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Deproteinization potential and antioxidant property of haloalkalophilic organic solvent tolerant protease from marine *Bacillus* sp. APCMST-RS3 using marine shell wastes

Thirumalai Maruthiah[a], Beena Somanath[b], Grasian Immanuel[a], Arunachalam Palavesam[c,*]

[a] Centre for Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam, 629 502 Kanyakumari District, Tamil nadu, India
[b] Department of Zoology, Rani Anna Government College for Women, Manonmaniam Sundaranar University, Tirunelveli, 627 012 Tamil nadu, India
[c] Department of Animal Science, Manonmaniam Sundaranar University, Tirunelveli, 627 012 Tamil nadu, India

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ABSTRACT

The current increase in the vast amount of marine crustacean shell waste produced by the fish processing industries has led to the need to find new methods for its disposal. Hence, the present study was carried out via marine shell wastes as substrate for protease production. The maximum production (4000.65 U/ml) from *Bacillus* sp. APCMST-RS3 was noticed in 3:1% shrimp and oyster shell powder (SOSP) as substrate. Purified protease showed 53.22% and 22.66% enzyme yield; 3.48 and 8.49 fold purity with 40 kDa molecular weight; whereas, its K_m and V_{max} values were 0.6666 g/l, 1111.11 U/ml. This enzyme showed optimum activity at pH 9 and 60 °C temperature. Also, it retained maximum protease activity in the presence of NaCl (2.5 M), surfactants (Tween 20, 40, 60, 80 and SDS) and metal ions ($MnCl_2$, $CaCl_2$, $HgCl_2$ and $BaCl_2$) and solvents. The candidate bacterium effectively deproteinized (84.35%) shrimp shell and its antioxidant potentials.

1. Introduction

Proteases are the commercially important groups of extracellular microbial enzymes widely used in several industrial sectors such as detergent, food, pharmaceutical, chemical, leather and silk, apart from waste treatment. Hence, the global industrial enzyme market is expected to reach 4.4 billion USD by 2015. Further, microbial enzymes meet out a maximum of 34% of the total enzyme demands in food and animal feed preparation and 29% in detergent and cleaner sectors. Further, these enzymes contribute 11% in pulp and paper industries and 17% in the textile, leather and other industrial sectors [1].

Despite this demand, the microbial protease production is limited due to want of cost effective substrate. On the other hand the fish processing industry produces above 60% byproducts such as head, skin, trimmings, fins, frames, viscera and roes, and only 40% were used for human consumption [2]. The left over fish wastes create environmental pollution minace in both developed and developing countries. In the real sense, these fish wastes contain considerable amount of protein rich material that are normally processed into low cost value added products, such as animal feed, fish meal and fertilizer [3]. In transformation of these proteins rich fish processing wastes, various techniques have been employed to recover the vital nutrients and bioactive compounds, which are in turn, help to improve the human health by way of enhancing resistance against several diseases. Also marine crustacean wastes are very rich in chitin, having true economic value because of its versatile biological activities and agrochemical applications [4,5]. Additionally, chitin in the exoskeleton of crustaceans is closely associated with proteins and minerals. The microbial enzymatic digestion of nutrient rich fishery wastes allows the recovery of the protein hydrolysate with a well balanced amino acid composition. Antecedently, microbial deproteinization was performed using purified proteases [6]. But the main drawback of this method is the high cost of purified enzyme. To solve these issues, attempts have been made to extract the active molecules from the fishery wastes with the low cost extraction process. Also, it could be considered as more efficient due to the presence of coexisting proteases.

Further, it was reported that, antioxidant substances in food play significant role as health-benefiting factors that protect the body from oxidative stress. In the current trend, fish protein

* Corresponding author. Fax: +91 4652 253078.
E-mail address: plavesh06@gmail.com (A. Palavesam).

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hydrolysate have gained much interest as potential antioxidative peptide sources, especially due to the availability of bulk quantities of fish processing wastes and underutilized species [7]. The preparation of chitin from various crustacean shells involves demineralization and deproteinization with the use of strong acids or bases [8,9]. Typically microorganisms and proteolytic enzymes were effectively used to deproteinze the crustacean wastes to overcome the scarcity of the chemical treatments [10–13]. Viewing the above, the present study was undertaken to purify and characterize HAOP (Haloalkalophilic Organic Solvent tolerant Protease) from marine sedimentary bacterial isolate and to investigate its deproteinization, stain removal and antioxidant potentials.

2. Materials methods

2.1. Isolation and identification of HAOP bacterium

The bacterium used in this study was isolated from the sediment samples collected from the marine environment of Rajakkamangalam coast (Lat. 8° 7′ 27.7″ N Long. 77° 23′28″ E), Kanyakumari District, Tamil Nadu, India by using organic solvent enrichment (10% cyclohexane) method with slight modification [13]. Initially the sediment samples were aseptically collected from the coast and serially diluted and plated in a halophilic medium (casein acid hydrolysate-1%; yeast extract-1%; peptone-0.5%; trisodium citrate-0.3%; potassium chloride-0.2%; magnesium sulphate-2.5%, NaCl-8%) and incubated at 40 °C, pH 9.0 for 72 h. The isolated (12 nos) colonies were individually streaked on skim milk agar plate and incubated for 48 h, after incubation the zone formation of protease production was noted. From this, the isolate showing maximum protease activity was taken as the candidate bacterium and characterized further. This particular isolate was identified based on morphological, physiological, biochemical and 16S rRNA gene sequencing.

2.2. Protease production using marine shell waste

For the current study, shrimp, crab, oyster and lobster shells were collected from the fish landing centers of Kanyakumari District, Tamil nadu, India. The collected shells were processed by following the method described by Maruthiah et al. [13]. Briefly, the shells were separated from the flesh, antennas and legs of crustaceans, washed thoroughly in running tap water to remove the soluble organic matter, adherent protein and existing impurities. The shells were then boiled in water for 1 h at 100 °C to remove the excess flesh. The shells free of flesh, were dried and then homogenized into fine powder; which was then used as sole carbon source for protease production.

To find out the protease production, 1.0% powdered shell wastes such as shrimp shell powder (SSP), oyster shell powder (OSP), lobster shell powder (LSP), crab shell powder (CSP) and also mixed shrimp and oyster shell powder (SOSP) in the ratio of 1:3, 1:1, 3:1% were initially screened using 50 ml mineral medium (Magnesium sulphate −0.05% and potassium dihydrogen ortho phosphate −0.1%). Then the inoculum (5 ml) was transferred into production medium containing 3:1% SOSP, magnesium sulphate −0.05%, potassium dihydrogen ortho phosphate −0.1%. The culture flask was then incubated in a shaker incubator (150 rpm) for 48 h at 50 °C. The cells were then harvested by centrifugation at 10,000 rpm for 15 min and the supernatant obtained was used as crude enzyme. The bacterial growth (OD) and protease production were studied for a period of 60 h in an interval of every 6 h.

2.3. Purification

Crude supernatant measuring 100 ml was precipitated using ammonium sulphate at 75% saturation level. After overnight incubation at 4 °C, the precipitate was centrifuged at 5000 rpm for 15 min and then dialyzed against Tris–HCl buffer (pH 7.2; 50 mM) at 4 °C with three buffer exchanges. The dialyzed sample was then loaded on the top of the gel filtration column (Sephadex G-75, Sigma, USA, 1 × 50 cm) and equilibrated with 5 bed volumes of 50 mM Tris–HCl buffer (pH 7.2). The unbound proteins were collected with same buffer and the bound proteins were eluted at the flow rate of 0.5 ml/min and each fraction (2.0 ml) was collected. All the elutions were monitored by measuring absorbance at 280 nm using UV–vis spectrophotometer (TECOMP 8500, Hong Kong). Then the fractions were also analyzed for protease activity and highly active fractions were pooled and concentrated by using Ultra centrifugal filters (Amicon, MWCO-10 kDa; Millipore Ireland Ltd., Ireland). The molecular mass of the purified protease was determined through 12% SDS-PAGE [14]. The proteins were visualized with coomassive brilliant blue R-250 and the molecular weight was determined (AlphaImager mini system, Cell Biosciences, USA). The purified protein band separated on SDS-PAGE was further confirmed by using casein substrate gel (Zymography) electrophoresis [15].

2.4. Determination of protease activity and protein content

The protease activity was assayed by following the method of Takami et al. [16]. The amount of protease produced was measured with the help of a tyrosine standard graph. The protein content in the samples was estimated following the method of Lowry et al. [17] using BSA as standard (Sigma, USA).

2.5. Characterization of purified protease

The effect of different pH (5–10) and temperatures (30–80 °C) on protease activity was studied. The effect of metal ions ($MgCl_2$, $ZnCl_2$, $MnCl_2$, $HgCl_2$, $ZnSO_4$, $MnSO_4$ and $BaCl_2$ at 5 ppm), surfactants (Poly ethylene glycol, SDS, triton X 100, tween 20 and tween 40 at 5 mM), NaCl (0.5–2.5 M), inhibitors (PMSF, DTT, iodoacetamide, mercaptoethanol, EDTA at 5 mM), substarte specificity (1% Casein, BSA, Gelatin), organic solvents (10 and 20%) and commercial detergents (ariel, tide, rin, surf, sunlight and henko at 7 mg/ml) were also examined by using above mentioned standard protease assay procedure.

2.6. Determination of K_m and V_{max}

The enzyme kinetics (K_m and V_{max}) was calculated by using Lineweaver Burk plot and here the assay was carried out in medium pH 9 at 60 °C. The substrate (casein) concentration used was 0.1–0.9% (w/v).

2.7. Stain removal

The application of protease as a detergent additive was studied on white cotton cloth pieces (5 × 5 cm) stained with human blood and natural colorants such as coffee, tea, pomegranate, tomato, green leaves, chocolate and beet root. After incubation at 60 °C for 15 min, the cloth pieces were taken out, rinsed with distilled water, and dried. The stain removal efficiency of the enzyme was assessed by visual examination. Untreated cloth pieces stained with human blood, coffee, tea, pomegranate, tomato, green leaves, chocolate and beetroot were taken as control.

2.8. Deproteinization of marine shells by proteolytic bacterium

The selected marine shells [SSP, CSP, LSP, OSP] each with 25.0 g were mixed with 100 ml of above mentioned halophilic production media (solid to liquid ratio was 1:3 w/v) except casein acid hydrolysate, peptone and yeast extract. To this, 5 ml of proteolytic bacterial cell suspension was added and incubated at 50 °C for 3 h [13]. After the fermentation, the product was centrifuged at 5000 rpm for 15 min. Then the solid fractions were dried. The dried fractions were analyzed for protein and dry weight analysis.

2.9. Antioxidant activity

2.9.1. DPPH radical scavenging assay

A known volume of (500 μl) culture supernatant, crude protease and commercial enzyme was mixed with different concentrations of deproteinized dried shrimp shell waste (0.25–2 mg/ml). To this mixture 375 μl of 99.5% ethanol and 125 μl of 0.02% DPPH in 99.5% ethanol were added. The mixtures were then incubated for 60 min in the dark at room temperature and the reduction of DPPH radical was measured at 517 nm using a UV–visible spectrophotometer. A control was also maintained in the same manner with distilled water in the place of sample [13].

DPPH radical-scavenging activity was calculated as follows:

$$\text{Radical} - \text{scavening activity}(\%) = \frac{\text{A control} - \text{A sample}}{\text{A control}} \times 100$$

where A is absorbance at 517 nm. The test was carried out in triplicate.

2.9.2. Reducing power assay

Each sample solution (1 ml) containing deproteinized dried shrimp shell waste at different concentrations of (0.25–2 mg/ml) was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% (w/v) potassium ferricyanide. The mixtures were then incubated for 30 min at 50 °C, followed by the addition of 2.5 ml of 10% (w/v) trichloroacetic acid. The reaction mixtures were then centrifuged for 10 min at 10,000 rpm. Finally, 2.5 ml aliquot of the supernatant solution, from each sample mixture was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% (w/v) ferric chloride. After 10 min incubation, the absorbance of the resulting solution was measured at 700 nm. Higher absorbance of the reaction mixture indicated maximum reducing power [13].

2.9.3. Metal chelating measurement

A known volume (1.1 ml) of diluted sample was mixed with 100 μl of $FeCl_2$. The reaction was initiated by the addition of 400 μl of ferrozine, and after 10 min when the mixture reached equilibrium, the absorbance was read at 562 nm. A control was maintained in the same manner, and here distilled water was used instead of sample [13]. The percentage of inhibition of the complex ferrozine–Fe^{2+} was calculated using the following equation:

$$\text{Inhibition}(\%) = \frac{\text{A control} - \text{A sample}}{\text{A control}} \times 100$$

A is absorbance at 562 nm.

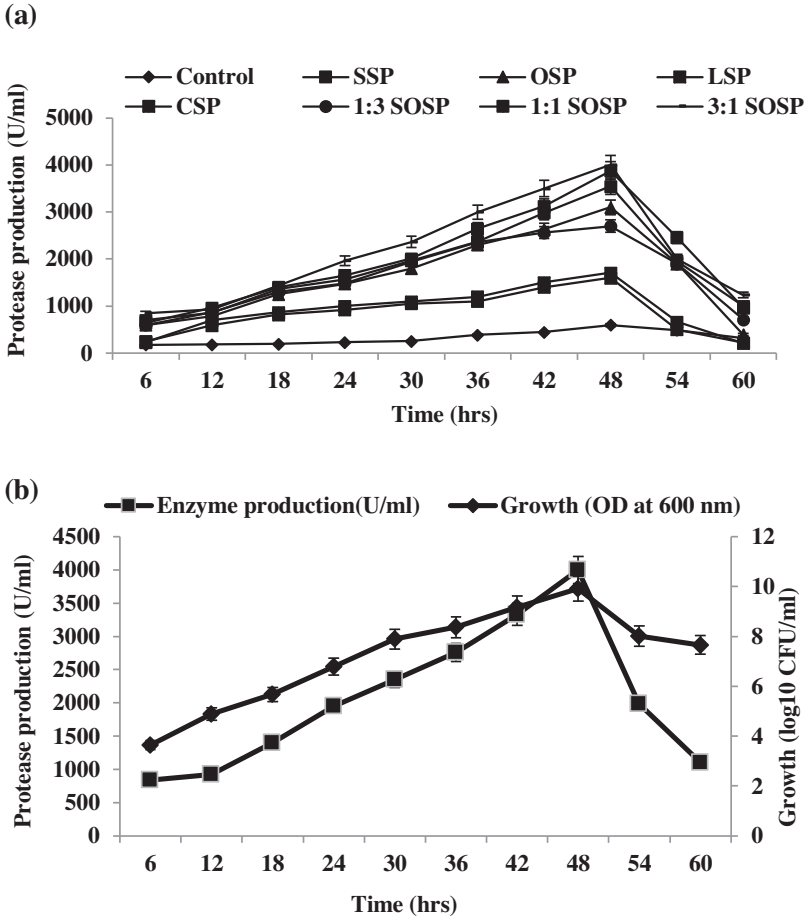

Fig. 1. Effect of protease production using marine shell wastes. (a) Protease production using marine shell wastes. (b) Growth and protease production by shrimp and oyster shell waste.

Table 1
Summary of purification of HAOP produced by *Bacillus* sp. APCMST-RS3.

Purification steps	Total Activity (U/ml)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Culture filtrate	1086.26 15.52	321.65 ± 12.02	3.37 ± 0.27	100 ± 0.00	1 ± 0.00
Ammonium sulphate precipitation	578.18 ± 12.59	49.17 ± 2.67	11.75 ± 1.32	53.22 ± 2.74	3.48 ± 0.55
Gel filtration (G-75)	246.15 ± 4.10	8.60 ± 1.09	28.62 ± 1.68	22.66 ± 1.27	8.49 ± 0.62

Each value represents the mean ± SD.
The total volume of fermentation medium was 100 ml.

3. Results and discussion

Halophilic alkalithermophiles are an unusual group of extremophiles capable of robust growth under the combined extremes of high salinity, alkaline pH and elevated temperature. They are potential sources for novel enzymes that are stable and function under multiple extreme conditions where other enzymes would be inactivated and/or denatured. The bacterium used in this study was isolated from the sediment samples collected from Kurumpanai estuary, Kanyakumari coast, Tamil nadu, India. The morphology, biochemical and 16S rRNA sequencing revealed that, the identified proteolytic bacterium was *Bacillus* sp. APCMST-RS3 (Gene Bank Accession number-KF009689). The alkaline protease from various strains of *Bacillus* sp. originated from saline environment is previously reported for their extracellular protease. The purification and characterization of protease from different soil *Bacillus* spp. such as *B. circulans* BM15, *B. laterosporus*, *B. cereus*, *B. subtilis* VSG4, *B. cereus* VITNO4, *B. flexus* APCMST-RS2P and *Bacillus* sp. APCMST-RS7 were reported [18,19,21–29,13].

To find out the suitable crustacean shell waste for protease production, fermentation was carried out with medium containing SSP, OSP, LSP, CSP and SOSP respectively. The results inferred that medium with 3:1 SOSP (4000.65 U/mL) had higher protease production than 1:1 SOSP (3877.21 U/ml), 1:3 SOSP (2700.55 U/ml), SSP (3547 U/ml), OSP (3100.70 U/ml), CSP (1708.41 U/ml) and LSP (1600 U/ml), respectively (Fig. 1a and b). In consistence with the present study, it was reported that the production by various *Bacillus* spp. was high when crustaceans shell wastes were used as sole carbon source [20,29–31].

Further HAOP from *Bacillus* sp. APCMST-RS3 was purified to the homogeneity by a combination of ammonium sulphate precipitation, dialysis, ultra filtration and gel filtration chromatography. The purified protease showed 8.49-fold purity with an overall yield of 22.66%. At this stage specific activity of the purified enzyme was 28.62 U/mg (Table 1). Results on SDS-PAGE and zymogram analysis inferred that, the molecular weight of the HAOP was 40 kDa (Fig. 2). The molecular masses of alkaline proteases generally fall in the range of 15–30 kDa [32]. Earlier studies also indicated the occurrence of alkaline protease with similar molecular mass range from *Bacillus* spp. In correlation with present study, the purified alkaline protease from *B. cereus* showed the molecular mass of 38 kDa [23]. Similarly alkaline protease with 42 kDa was also reported from *B. thermantarcticus* M1 [33]. Also, an alkaline protease from *B. cereus* TKU006 having 39 kDa with 0.07% yield and 0.3% purity after gel filtration chromatography was reported [20]. The purified protease from *B. flexus* APCMST-RS2P isolated from marine sediment also showed molecular weight of 44.3 kDa with 9.83% yield and 10.62% purity [28].

Effect of pH on protease activity and stability was investigated in the pH range of 5–10. The results indicated that pH range of 7–9 was optimum and the enzyme activity was obviously high at pH 9 and at pH 10, the low activity was noticed (Fig. 3a). Further this result confirmed that this enzyme is an alkaline protease. In recent days, the alkaline protease has been widely studied by many researchers due to its importance in various industries. Supporting the present findings, strains like *B. majovensis* A21 [34] and *B. laterosporus*-AK1 [19] have reported to produce alkaline protease with the optimum pH range of 8.5–9.0. Also supporting this study, the protease from *B. cereus* TKU006 had an optimum pH of 9.0 [20]. Likewise, the alkaline proteases from *B. subtilis* VSG-4 and *B. firmus* CAS7 were also registered maximum activity at pH 9.0 [24,31].

Among the environmental variables, temperature plays an important role in activation and inactivation of enzymes. In the present study, the effect of temperature on protease activity revealed that 60 °C was the optimum temperature for maximum (100%) protease activity (Fig. 3b), and it evidented that this enzyme was moderate thermophilic in nature. Similar to the present study, the protease from *Bacillus* sp. showed maximum activity at 60 °C [35]. In agreement with the current study, *B. mojavensis* A21 protease was active between 50 and 70 °C with an optimum of around 60 °C [34]. The solvent tolerant alkaline protease from *B. firmus* CAS 7 had maximum activity at 60 °C [31]. The alkaline protease from *B. cereus* had retained maximum activity at pH 8–9 and 60 °C [23]. However, protease from marine *Alkalibacillus* showed optimum activity at 52 °C [36].

The activity of HAOP from *Bacillus* sp. APCMST-RS3 was accelerated much by the tested concentrations of NaCl (0.5–3.5 M) and accordingly it required 2.5 M NaCl for maximum activity. At higher concentrations (>2.5 M), the protease activity decreased considerably and it retained 50% activity (Fig. 4). It inferred that, the protease from *Bacillus* sp. APCMST-RS3 was halophilic in nature. The protease from halophilic organism is most preferred for biotechnological applications especially in detergent industries. In accordance with the present finding, the protease from *B. aquimaris* had retained maximum activity at 2.0 M NaCl concentration [21]. Also purified proteases from marine sedimentary proteolytic bacterium such as *B. halodurans* CAS 6 and *B. firmus*

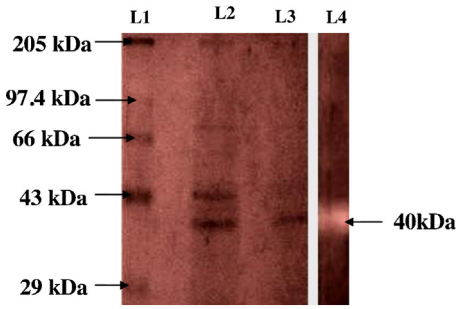

Fig. 2. SDS-PAGE and zymography analysis of purified protease produced by *Bacillus* sp. APCMST-RS3.

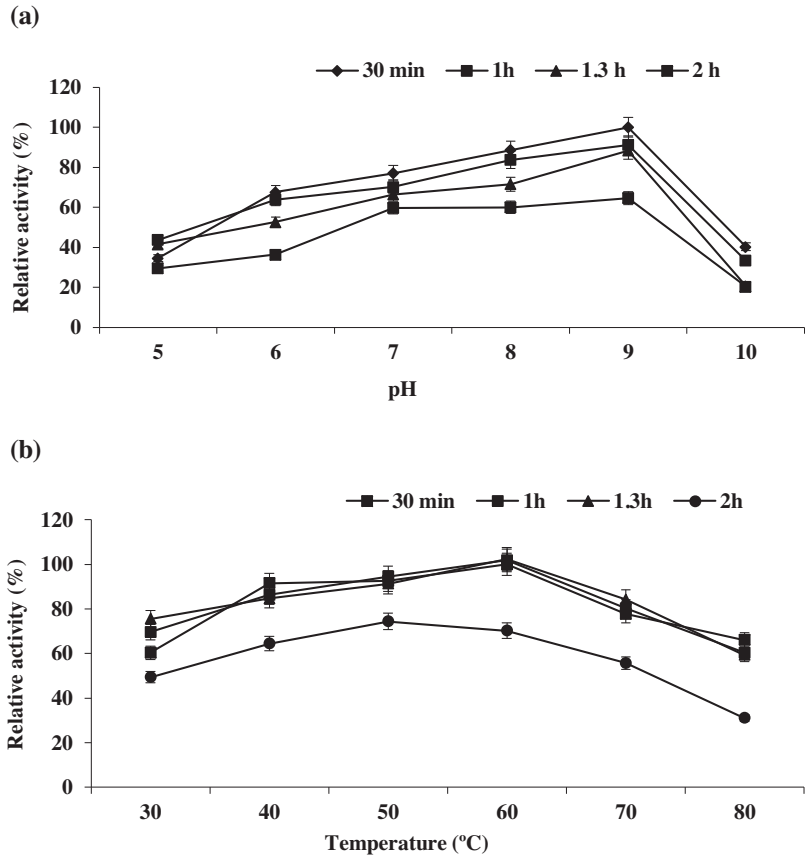

Fig. 3. Effect of pH and temperature on protease stability (a) the effect of pH stability was tested in different pH (5–10) using Tris–HCl (pH 9.0) at 60 °C. (b) The thermo stability of enzyme was studied by incubation of the enzyme at 30–80 °C and further determination of the residual activity at 60 °C, pH 9.0.

CAS 7 were retained its maximum enzyme activity in the presence of 30% NaCl concentration [30,31]. Likewise, the alkaline protease from coastal isolate *Oceanobacillus* sp. can withstand at 2 M NaCl and registered maximum protease activity [37]. Marine *Alkalibacillus* protease also recorded higher activity at 1 M and it retained 61% activity at 3.1 M NaCl concentration [36]. Our recent study had also showed that the halophilic alkaline protease from *Bacillus* sp. APCMST-RS7 require 1.5 M NaCl [13].

This present study also inferred that the metals ions like $CaCl_2$, $CuSO_4$, $MnCl_2$, $BaCl_2$ and $MgCl_2$ were found to enhance the protease activity of *Bacillus* sp. APCMST-RS3 and other metals (Zn^{2+} and Hg^{2+}) inhibited the protease activity when compared to control (Table 2). In accordance with the present study, the alkaline protease from *Halobacillus* sp. SCS10 20089 required Mn^{2+}, Ca^{2+}, Mg^{2+} and Ba^{2+} for its optimum activity [27]. Our earlier studies are also proved that, the purified protease from *B. subtilis* AP-MSU 6, *B. flexus* APCMST-CS4 and *Bacillus* sp. APCMST-RS7 showed higher activity in the presence of Ba^{2+}, Cu^{2+}, Mn^{2+} [38,28,13]. Similarly, alkaline protease from *B. firmus* CAS7 showed maximum activity in medium added with Ca^{2+}, Mn^{2+}, Mg^{2+} [31]. Various researchers reported that the alkaline protease from different *Bacillus* spp. performed well in the presence of Ca^{2+} & Mg^{2+} [19,24,36]. The effects of surfactants like tween 20, tween 40, tween 80 and SDS were found to increase the protease activity. Others like Triton X-100 and PEG (poly ethylene glycol) had inhibited the enzyme activity (Table 2). The tolerance showed by the protease synthesized by *Bacillus* sp. APCMST-RS3 towards the tested detergents in particular against SDS is indicative of the possibility of utilizing it as enzyme in detergent formulation. Supporting the present study, various authors prove the protease from *Bacillus* sp. showed the

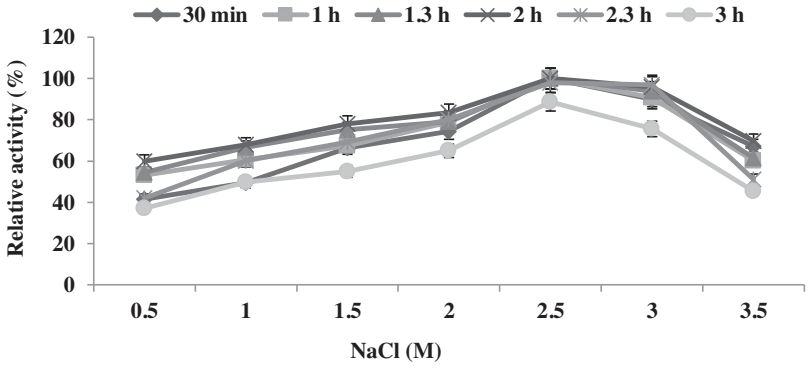

Fig. 4. Effect of different concentration of NaCl on protease activity and stability. Further, the residual activity was measured at 60 °C, pH 9.0.

Table 2
Effect of different metal ions and surfactants on protease activity.

Metal ions (5 mM)	Relative activity (%)	Surfactants (5 mM)	Relative activity (%)
Barium chloride	133.65 ± 5.56	Tween 20	135.65 ± 5.46
Zinc sulphate	0.00 ± 0.00	Tween 40	129.67 ± 5.43
Magenesium chloride	132.00 ± 4.00	Tween 60	125.21 ± 4.81
Manganese chloride	133.33 ± 4.62	Tween 80	119.65 ± 5.70
Mercuric chloride	1.65 ± 0.36	Triton X 100	63.68 ± 3.68
Zinc chloride	2.97 ± 0.61	PEG	44.00 ± 3.64
Copper sulphate	135.32 ± 4.81	SDS	121.65 ± 04.81
Calcium chloride	147.32 ± 5.56	Control	100 ± 0.00
Control	100 ± 0.00		

Each value represents the mean ± SD.

maximum protease activity in the presence of non ionic detergents such as tweens and SDS [33,20,39,11,27,28,13].

The nature of protease was studied using specific class of protease inhibitors. In protease from *Bacillus* sp. APCMST-RS3 was completely inhibited by PMSF, thus it is confirmed as seine protease type (Table 3). However, serine type of proteases are reported in different *Bacillus* sp. such as *B. laterosporus*-AK1, *B. mojavensis* A21, *B. cereus*, *B. subtilis* AP-MSU 6, *B. flexus* APCMST-CS4 and *Bacillus* sp. APCMST-RS7 [19,34,23,26,27,13]. In the present study, the protease was able to degrade proteinaceous substrates like casein, BSA and gelatin to varying degrees. It was reported that HAOP was more active against casein, when compared to gelatin and bovine serum albumin (Table 3). In support of this study, many researchers reported that the alkaline proteases from *B. laterosporus*, *B. cereus*, *B. subtilis* AP-MSU 6, *B. flexus* APCMST-CS4 and *Bacillus* sp. APCMST-RS7 [19,23,26,27,13] also exhibited the highest activity towards casein.

The use of enzymes in organic media has been one of the most novelties of catalysis in the last few years. One major concern in this regard has been their instability/low-activity in organic media, since proteases are suitable for peptide and ester synthesis under non-aqueous conditions [40]. In the present study, all the tested water soluble and insoluble organic solvents enhanced the protease activity except few (Table 4). The results of the current study were in good agreement with those confirmed earlier for the thermostable protease from *Bacillus* sp. against methanol, petroleum ether and ethanol [41]. Also, protease from *Bacillus* sp. showed higher activity in the presence of both water miscible and water immiscible organic solvents including ethylene glycol, ethanol, butanol, DMSO, xylene and perchloroethylene [42]. These studies suggested that specific proteases are reported to tolerate organic solvents at higher levels and in this regard protease from *Bacillus licheniformis* was stable with organic solvents like ethanol, diethyl-ether, methanol and hexane and DMSO [11]. The alkaline protease from *Alkalibacillus* sp. also recorded maximum protease activity in the presence of ethanol and methanol [36]. Similarly, our recent study evidenced that, HAOP from *Bacillus* sp. APCMST-RS7 had maximum tolerance in the presence of hexane, petroleum ether and *N*-butanol [13].

The influence of commercial detergents on protease activity was also screened in the present study. From the tested commercial detergents, HAOP form *Bacillus* sp. APCMST-RS3 retained much more activity in the presence of ariel (Table 3) and also exhibited better stain removal efficiency, and this property explored its use in detergent industry. In accordance with this, it was shown that protease from different *Bacillus* spp. tolerated well in the presence of ariel [19,24]. In our earlier study, the protease from *Bacillus* spp. was highly stable in the presence of all the tested commercial detergents including ariel [26,27]. In the present study, HAOP enzyme from *Bacillus* sp. APCMST-RS3 showed better stain removal efficiency. Earlier studies also reported the effectiveness of alkaline protease from *Bacillus* spp. on blood stain removal from cloth in the presence and absence of detergents [11,13,34,43].

The extraction of chitin from shrimp shells is being carried out with 4% NaOH for deproteinization and also with 4% HCl for demineralization by the traditional chemical treatment. This process is having several bottle necks since it is expensive and environmentally hazardous and that necessitate, finding of alternative processes, which would be really helpful for the

Table 3
Effect of different inhibitors, substrate, commercial detergents and surfactants on protease activity.

Inhibitors (5 mM)	Relative activity (%)
PMSF	10.25 ± 0.74
EDTA	112.32 ± 6.41
DTT	122.65 ± 8.37
Iodoacetamide	125.06 ± 8.03
Mercapto ethanol	131.55 ± 6.79
Control	100 ± 0.00
Substrates (1%)	
Casein	100 ± 0.00
BSA	29.65 ± 1.80
Gelatin	52.32 ± 2.34
Commercial detergents (7 mg/ml)	
Surf excel	111.11 ± 4.20
Ariel	129.66 ± 8.44
Tide	124.58 ± 7.78
Rin	99.65 ± 6.92
Technobright	94.87 ± 7.23
Henko	98.65 ± 5.40
Control	100 ± 0.00

Each value represents the mean ± SD.

Table 4
Effect of different organic solvents on protease activity.

Organic solvents (v/v)	Relative activity (%)	
	10%	20%
Chloroform	30.00 ± 0.84	20.20 ± 0.52
Acetone	40.65 ± 1.65	32.64 ± 1.52
Hexane	140.88 ± 6.25	133.35 ± 5.02
Benzene	96.65 ± 3.61	114.20 ± 5.24
2-Propanol	100.25 ± 3.55	86.35 ± 2.00
Ethanol	131.64 ± 5.02	128.65 ± 2.56
Methanol	111.21 ± 5.12	129.00 ± 5.09
Xylene	114.58 ± 3.71	100.54 ± 3.30
N-butanol	129.65 ± 3.08	109.65 ± 3.05
Petroleum ether	133.33 ± 3.86	119.88 ± 2.24
Control	100	100

Each value represents the mean ± SD.

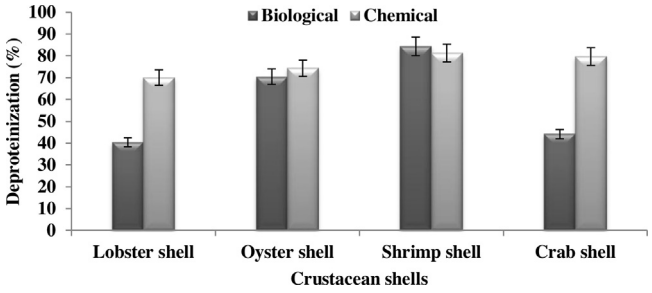

Fig. 5. Effect of deproteinization on marine shells wastes.

seafood industry. The reclamation of processing waste of crab shell by bioconversion emerged as an alternative solution for the environmental problems and associated with crustacean processing [44]. Suggesting the efficiency of the bio-deproteinization process, *Bacillus* sp. APCMST-RS3 effectively deproteinized the crustacean shell wastes to the maximum of 84.35% of the shrimp shell protein within 7 days of fermentation when compared to the chemical process (Fig. 5). In accordance with the present findings, six different types of protelolytic *Bacillus* spp. were reported to deproteinize the crustacean waste with the efficiency of more than 83% [12]. Likewise, the Taiwan soil isolates such as (*B. subtilis* Y-108) and *B. subtilis* CCRC-10029 also effectively deproteinized the crustacean shell wastes in the preparation of chitin [45]. Our most recent study authenticated that the halophlic organic solvent tolerant proteolytic bacterium had effectively deproteinized 77.26% shrimp shell waste [13]. Thus earlier studies clearly suggest that 100% deproteinization could not be achieved which may be due to non-accessibility of enzymes to some proteins protected by chitin and minerals.

Free radical-scavenging is a primary mechanism by which antioxidants inhibit oxidative processes. Further it is a widely used method for evaluating the ability of hydrolysates to scavenge free radicals generated from DPPH reagent. DPPH is a stable free radical that shows maximum absorbance at 517 nm. When DPPH radical encounters a proton donating substrate such as an antioxidant, the radical is scavenged and the absorbance is reduced [45]. Peptides in the culture supernatant incubated with shrimp shell powder may be responsible for the antioxidative properties and also contains natural antioxidants, mainly phenolic compound [46,47]. The antioxidant materials may contain oligopeptides that are electron donors, which are able to react with free radicals to terminate the radical chain reaction [20].

The maximum deproteinized crustacean shell (shrimp shell) by *Bacillus* sp. APCMST-RS3 was taken for further antioxidant analysis. The DPPH radical scavenging activity of culture supernatant, crude protease and commercial protease was determined by using shrimp shell hydrolysate as substrate. Screening of the available literature evidenced the scarcity of report pertaining to antioxidant potentials of combination of protease using crustacean wastes as substrate. In general, shrimp waste hydrolysates containing peptides/chitooligosaccharides react with free radicals to convert them into more viable and quench the radical chain reaction [39,12]. Antioxidant assay results of current study inferred that, the DPPH radical scavenging activity showed enhancement with respect to increase in concentrations of shrimp shell hydrolysate along with protease. For instance at 0.5 mg/ml concentration of shrimp shell protein hydrolysate, the DPPH radical scavenging activity was found to be 30.14%; but this level rose to the maximum of 79.14% at 2.0 mg/ml concentration using culture supernatant. The observed result is markedly higher than the control samples (Fig. 6a). In consistence with the present study, culture supernatant of proteolytic bacterium *B. halodurans* CAS6 fermented with

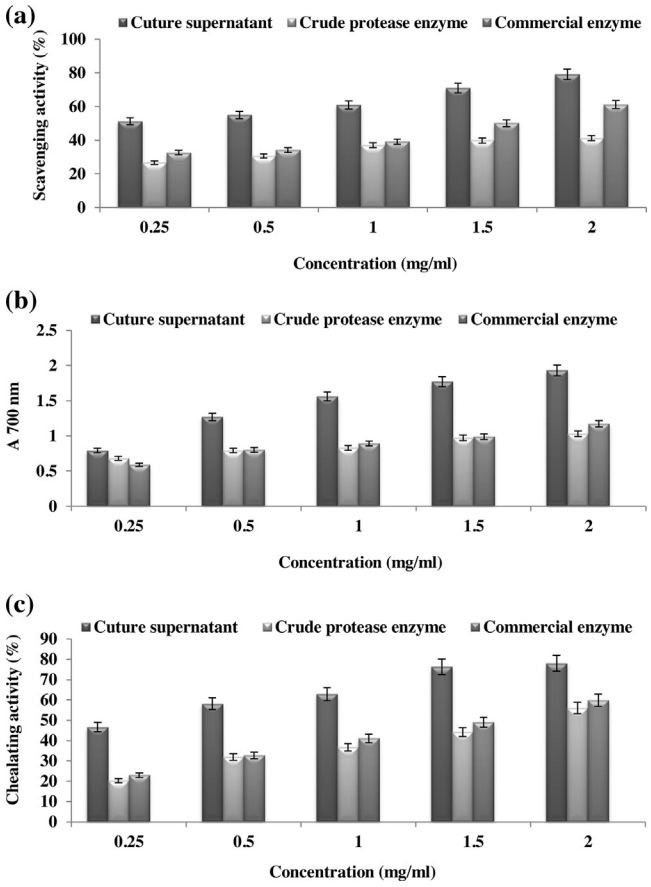

Fig. 6. Effect of antioxidant activity against shrimp shell waste using culture supernatant, crude protease, commercial protease enzyme. (a) DPPH activity (b) Reducing power. (c) Chelating activity.

marine crustacean wastes such as shrimp shell powder (94%), crab shell powder (67%), squid pen powder (72%) and shrimp & crab shell powder (3:1–90%, 1:1–83%, 1:3–76%) showed maximum antioxidant activity [30]. Also in agreement with our results, investigation on antioxidant profiles of *Bacillus* sp., *B. pumilus* and *B. mojavensis* using shrimp shell wastes and sardine muscles had pronounced antioxidant activity [48,12,13].

Results on reducing power also inferred that it increased steadily with the progressive increase in concentration of shrimp shell hydrolasate (0.25–2 mg/ml). Here, maximum reducing power (OD = 1.93) was registered at 2.0 mg/ml of shrimp shell hydrolysate using culture supernatant from APCMT-RS3 compared to other test samples (Fig. 6b). In correlation with the present study, protease from several proteolytic *Bacillus* spp. showed better reducing power activity with increase in concentration of shrimp shell hydrolysate [48,12,13].

Thus the results of the present study confirmed that, culture supernatant along with shrimp shell hydrolysate displayed maximum chelating ability (78.04%), when compared to crude protease (56.07%) and commercial protease (59.87%) at 2.0 mg/ml concentration (Fig. 6c). Similar to the present study, the proteolytic bacterium *B. licheniformis* RP1 showed maximum chelating activity (98%) by using shrimp shell hydrolysate [12]. Also in agreement with the results of present study, our earlier findings showed that culture supernatant from halophilic organic solvent tolerant proteolytic bacterium had excellent antioxidant activity (chelating activity—85.14%) with deproteinized shrimp shell powder [13].

4. Conclusion

The HAOP was purified from the marine isolate *Bacillus* sp. APCMST-RS3 and on characterization it revealed that this protease was alkaline halophilic and organic solvent tolerant, which could utilize shrimp and oyster shell waste as sole carbon source during bioconversion process. The vital efficiency was also proved, and such as the candidate bacterium is capable of removing 84.35% proteins from the shrimp shells. HAOP from *Bacillus* sp. APCMST-RS3 had excellent industrial application properties such as detergent, stain removal and deproteinization with proven antioxidant potential. Relatively very little information is available for the isolation and purification of HAOP from estuarine sediment bacterium with unique applications in bioconversion process.

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