent physiological properties [7]. To overcome the hazards from chemical treatments, alternative methods on use of microorganisms [8–10] or proteolytic enzymes [11] for the deproteinization of crustacean wastes have been reported.

The bioconversion of shellfish chitin wastes for the production of proteases [12–15] and/or chitinases [16] has been proposed in order to enhance the utilization of chitin containing marine crustacean wastes. Proteases are by far the most important group of enzymes produced commercially and are used in many areas of applications such as the detergent, food, agrochemical, and pharmaceutical industries [17].

Reports on enzymes that are naturally stable and also exhibiting high activities in the presence of organic solvents and detergents have got significant importance in the present area [18]. In a previous study, Manni et al. [19] reported the purification and molecular characterization of *Bacillus cereus* SV1 protease. The present paper describes the production of a novel surfactant- and oxidant-stable alkaline protease from *B. cereus* SV1 on shrimp wastes powder (SWP). The organic solvent and surfactant stabilities of the protease and its potential application in the deproteinization of shrimp wastes are investigated.

Materials and Methods

Bacterial Strain

The strain used in this study was isolated from an oil sewage station from a fishing port in Sfax, Tunisia. It was identified as *B. cereus* SV1 based on its morphological and physiological characteristics and 16S rRNA sequence analysis [19]. For a short-term maintenance and use, the strain was streaked on Luria–Bertani (LB) agar petri plates composed of (g/L) casein peptone 10.0, yeast extract 5.0, NaCl 5.0, and agar 18.0 [20]. For a long-term storage, the strain was conserved at −80 °C in LB medium supplemented with 30% (*v*/*v*) glycerol.

Preparation of Shrimp Wastes Powder

The SWP, used in the experiments, was prepared in our laboratory. Briefly, wastes from *Metapeneaus monoceros* shrimps, collected from the marine food processing industry, were washed thoroughly with tap water and then cooked for 20 min at 100 °C. The solid material obtained was dried, ground to obtain a fine powder, and then stored in glass bottles at room temperature. The chemical composition (proteins, chitin, lipids, and ash) was determined.

Chemical Analysis of Shrimp Wastes Powder

The moisture and ash content were determined according to the AOAC (1995) standard methods 930.15 and 942.05, respectively [21]. Total nitrogen content of shrimp protein hydrolysates was determined by using the Kjeldahl method. Crude protein was estimated by multiplying total nitrogen content by the factor of 6.25. Lipids were determined gravimetrically after soxhlet extraction of dried samples with hexane. The ionic composition was measured by atomic absorption spectrophotometer (Fe, Mn, Cu, and Zn) and by HPLC (Ca, Na, K, and Mg).

Enzyme Production

The initial medium M1 used for the production of proteases by *B. cereus* SV1 consists of (g/L) SWP 10.0, ammonium sulfate 2.0, K_2HPO_4 0.5, KH_2PO_4 0.5, and $MgSO_4$, 7 H_2O 0.1 (pH 8.0). Cultures were performed on a rotatory shaker (150 rpm) for 72 h at 30 °C in 250-mL conical flasks with a working volume of 25 mL. Media were autoclaved at 121 °C for 20 min. The growth of the microorganism was estimated by total plate count on nutrient agar. The culture medium was centrifuged at 12,000×*g* for 15 min at 4 °C, and the cell-free supernatant was used for estimation of proteolytic activity. Inocula were routinely grown in LB.

Protease Assay

The protease activity was measured by the method described by Kembhavi et al. [22] using casein as a substrate. A 0.5 mL aliquot of the culture supernatant, suitably diluted, was mixed with 0.5 mL Tris–HCl buffer (pH 8.0) containing 2 m*M* $CaCl_2$ and 1% (*w*/*v*) casein and incubated for 15 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 rest 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.

pH Optimum and pH Stability

The optimum pH of the crude protease was studied over a pH range of 6.0–12.0. For the measurement of pH stability, the enzyme was incubated for 1 h at 40 °C in different buffers, and the residual proteolytic activity was determined under standard assay conditions. The

following buffer systems were used: 100 m*M* phosphate buffer, pH6.0–7.5; 100 m*M* Tris–HCl buffer, pH8.0–8.5; 100 m*M* glycine-NaOH buffer, pH9.0–11.0; 100 m*M* KCl-NaOH buffer, pH12.0.

Effect of Temperature on Activity and Stability

The optimum temperature of protease activity was determined with reaction mixture incubated at different temperatures between 40 and 70 °C for 15 min in the presence of 2 m*M* $CaCl_2$. Thermal stability was examined by incubating the enzyme preparation at different temperatures for 60 min in the presence of 2 m*M* $CaCl_2$. Aliquots were withdrawn at desired time intervals to measure the remaining activity at pH8.0 and 60 °C. The non-heated enzyme was considered as control (100%).

Effect of Enzyme Inhibitors and Metal Ions

The effect of enzyme inhibitors on protease activity was studied using phenylmethylsulfonyl fluoride (PMSF), ethylene-diaminetetraacetic acid (EDTA), and dithio-nitrobenzene (DTNB). The crude enzyme was preincubated with inhibitors for 30 min at 25 °C, and then the residual enzyme activity was measured using casein as a substrate at pH8.0 and 60 °C. The activity assayed in the absence of inhibitors was taken as 100%.

The influence of various metal ions (5 m*M*) on enzyme activity was investigated by adding the monovalent (Na^+ and K^+) or divalent (Mg^{2+}, Hg^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, Ba^{2+}, and Mn^{2+}) metal ions to the reaction mixture. The activity of the enzyme without metallic ions was taken as 100%.

Effects of Oxidizing Agents and Surfactants on Protease Stability

The effect of some surfactants (SDS, Triton X-100, Tween 20, and Tween 80) and oxidizing agents (H_2O_2 and sodium perborate) on enzyme stability were studied by incubating the crude enzyme for 1 h at 40 °C and the residual activities were measured. The activity of the enzyme without any additives was taken as 100%.

Detergent Compatibility

The stability of the crude enzyme in the presence of liquid laundry detergents was examined by incubating enzyme preparation (300 U/mL) for 1 h at 30, 40, and 50 °C with various detergent preparations, and the residual activities were determined. The enzyme activity of a control sample (without detergent), incubated under similar conditions, was taken as 100%. The liquid detergents used were Dixan (Henkel-Spain), Nadhif (Henkel-Alki-Tunisia) and Lav^+ (Tunisia), and were diluted 100-fold in tap water to simulate washing conditions. The endogenous enzymes contained in these detergents were inactivated by incubating the diluted detergents at 70 °C for 1 h prior to the addition of the enzymes.

Organic Solvent-Stability Assay

The organic solvent stability of the enzyme was studied by incubating the crude enzyme with various organic solvents (50%; *v*/*v*; methanol, ethanol, butanol, *N*,*N*-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), diethyl ether, ethyl acetate, hexane, acetone, and

acetonitrile) at 30°C with shaking (150 rpm) for 60 days. Aliquots were withdrawn at desired time intervals to test the remaining activity.

Deproteinization of Shrimp Wastes by SV1 Protease

Shell wastes (1:2; *w*/*v*) were minced then cooked at 90°C for 20 min to inactivate endogenous enzymes. The cooked sample was then homogenized in a Moulinex® blender for about 2 min. The pH of the mixture was adjusted to 8.0, then the shrimp waste proteins were digested with the crude enzyme of SV1 strain. After 3-h incubation at 40°C, the reaction was stopped by heating the solution at 90°C during 20 min to inactivate the enzyme. The shrimp waste protein hydrolysates were then centrifuged at 5,000×*g* for 20 min to separate insoluble and soluble fractions. The solid phase was washed and then dried for 1 h at 60°C.

Deproteinization (DP) was expressed as percentages and computed by the following equation as described by Rao et al. [23].

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

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

Results and Discussion

Chemical Composition of Shrimp Wastes

The content of the three main components, i.e., minerals (ash), protein, and carbohydrates given as percentage of the dry weight of shrimp shells, was determined. The shrimp wastes used in the study had a moisture content of 66.2±1%. The protein content was found to be 40.83±1.22% of the dry weight, similar to shell wastes from shrimp *Crangon crangon* (40.6±5.43%, [24]). The fat, ash, and carbohydrate content of the material were 6.26± 0.22%, 34.69±0.19%, and 20±1.41%, respectively. Similar results were reported by Percot et al. [25] (ash, 34.49±0.04%; fat, 6.00±2%). The major mineral elements were Ca^{2+} (13.45%), Na^{+} (0.58%), Mg^{2+} (0.12%), and K^{+} (0.07%). The level of calcium was found to be in the same range as those reported in many works [15, 26]. This is due to the fact that the main constituent of minerals in shrimp shell is calcium carbonate [27]. The high amount of inorganic matter indicates that the demineralization step cannot be avoided during the chitin extraction process.

Protease Production

In a previous study, we reported the production of Ca-dependant protease by *B. cereus* SV1; no protease activity was detected in the absence of $CaCl_2$ [19]. In this study, protease production by SV1 strain was first tested in M1 medium containing different carbon sources at a concentration of 10 g/L. SV1 strain exhibited a higher production level of protease in culture media containing SWP (2,287 U/mL) as carbon source followed by

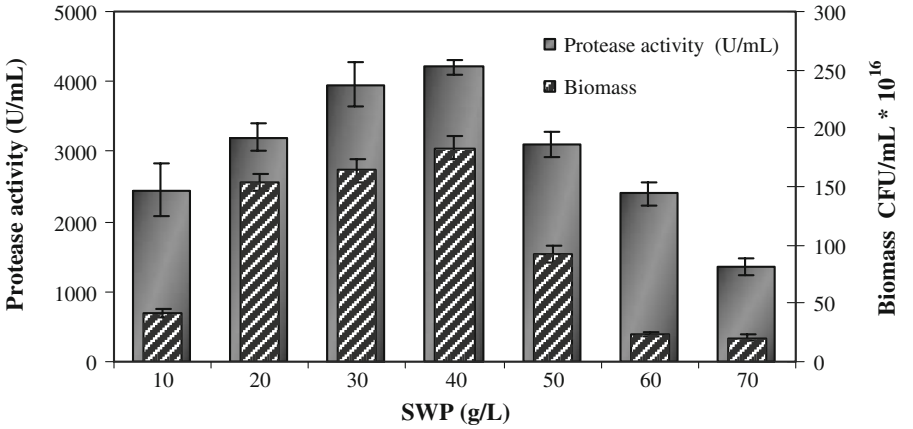

Fig. 1 Effect of different concentrations of SWP on protease production by *B. cereus* SV1

starch (2,173 U/mL) and *Mirabilis jalapa* tubers powder (1,876 U/mL). On the contrary, protease production between 280 and 1,124 U/mL was obtained with the other carbon sources tested (chicken feather meal, maltose, glucose, lactose, saccharose, fructose, and hulled grain of wheat).

Since SWP was a better carbon source, the effect of its concentration on protease production was studied. As shown in Fig. 1, the highest protease production (4,200 U/mL) was achieved at 40 g/L. These results show that the strain can obtain its carbon and salts requirements (in particular Ca^{2+}) directly from the SWP. In fact, as reported earlier, the mineral content in the SWP prepared from the shrimp wastes shows clearly that calcium content is the major element (13.45%).

The effect of various organic and inorganic nitrogen sources, at a concentration of 2 g/L, was examined in M1 medium containing 40 g/L of SWP (Fig. 2). Protease production in

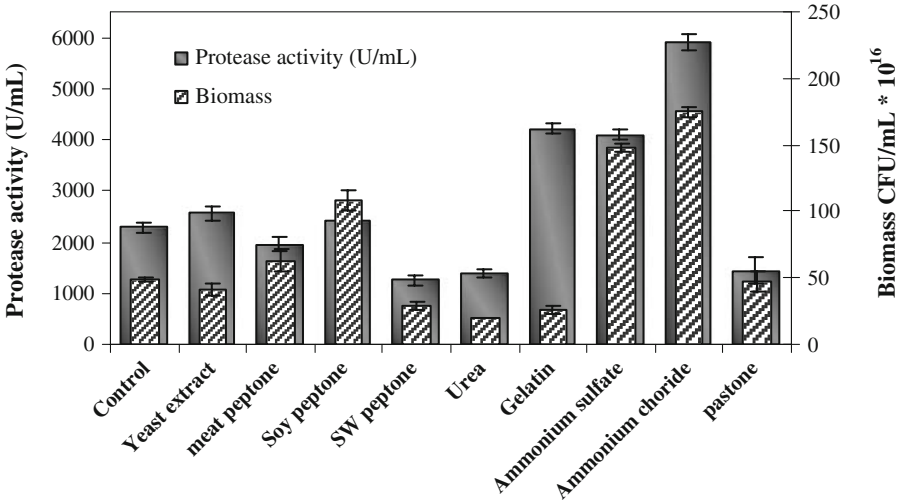

Fig. 2 Effect of different nitrogen sources on protease production by *B. cereus* SV1. SW: Shrimp Waste

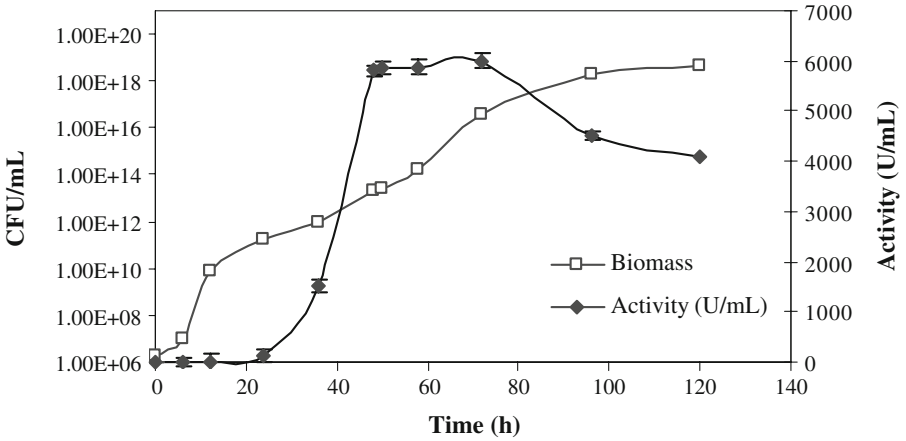

Fig. 3 Time course of protease production and growth of *B. cereus* SV1 in the optimized medium

the absence of nitrogen source was relatively high, about 2,280 U/mL. This indicates that the strain can grow well on SWP as the sole organic complex substrate and can obtain its carbon and nitrogen requirements directly from this material. Among the various organic and inorganic nitrogen sources tested, the maximum enzyme activity was obtained with ammonium chloride (NH_4Cl). A 2.5-fold higher production of protease by SV1 strain (5,900 U/mL) was achieved when NH_4Cl was used at a concentration of 2 g/L in comparison with that obtained in the absence of nitrogen source. Interestingly, among the organic nitrogen sources, gelatin gave a higher protease production but a lower biomass. Since NH_4Cl was the best nitrogen source for protease synthesis by *B. cereus* SV1, the effect of its concentration on the enzyme production was studied, and the maximum occurred at 2 g/L (data not shown).

Time course of protease production and growth of *B. cereus* SV1 in optimized medium were also studied. As reported in Fig. 3, the maximum protease activity reached 6,000 U/mL within 72 h of fermentation when the cell growth reached the end of the exponential phase and then decreased gradually.

The obtained results indicate that SWP is an excellent substrate for the growth and for protease production by *B. cereus* SV1. Since SWP is cheaply obtained, its use as a carbon source instead of commercial substrates may reduce considerably the cost of enzyme production and could promote new industrial applications.

Biochemical Properties

Zymographic analysis of the crude enzyme revealed only one clear band of casein hydrolysis, suggesting the presence of at least one protease (results not shown).

The relative activity values at various pHs from 6.0 to 12.0 are shown in Fig. 4. The enzyme was highly active between pH6.0 and 9.0 with an optimum at pH8.0 when incubated for 15 min at 60°C. The relative activity at pH9.0 was about 80%. The pH stability profile showed that the enzyme is highly stable in the pH range of 6.0–9.5 as shown in Fig. 4, retaining more than 80% of its initial activity. At pH10.0 and pH11.0, the enzyme retained about 71.8% and 50.7% of its initial activity, respectively. The SV1 protease had an optimum pH and a broad pH stability range (pH6–9.5) similar to other *Bacillus* proteases [26, 28–30].

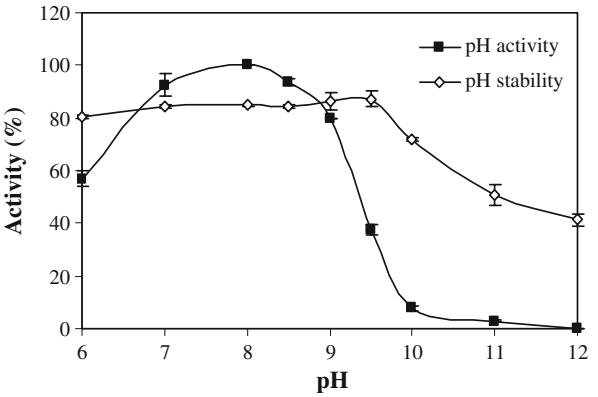

Fig. 4 Effect of pH on activity and stability of the SV1 protease

The effect of temperature on the activity of SV1 protease is shown in Fig. 5a. Optimum temperature was 60 °C in the presence of 2 m*M* $CaCl_2$. Thermal stability of SV1 crude enzyme was also determined by incubating the enzyme for 60 min at temperatures from 50 to 70 °C in the presence of 2 m*M* $CaCl_2$. The temperature stability profiles, presented in Fig. 5b, show that SV1 protease was highly stable at temperatures below 50 °C, retaining nearly 100% of its activity. At 55 and 60 °C, the enzyme retained about

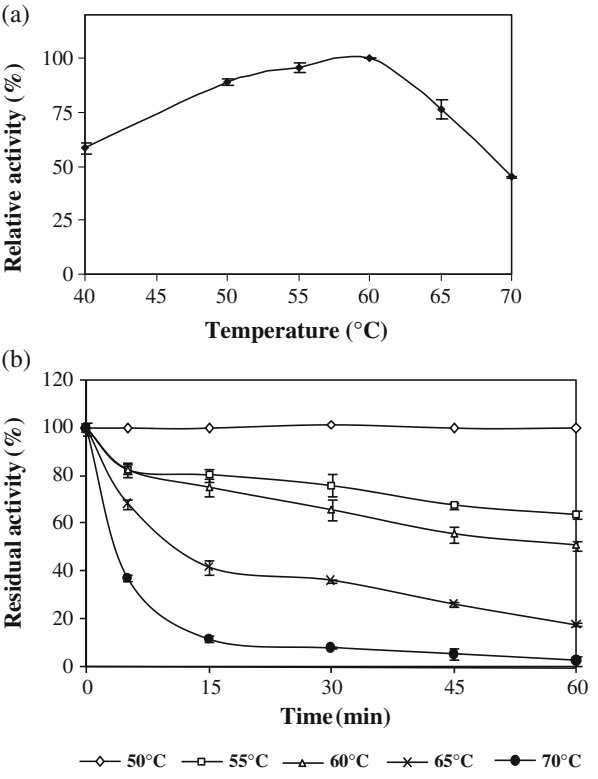

Fig. 5 Effect of temperature on activity (**a**) and stability (**b**) of the SV1 protease

64.1% and 51% of its initial activity, respectively. However, the enzyme was completely inactivated after 30 min incubation at 70 °C. Half-lives of the crude protease at 55, 60, 65, and 70 °C were estimated to be 83, 60, 12, and 4 min, respectively. In the absence of calcium, all calcium-free enzyme activity was lost after incubation of the protease for 15 min at 60 °C.

The effect of various enzyme inhibitors such as chelating agents and group-specific reagents on the activity of the SV1 protease was determined (Table 1). Serine enzyme

Table 1 Effect of some inhibitors, metal ions, surfactants, and oxidizing agents on protease activity and stability.

	Concentration	Activity (%)
Inhibitors		
None	–	100
PMSF	2 mM	100
	5	80
DTNB	2 mM	100
	5	100
EDTA	2 mM	12
	10	0
Metal ions		
Ca^{2+}	5 mM	300.5±14.8
Ba^{2+}	5 mM	84.5±1.5
Zn^{2+}	5 mM	14.2±1.2
Cu^{2+}	5 mM	0
Mg^{2+}	5 mM	133.8±0.9
Mn^{2+}	5 mM	73.2±1.9
Hg^{2+}	5 mM	0
K^{+}	5 mM	99.5±0.7
Surfactants		
SDS	0.1 (*w*/*v*)	90.6±1.9
	0.5	60.4±3.6
	1	35.2±3.1
Triton X-100	1 (*v*/*v*)	100
	5	100
Tween 20	1 (*v*/*v*)	123±1.7
	5	107.7±1.3
Tween 80	1 (*v*/*v*)	100
	5	100
Oxidizing agents		
H_2O_2	1 (*v*/*v*)	175.6±9.2
	5	151.9±6.2
	10	138.1.7±0.8
Sodium perborate	0.1 (*w*/*v*)	178.45±2
	0.5	159.4±0.8
	1	152.6±0.2

inhibitor (PMSF) and thiol reagent (DTNB) were practically without influence on the activity of the enzyme. However, the enzyme was strongly inhibited by the chelating agent (EDTA; 10 m*M*), with 100% of its original activity being lost. This confirms that the protease from the *B. cereus* strain belongs to the family of metalloproteases.

The effect of various metal ions, at a concentration of 5 m*M*, on the activity of the SV1 protease was studied at pH8.0 and 60°C by the addition of the respective cations to the reaction mixture (Table 1). Of all the metal ions tested, Ca^{2+} and Mg^{2+} were particularly effective in improving the enzyme activity causing approximately 300% and 134% stimulation, respectively. These cations have also been reported to increase activity of other *Bacillus* proteases [30–32]. The enzyme was not affected by K^{+}. However, Ba^{2+}, Mn^{2+}, and Zn^{2+} inhibited the enzyme activity by 15%, 27%, and 85%, respectively. Cu^{2+} and Hg^{2+} affect greatly the enzyme activity causing 100% inhibition.

Effect of Surfactants and Oxidizing Agents on Protease Stability

In order to evaluate the stability with surfactants and oxidizing agents, the crude enzyme was preincubated 60 min at 40°C in the presence of SDS, Tween 20, Tween 80, Triton X-100, sodium perborate, and H_2O_2, and the residual activity was assayed under the standard assay conditions.

The enzyme was highly stable in the presence of the non-ionic surfactants like Tween 20, Tween 80, and Triton X-100, as shown in Table 1. Furthermore, the SV1 protease was stable in the presence of the strong anionic surfactant (SDS), retaining approximately 90%, 60%, and 35% of its initial activity after incubation 1 h at 40°C in the presence of 0.1%, 0.5%, and 1% SDS, respectively. The stability toward SDS is important because SDS stable enzymes are not generally available [33]. The stability of SV1 protease against SDS was similar to the alkaline protease from *Bacillus mojavensis* A21 [32] and *Bacillus licheniformis* RP1 [34] and nattokinase from *Pseudomonas* sp.TKU015 [13]. However, SV1 protease was less stable than alkaline protease from *B. cereus* [35], which retained 100% of its activity when incubated 1 h at 60°C in the presence of 0.5% SDS.

Interestingly, the activity of SV1 enzyme was highly enhanced when preincubated in the presence of oxidizing agents. The activity after 1 h incubation at 40°C was 175% and 138% in the presence of 1% and 10% H_2O_2 and 152% in the presence of 1% sodium perborate.

The stability of the crude enzyme in the presence of oxidizing agents and surfactants is particularly attractive for its eventual use in detergent formulation.

Compatibility of the Crude Enzyme with Liquid Commercial Detergents

The high activity and stability of the SV1 protease in the pH range from 7.0 to 9.0, and its stability in the presence of detergent components such as surfactants and oxidizing agents are very useful for its potential application as liquid detergent additive. To check the compatibility and stability of the crude enzyme with liquid detergents, the enzyme preparation was preincubated in the presence of various liquid commercial laundry detergents of different composition for 1 h at 30, 40, and 50°C. The liquid detergents were diluted 100-fold to simulate washing conditions.

The data presented in Fig. 6 show that the crude enzyme is highly stable in the presence of all liquid detergents tested at 30 and 40°C. The enzyme retained 100% of its activity in the presence of Dixan, Nadhif, and Lav+ after 1-h incubation at 30°C. The enzyme was relatively stable at 50°C, retaining 85%, 84%, and 83% in the presence of Nadhif, Dixan, and Lav+, respectively.

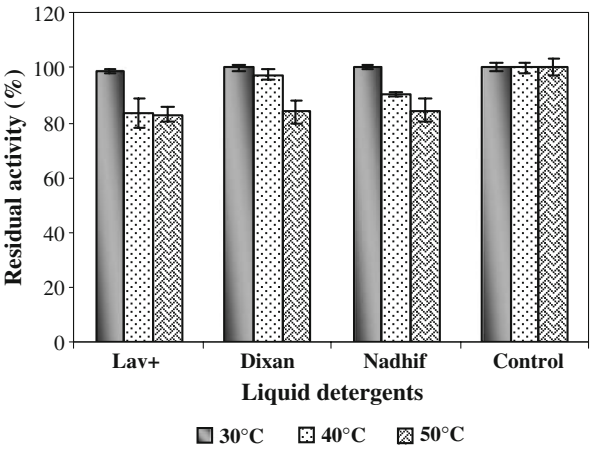

Fig. 6 Stability of the crude protease from *B. cereus* SV1 in the presence of various commercial liquid detergents. The enzyme at 300 U/mL was preincubated with commercial liquid detergents diluted 100-fold in tap water

Effect of Organic Solvents on Enzyme Stability

A previous study showed that peptide synthesis could be enhanced by the addition of organic solvents in the reaction mixture [36]. To achieve a high yield in protease-catalyzed peptide synthesis, the stability of protease is very important because enzymes are usually denatured or inactivated in the presence of organic solvents before completing the reaction. Therefore, proteases, which are naturally stable in the presence of organic solvents, are very useful for synthetic reactions.

The solvent stability of reported solvent-stable proteases is generally tested at the concentration of 25% [12, 26, 37]. In this study, the effects of various organic solvents with different Log*P* (50%, *v*/*v*) on the stability of the crude protease were investigated. Half-lives of the activity of the crude protease are shown in Table 2. In the absence of organic solvents, the half-life of the SV1 protease was approximately 34 days. In the presence of ethyl acetate, DMF, acetontrile, and butanol, the half-lives of SV1 protease were about 51, 48, 27, and 18 days, respectively. Interestingly, the stability of the enzyme was highly enhanced by diethyl ether and DMSO, and no loss of activity was observed after 60 days of incubation, while in the absence of an organic solvent, the remaining activity was only

Table 2 Half-lives of the activity of the SV1 protease in the presence of 50% (*v*/*v*) organic solvents.

Organic solvent	Half-life (days)	Residual activity (%) after 60 days
None	34	34.45±0.77
Methanol	≥60	75.8±1.69
DMSO	≥60	100±0.98
Ethanol	≥60	69.6±1.27
DMF	48	32.7±0.98
Butanol	18	25±0.61
Acetonitrile	27	0
Hexane	1.5	0
Acetone	0.8	0
Diethyl ether	≥60	100±1.83
Ethyl Acetate	51	45±0.55

34.45%. Additionally, the half-lives of the enzyme in the presence of methanol and ethanol were more than 60 days.

Therefore, SV1 protease is quite stable in the presence of 50% (*v*/*v*) hydrophilic solvents with the Log*P*≤0 except for acetone (Log*P*=−0.23). However, the enzyme was less stable in hydrophobic solvents with Log*P* values above 1 such as hexane (Log*P*=3.5).

Enzymatic Deproteinization of Shrimp Wastes by SV1 protease

Chitin in the exoskeleton of shrimp shells is closely associated with proteins. Therefore, deproteinization in chitin extraction process is crucial. Chemical treatment requires the use of HCl and NaOH, which can cause deacetylation and depolymerization of chitin. Many reports have demonstrated the application of proteolytic microorganisms for the deproteinization of marine crustacean wastes to produce chitin [9, 10, 30, 38]. However, few studies on the use of proteolytic enzymes for the deproteinization of crustacean wastes have been reported.

The SV1 strain was found to grow well and produce proteases when it was cultivated in media containing only shrimp waste powder, indicating that it can deproteinize crustacean wastes to obtain its carbon and nitrogen requirements. In this study, the crude enzyme from *B. cereus* SV1 was also applied for the deproteinization of shrimp waste to produce chitin. The *E*/*S* ratios between 5 and 30 were used to compare the deproteinization efficiency. As shown in Fig. 7, the deproteinization rate with a ratio of 5 was only 32.64±0.38%. The percentage of protein removal increased with increasing *E*/*S* ratio and reached about 88.8± 0.42% with *E*/*S*=20. Beyond a ratio of 20, no significant increase in the deproteinization rate was observed

The deproteinization activity of SV1 crude protease was better than many proteases reported in many previous studies. The percentage of protein removal from natural shrimp waste was 78% after a 7-day incubation at 37°C with the culture supernatant from *Pseudomonas aeruginosa* K-187 [11]. Bustos and Healy [8] compared the effects of microbial and enzymatic deproteinization. A maximum value of 82% of deproteinization was achieved with *Pseudomonas maltophilia* after 6 days of incubation, but no more than 64% deproteinization was achieved by using purified microbial protease under the same condition. With *Bacillus* sp. TKU004 which produce a solvent-stable metalloprotease, the percentage of protein removal reached only 73% after 3 days of incubation [14]. The fact that deproteinization cannot reach 100% may be explained by the non-accessibility of enzymes to some proteins protected by chitin.

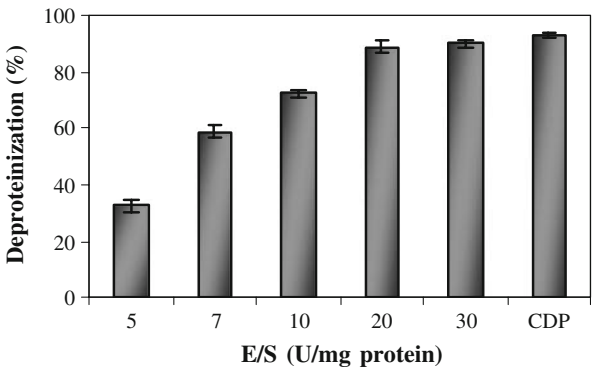

Fig. 7 Effect of the *E*/*S* ratio on the deproteinization of shrimp waste

The obtained results demonstrated that the protease produced by *B. cereus* SV1 could be used effectively in the deproteinization of shrimp wastes. Furthermore, the application of proteases or proteolytic bacteria for the deproteinization would be a good solution for the environmental problems associated with crustacean processing.

Conclusion

In this report, SV1 was found to grow well and produce proteases when grown in media containing only SWP as carbon source. In addition, we demonstrated that the culture supernatant can deproteinize shrimp wastes; 88.8%±0.42 of the protein was removed when *E*/*S* of 20 was used.

The crude protease was active in a broad range of pH7.0–9.5 and the optimum temperature was 60 °C. The protease SV1 showed a significant activity with several organic solvents. The enzyme retained 100% of its initial activity after a 60-day incubation at 30 °C in the presence of 50% (*v*/*v*) DMSO and ethyl ether. These properties make this organic solvent-stable protease a promising biocatalyst for enzymatic synthesis in the presence of organic solvents.

Another remarkable feature of our protease is its high stability toward anionic and non-ionic surfactants. Additionally, the activity was significantly enhanced by oxidizing agents, retaining more than 130% and 150% of its initial activity after 1-h incubation in the presence of 10% H_2O_2 and 1.0% sodium perborate, respectively. The crude enzyme showed an excellent stability and compatibility with three commercial liquid detergents tested.

The stability of the crude protease in presence of organic solvents, surfactants, and detergents makes the enzyme an ideal choice for applications in liquid detergent formulation, leather industries, and peptide synthesis. Eventually, the enzymatic deproteinization is suitable for isolation of chitin and also for the production of protein hydrolysates.

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