International Research Journal of Biological Sciences _____ ISSN 2278-3202
Vol. **10(2),** 1-7, May **(2021)** *Int. Res. J. Biological Sci.*

Extraction and characterization of fish visceral protease from *carangoides malabaricus* and its potential application in detergent and pharmaceutical industries

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Available online at: www.isca.in, www.isca.me
Received 8th March 2020, revised 16th August 2020, accepted 15th February 2021

Abstract

Fish viscera have innumerable potential applications being the rich source of digestive enzymes, especially proteases. In order to assess the bio prospecting of fish processing wastes as natural wealth to obtain value added bioactive compounds, the visceral wastes of Carangoides malabaricus were characterized. This study involves the characterization of crude visceral protease extract from C. malabaricus and its potential application as a destainer. The optimum activity and stability of the crude visceral protease was observed at pH 9.0 and 50℃. This alkaline proteolytic crude extract was then tested for its potential application as destainer and it showed better stain removing efficiency. Characterization studies revealed that metal ions like Calcium chloride, surfactants like Tween 20 and SDS, inhibitors like PMSF influenced the activity and stability of the crude Visceral Protease. The present study also inferred that, crude visceral protease enzyme from C. malabaricus along with shrimp shell hydrolysate displayed higher DPPH radical scavenging activity (58.11%), Reducing activity (1.89mg/ml) and Chelating ability (73.6%). As a whole, this study confirmed possible application of Visceral Protease from C. malabaricus in detergent and pharmaceutical industries.

Keywords: Fish viscera, alkaline proteases, characterization.

Introduction

India is the third largest producer of fish and the second largest producer of fresh water fish in the world[1]. Discards from fish processing industries account for nearly 70–85% of the total weight of the catch and these are generally dumped into land or hauled into the ocean. Moreover, fish waste is considered as worthless garbage and is generally discarded without recovery of any useful product[2]. Similar to most of the food manufacturers, fish processing operations engender wastes in solid forms like fish carcasses, viscera, skin and heads or in liquid forms like washing and cleansing water discharges, blood, and water from drained fish storage, tanks and brine[3]. Among the by-products of fish, the viscera are a good source of digestive enzymes that may have some unique properties of interest[4]. A number of enzymes have been isolated from fish and shell fish processing wastes such as hyaluronidase, alkaline phosphatase, acetyl glucosaminidase, chitinase and proteases. A group of enzymes whose catalytic function is to hydrolyze (breakdown) proteins are called Protease. For marine animals, proteases are chiefly produced by the digestive glands. The fish visceral waste is regarded as one of the affluent sources of proteolytic enzymes. The recovery of protease from fish visceral waste might pave the way to mitigate pollution problems[5]. The industrial demand for highly active proteolytic enzymes with appropriate specificity and stability in terms of temperature, pH, surfactants and metal ions continues to stimulate the search for new enzyme sources[6]. Proteases have diversified applications in a wide variety of industries such as pharmaceutical, food, detergent and leather industries, peptide synthesis and for the recovery of silver from used X-ray films[7]. Therefore, this study aimed to characterize crude fish visceral protease from *C. malabaricus* so as to evaluate its stain removing, antioxidant, reducing and chelating efficiencies.

Materials and methods

Selection and Collection of Fish waste: The visceral organ waste of *Carangoides malabaricus* was collected from Tirunelveli local market, soon after collection they were kept at 10˚C in an ice box and brought to the laboratory. The samples were then cleaned thoroughly and used for enzyme extraction.

Preparation of Crude Homogenate: The fish visceral wastes were weighed and were chopped into small pieces. The chopped pieces were then homogenized with 7.8 pH; 20mM Tris-HCl buffer using a homogenizer. The homogenate was centrifuged at 10000 rpm for 15 min at 4˚C and the supernatant was collected.

Protease Assay: The substrate used was 1% aqueous casein in Tris HCl buffer (100mM; pH8.0). The reaction mixture contained 0.9ml substrate solution and 0.1ml enzyme source and it was incubated at room temperature for 30 minutes. Then, the reaction was stopped by the addition of 3ml of trichloroacetic

acid (5%). Further the precipitate formed was centrifuged at 5000 rpm. From this, 0.5ml of supernatant was pipetted out and mixed with 2.5ml of sodium carbonate (0.5M) and was incubated for 20 minutes. Finally, the incubated mixture was added with 0.5ml of Folin phenol reagent and the optical density read at 660 nm[7].

Effect of pH on activity and stability of the crude alkaline protease: The optimum pH of the crude visceral protease enzyme activity was observed over a pH range of 5.0–12.0. For the measurement of pH stability, the visceral enzyme preparation was incubated at 60°C for about 2.5h in different buffers such as (100mM) citrate-phosphate buffer (pH 5.0 and 6.0), Tris-HCl buffer (pH 7.0 and 8.0), glycine-NaOH buffer (pH 9.0 to 11.0), KCl–NaOH buffer (pH 12.0). Then the residual proteolytic activity was determined under standard assay conditions, for every 30 minutes interval.

Effect of temperature on proteolytic enzyme activity and stability: The effect of temperature on the proteolytic activity of crude visceral protease enzyme was tested at different temperatures (10°C to 80°C) for 30 min at pH 9.0. Further, the thermo stability was also examined by incubating the crude visceral protease enzyme preparation at different temperatures for 120min. During the experimentation, aliquots were withdrawn at desired time intervals (30min to 120min), and the retaining proteolytic activity was determined.

Effect of Metal ions on activity and stability of the crude alkaline protease: Metal ions such as $MgCl_2$, $MnCl_2$, $BaCl_2$, $ZnSO_4$, $ZnCl_2$ and $CuSO_4$ were taken at the concentration of 5 mM each. They were individually mixed with the standard assay buffer (Tris-HCl buffer: 100 mM; pH 8.2) and the protease assay was carried out. The protease activity of the control sample (without any metal ions) was taken as 100%.

Effect of surfactants on activity and stability of the crude alkaline protease: The effect of surfactants such as Tween 20, Tween 40, Tween 60, Triton X 100, Poly ethylene glycol (PEG) and SDS on visceral protease activity was studied at a concentration of 5mM each. It was studied by preincubating each surfactant in Tris-HCl buffer (100 mM; pH 8.2) and the assay was carried out. The protease activity of the control sample (without any surfactants) was taken as 100%.

Effect of inhibitors on activity and stability of the crude alkaline protease: The effect of protease inhibitors and chelators on visceral protease activity was studied using PMSF, DTT, Iodoacetamide, Mercaptoethanol and EDTA at the concentration of 5mM each. This crude visceral protease was preincubated with selected inhibitors individually for 30min at room temperature. Then the assay was carried out. The protease activity of the control sample (without any inhibitors) was taken as 100%.

Stain removal efficiency of the crude alkaline protease: To assess the application of visceral protease as detergent additive, white cotton clothe pieces (5x5cm) stained with Ink, Grease, Human blood, Coffee and Tea were used. For comparison, commercial detergent surf excel was used. For this study, in total 3 set of experiments were carried out. Set 1 (Control), Set 2 (Distilled water + Detergent), Set 3 (Distilled water + Detergent + Enzyme). The stained clothes were air dried for 10 min and incubated for 60 min at room temperature. The clothes were taken out and rinsed with components mentioned above and dried. Untreated clothe pieces were taken as control.

Determination of Antioxidant activities of shrimp shell chitin: Antioxidant activities such as DPPH radical scavenging activity, Reducing power assay and Metal chelating measurement were carried out to evaluate the antioxidant activity against the deproteinized shrimp shell waste by crude visceral protease.

Results and discussion

Effect of pH and temperature on protease activity and stability: The effect of pH on visceral protease activity and its stability were investigated in the pH range of 5.0 to 12.0. In this experiment, Visceral Protease exhibited maximum activity at pH 9.0; therefore the nature of the visceral protease was confirmed as alkaline. The visceral protease activity began at pH 8.0 (75% activity) and attained maximum at pH 9 (100% activity). At higher pH level (pH 10.0 to pH 12.0) the visceral protease activity decreased during 1hr and 30min of incubation. It was inferred that, the activity and stability of visceral protease varies from one organism to other. Alexander *et al.* reported that pH optimum for the fish visceral protease of *Symphysdon aequifasciata* was 2.0[8]. Similarly, protease from *Sphyraena barracuda* visceral waste was stable in a broad pH range of 7.0-9.0[9]. Sabtecha *et al*. reported that crude visceral protease obtained from *Lutjanus campechanus* and *Sardinella longiceps* showed optimum activity at pH 10.0[9]. Similar pH optimum (pH 10.0) was also reported for the visceral protease of *Scorpaena scrofa*[10].

Effect of temperature on protease activity: Temperature effect on visceral protease activity was investigated within the temperature range of 30°C to 90°C and the optimum protease activity was registered at 50°C after 2h of incubation time. Beyond 50°C, the visceral protease activity decreased obviously. In consistence with the present study, the optimum temperature of 50ºC was reported for the alkaline visceral protease from species such as *Raja clavata* and *Salaria basilica*[11,12]. Also, Younes *et al*. reported a higher optimum temperature of 55ºC for the visceral protease obtained from *Saurida undosquamis* and *Scorpaena scrofa*[10].

Effect of Metal ions on activity and stability of the crude alkaline protease: In the present study, metals like calcium chloride (117.19%), barium chloride (109.22%), copper sulphate (100.11%), magnesium chloride (69.54%) and manganese chloride (33.13%) were found to influence the

visceral protease activity. On the other hand, $ZnCl_2$ (15.15%) and $HgCl_2$ (13.02%) were inhibited the visceral activity of protease when compared to the control.

Further, addition of Zn^{2+}, Cu^{2+} and Hg^{2+} were also found to decrease the visceral protease activity by 63%, 59% and 74%, respectively. Torrissen, and Christiansen reported that metal ion like Hg^{2+} can act upon the activity and stability of sulfhydryl residues in amino acid cystine[13]. Inconsistence with these studies, Bezerra *et al*. suggested the relevance of inhibition caused by metal ion like Hg^{2+} in the catalytic action of proteases[14].

Effect of surfactants on activity and stability of the crude alkaline protease: Among six different surfactants tested, Tween 20 and SDS were positively influenced the activity of visceral protease and the activities observed were 133% and 126.02% respectively. On the other hand, the other tested surfactants like triton X 100 and polyethylene glycol (PEG) were inhibited the visceral protease activity when compared to the control.

Effect of inhibitors on activity and stability of the crude alkaline protease: Experimentation on the effect of inhibitors on visceral protease activity showed that it was strongly inhibited by PMSF (1.19%) and hence inferred that it is a serine protease. However, the other inhibitors such as DTT (130.96%), mercaptoethanol (131.84%), iodoacetamide (129.65%) and EDTA (111.36%) enhanced the protease activity, when compared to the control. El-Adawy *et al*. reported that SBTI inhibited 90.9% activity of visceral protease from *Tilapia nilotica*[15]. Likewise, visceral proteases from *Liza aurata* and *Scorpaena scorfa* were inhibited by PMSF to an extend of 49%[10,16].

Stain removal efficiency of the crude alkaline protease: The crude visceral proteases from *C. malabaricus* showed good stain removal efficiency. Visual examination of stained cotton cloth pieces obviously indicated the stain removal efficiency of visceral protease enzyme. The present study reveals that the clothes rinsed with detergent and distilled water along with crude visceral protease showed high stain removal efficiency than control and remaining sets. Similarly, Jayapriya *et al*. reported that crude visceral enzyme extract of the Red snapper and Great barracuda have the capacity to remove blood stains effectively[17]. Likewise Lassoued *et al*. reported that crude alkaline visceral protease from *Raja clavata* removed cotton cloth stained with blood[11].

Determination of Antioxidant activities of shrimp shell chitin: In the present study, the DPPH radical scavenging activity of shrimp shell protein hydrolysate was found to be concentration dependent. Assessment was also made on the antioxidant activity of shrimp shell protein hydrolysate procured through visceral protease. At low concentration of 0.5mg/ml, the DPPH radical scavenging activity was 58.1%. However, its activity was rose to 76.3% at 2.0mg/ml concentration. The highest reducing activity registered was 1.89 at 2.0mg/ml concentration. Thus the results of present study confirmed that, crude visceral protease enzyme from *C. malabaricus* along with shrimp shell hydrolysate displayed higher (73.6%) chelating ability when compared to commercial (55.45%) and culture supernatant preparation (46.89%) at 2.0mg/ml concentration. Sivasubramanian *et al.* reported that protein hydrolysate obtained from Squid exhibited good radical scavenging activity of 96.50%, reducing power of 0.71% and metal chelating activity of 52.04%[18]. Jeevitha *et al.* also reported that hydrolysate obtained from *Sardinella longiceps* showed a higher radical scavenging activity and metal chelating activity at the concentration of 5mg/ml[19].

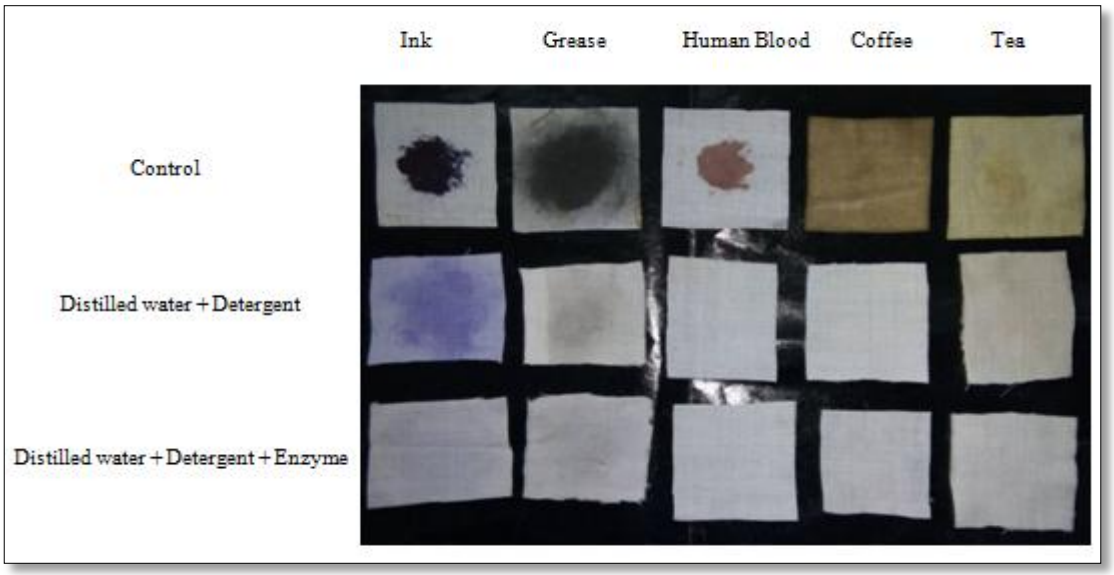

Figure-1: Stain removal efficiency of the crude alkaline protease.

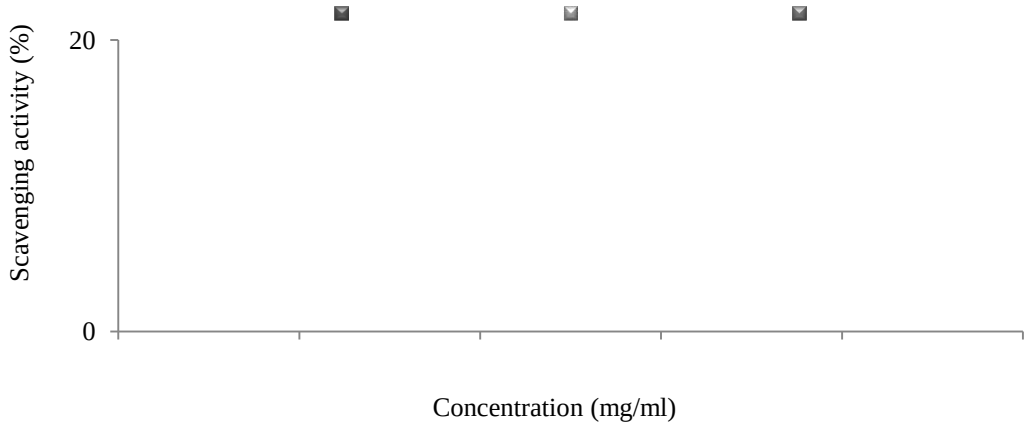

Figure-2: Effect of DPPH radical scavenging activity by different concentrations of SSPH using crude visceral protease.

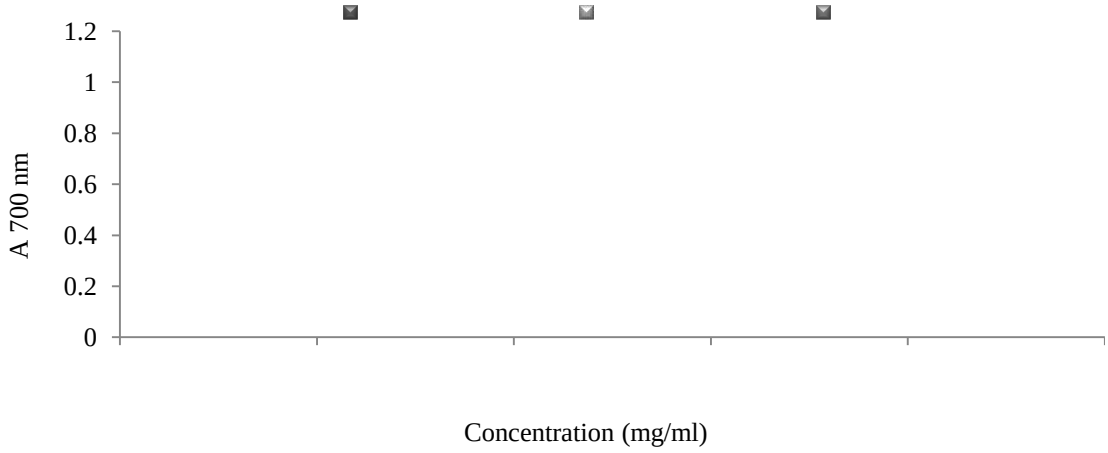

Figure-3: Effect of reducing power activity by different concentrations of SSPH using crude visceral protease.

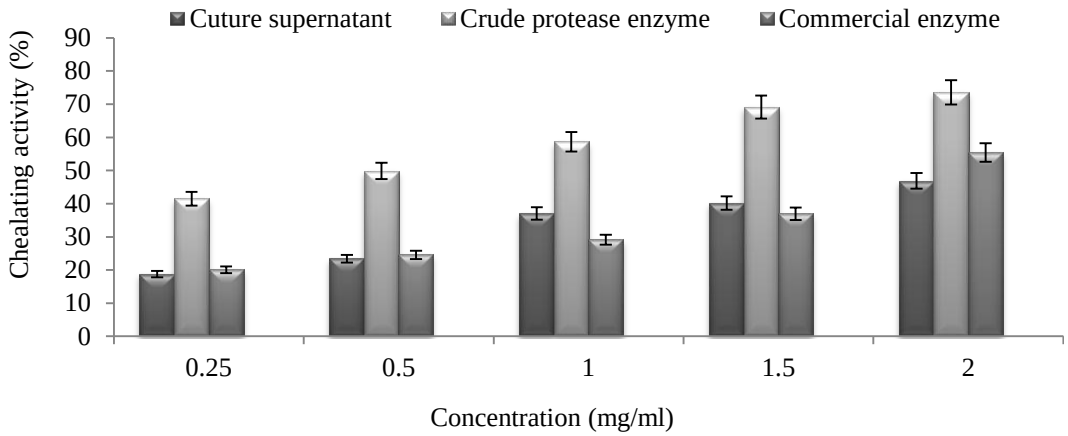

Figure-4: Effect of Metal chelating activity by different concentrations of SSPH using crude visceral protease.

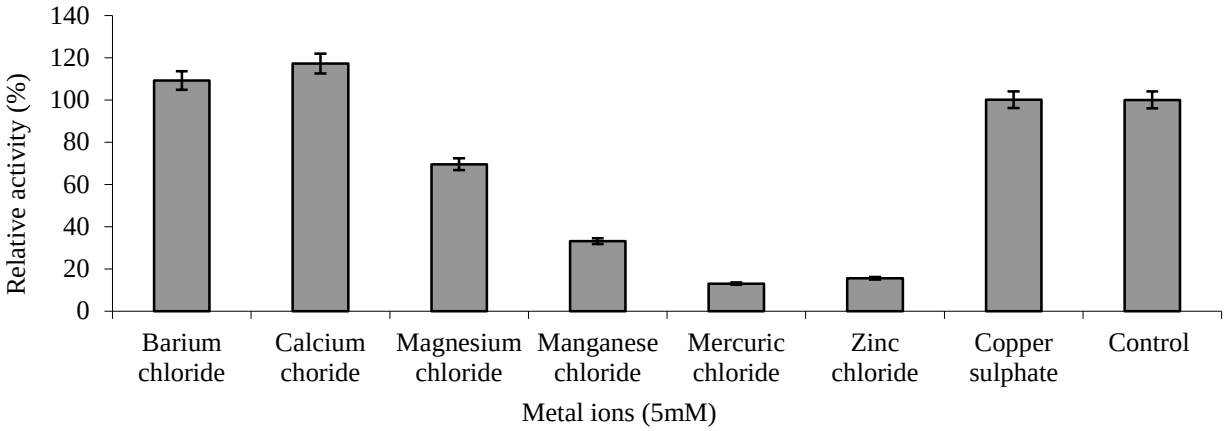

Figure-5: Effect of metal ion on the activity of crude visceral protease of C. malabaricus.

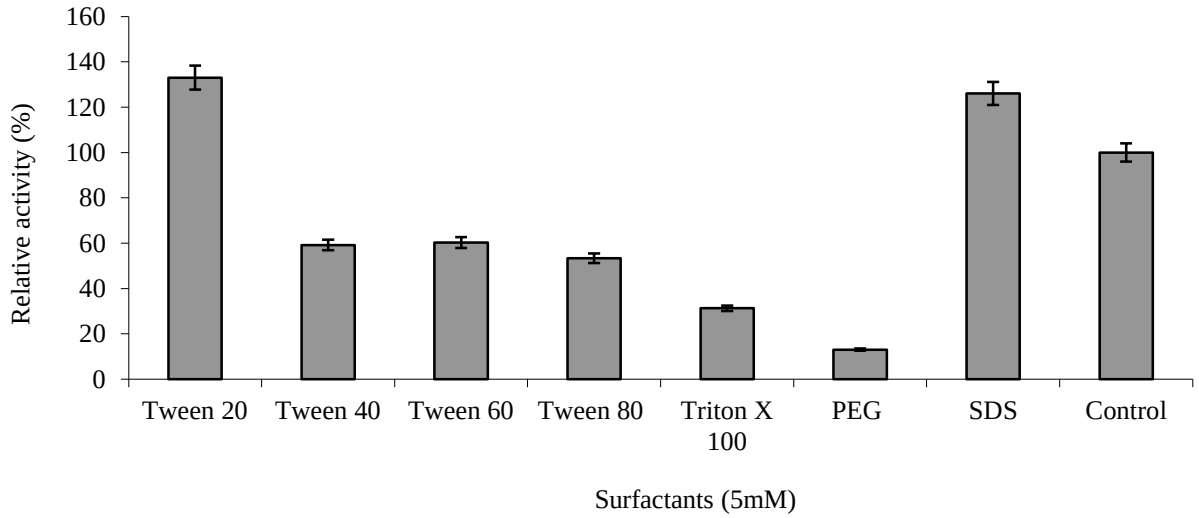

Figure-6: Effect of different surfactants on the activity of crude visceral protease of C. malabaricus.

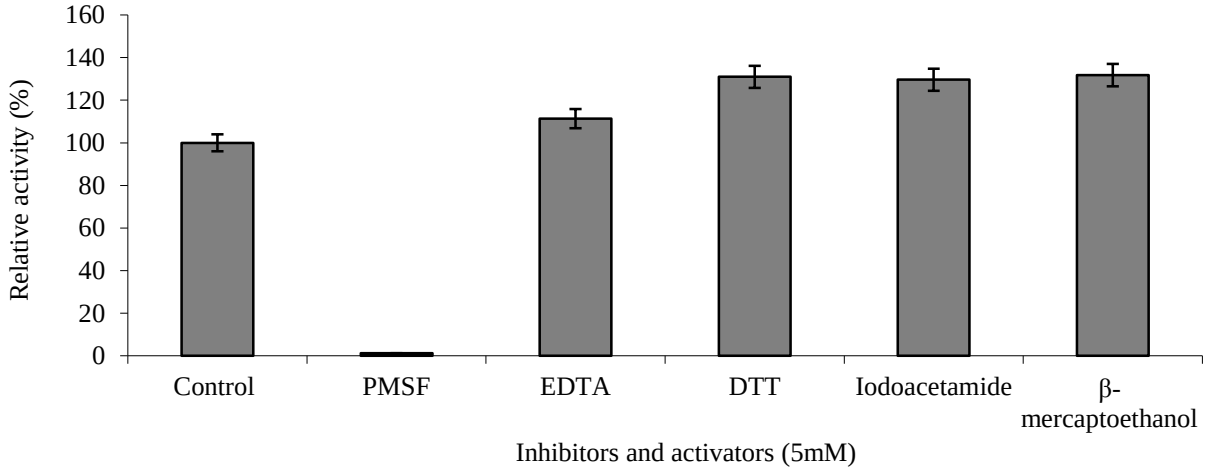

Figure-7: Effect of inhibitors on the activity of crude visceral protease of C. malabaricus.

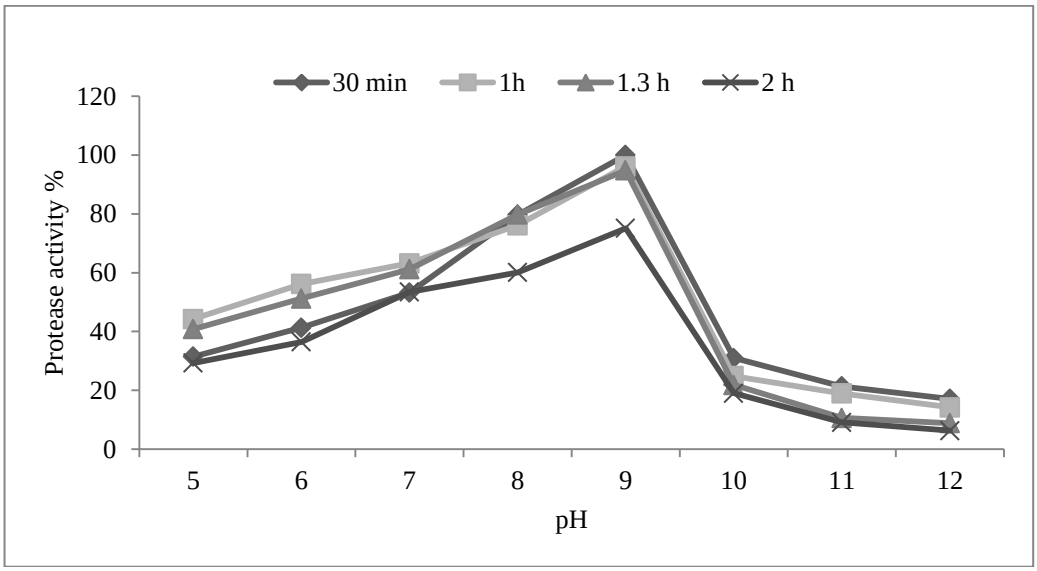

Figure-8: Effect of pH on the activity of crude visceral protease of C. malabaricus.

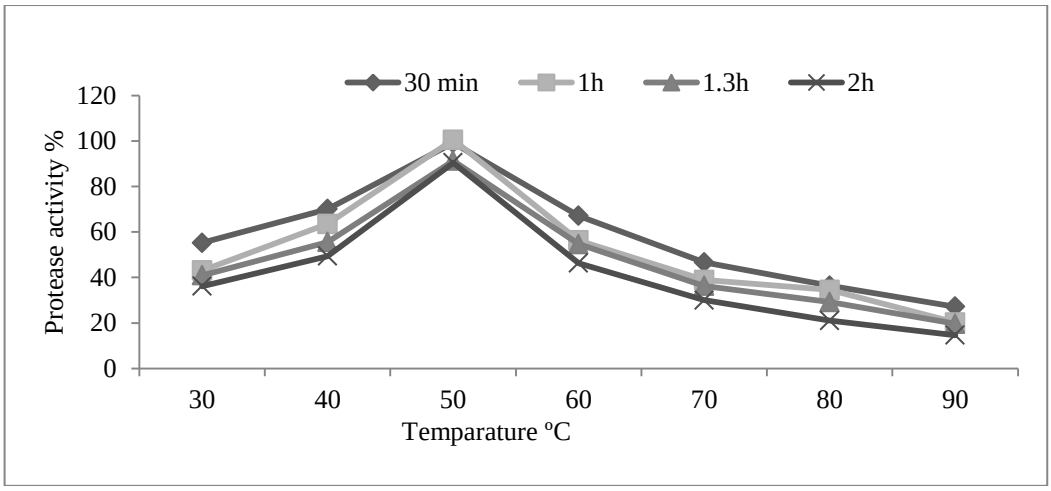

Figure-9: Effect of temperature on the activity of crude visceral protease of C. malabaricus.

Conclusion

The present study was undertaken to extract and characterize the visceral proteases from *Carangoides malabaricus*, as well as its industrial applications. The optimum pH and temperature for maximum activity of visceral protease enzyme was found to depend on host fish species. Crude visceral proteases from *C. malabaricus* showed optimum activity at pH 9.0 and exhibited its alkaline nature. Also the visceral protease was stable at 50°C and displayed better activity. With the optimal activity at higher pH (9.0) and temperature (50°C) the crude visceral protease of *C. malabaricus* was also showed its destaining efficiency and it was emerged as a potential detergent additive. Also, shrimp shell protein hydrolysate exhibited better DPPH scavenging activity. This study concluded that, fish visceral protease is an untapped bio resource with multiple biotechnological applications. Efficient utilization of fish visceral wastes will not only pave the way for pollution menace due to indiscriminate disposal of fish visceral wastes but also provide bioactive lead molecules with potential applications.

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