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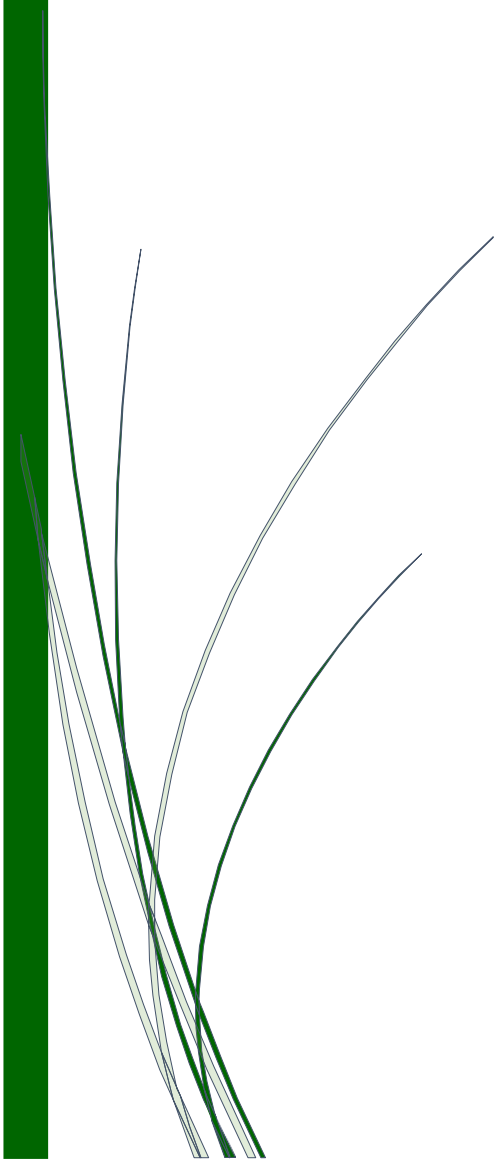

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Research article

Recovery of Silver from Waste X-Ray film Using Crude Digestive Alkaline Fish Visceral Protease of *Sphyraena obtusata*

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***Key words*:** Gelatin, Protease, Silver, Visceral waste

Among the by-products of fish, the viscera are a strong resource of digestive enzymes that may have some characteristic properties of interest to both basic and industrial applications [1]. India alone generate more than 2 metric million tons of waste throughout fish processing, of which 300,000 tons contribute to visceral waste alone [2]. This waste is contemplated as one of the wealthiest sources of proteolytic enzymes. Protease have assorted applications in an extensive variety of industries such as detergent, food, pharmaceutical and leather industries, peptide synthesis and for the recovery of silver from used X-ray films [3].

Silver is a precious metal used in photographic and X-ray film, which is considered as a significant source of silver metal after recycling of used films compared to other types of film. X-ray films enclose about 1.5 to 2% ratio of silver in gelatin-coated film made from polyester layer. The quantity of silver can be restored by dissolving gelatin layer in alkaline protease to be used for other purposes [4]. With an increasing demand for silver in the world, the focus concentrated on X-Ray/photographic films as one of the secondary source of silver owing to the considerable amount present in them [5].

The conventional silver recovery methods embrace burning the films directly [6], oxidation of metallic silver followed by electrolysis, stripping the silver-gelatin silver layer using microbial enzymes specifically alkaline proteases and stripping the gelatin silver layer using different chemicals [7].

Gelatin is a protein from animal collagen, which contains a large number of glycine, proline and 4-hydroxyproline residues [8]. The enzyme from microbial source breaks the gelatin layer embedded with silver in films creating pollution free stripping. Despite the fact that, the enzymatic method is slow, it is cost effective too [6]. Moreover, the viscera are a good source of digestive enzymes that may have some inimitable properties of interest to both basic and industrial applications [9] and the waste is regarded as one of the richest sources of proteolytic enzymes. Hence, the present study was undertaken to recover silver from waste X-ray film using fish visceral protease as a potential gelatin hydrolyser.

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Collection and preparation of enzyme extract

The visceral wastes of *Sphyraena obtusata* were collected from the local fish market and transported to the laboratory. The tissue samples were homogenized using 0.02M Tris-HCl, pH 8.0 and was centrifuged to obtain the crude extract.

Protease assay

Determination of protease activity was carried out by Anson described by Takami *et al.* [10]. The assay mixtures consist of 1.25ml Tris buffer (100mM, pH-7.2), 0.5ml of 1% aqueous casein solution and 0.25ml of culture supernatant. The mixture was incubated for 30min at room temperature and 3ml of 5% TCA was added to this mixture. Further it was placed in a freezer at 4°C for 15 min. From this, 0.5ml of supernatant was taken, to this 2.5ml of Na_2CO_3 (0.5M) was added, mixed well and incubated for 20min at room temperature. To this mixture, 0.5ml of folin-phenol reagent was added and the absorbance was read at 660nm using UV-Vis spectro-photometer.

Effect of pH on protease activity and stability

The optimum pH of the enzyme activity was studied over a pH range of 5.0–12.0. For the measurement of pH stability, the enzyme preparation was incubated at 60°C for about 2.5 h in different buffers such as (100 mM): 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 protease activity and stability

To investigate the effect of temperature, the proteolytic activity was tested at different temperatures

between 10°C to 80°C for 30 min at pH 9.0. The thermostability was examined by incubating the enzyme preparation at different temperatures for 120 min. Aliquots were withdrawn at desired time intervals (30 min to 2.5 h), and the remaining proteolytic was determined by the method described earlier.

Hydrolysis of Gelatin layer on X-ray film and silver extraction

The waste X-ray film was washed with distilled water and wiped with cotton impregnated with ethanol further it was dried in an oven at 40°C, for 30 min. The film were cut into 4 × 4 cm^2 pieces and each of the film was submerged in 20 ml of crude protease with pH 9 and incubated at 50°C (optimum condition) with continuous shaking until the gelatin-silver layer was stripped completely. Untreated x-ray film piece were taken as control. Turbidity of reaction and progress of hydrolysis was observed. Presence of silver was checked by qualitative tests.

Presence of silver

To the gelatin hydrolysate 6M HCL were added drop wise with shaking, until precipitation is complete. The precipitates formed were centrifuged and the pellets were discarded. Suspend the silver chloride precipitate in 1 ml of water and 6M NH3 (aq) drop were added drop wise until the precipitate dissolves. Further, the solution was acidified with 6M HNO_3 and the white precipitate should reappear.

Table 1 Protease activity of hydrolysate (Mean ± SD)

Time interval (Min)	Optical density (660nm)	Protease activity (U/ml)
15	0.812 ± 0.02	170.50 ± 0.06
30	0.775 ± 0.01	162.73 ± 0.05
45	0.726 ± 0.02	152.44 ± 0.05

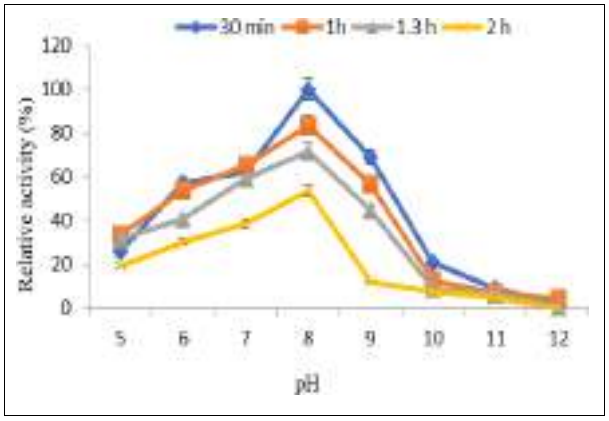


Fig 1 Effect of pH on protease activity and stability

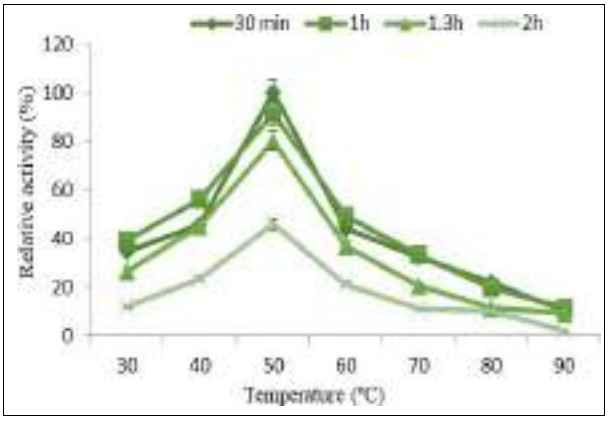
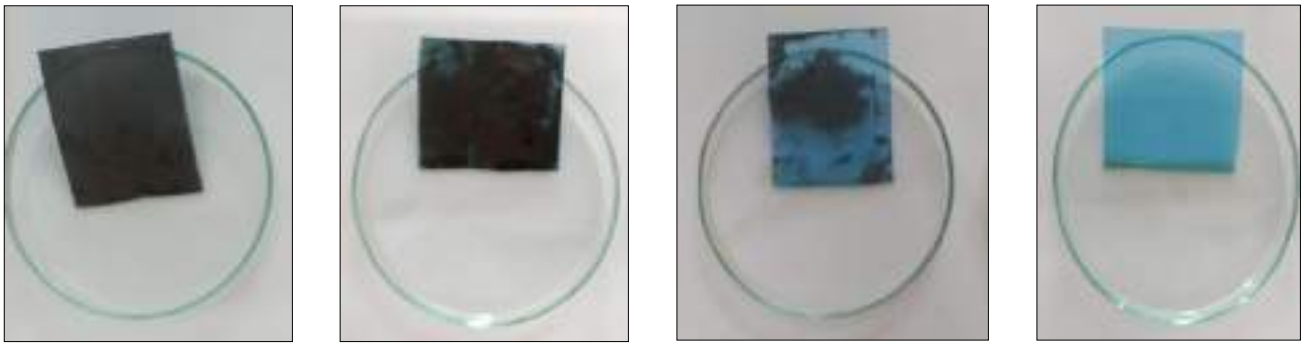


Fig 2 Effect of temperature on protease activity and stability



a) Control b) FVP treated-15 Min c) FVP treated-30 Min d) FVP treated-45 Min

Fig 3 a) X-Ray film used in this study (4x4cm2) as, X-Ray film treated with alkaline crude fish visceral protease (FVP) after b) 15 Min, c) 30 Min, d) 45 Min

From the result, it was revealed that maximum enzyme activity was observed at pH 8.00 and the nature of enzyme was confirmed as alkaline protease. 75% of activity was found at pH 6.00 and above, later the activity was raised up to pH 8.00 during 1 hour of incubation time. Similarly, the optimum temperature of protease activity was observed at 30°C to 60°C, in which maximum activity was registered at 50°C.

From the experiment it was observed that, the protease activity of gelatin hydrolysate was decreased with increase in incubation. The decline of protease activity was due to the exposure of enzyme to a temperature of 50°C, which is the optimum temperature in the different periods of time. Protease activity obtained at 15 minutes of incubation was 170.5 U/ml, at 30 minutes of incubation was 163.7 U/ml followed by 152.4 U/ml at 45 minutes of incubation.

The gelatin layer was stripped entirely within 45 minutes under optimum condition (pH 8, 50°C). Gelatin hydrolysis was found to be best when Tris-HCl buffer of pH 9 was used indicating that it is the most ideal pH for hydrolysis of Gelatin. A loss in weight of X-ray film was observed (0.51mg) after the treatment when compared with the initial weight of the film (0.65 mg). The silver was recovered in the hydrolysate and the presence of silver was confirmed by qualitative test.

According to the studies, the activity and stability of protease varies from one organism to other. The optimum pH for the visceral protease of *Argyrosomus argentatus* was 7 [11]. Likewise, the optimum pH of 8 reported for the crude visceral protease of Raja clavate [12] was similar to the report of [13-14] for *Oreochromis niloticus* and *Zosterisessor* ophiocephalus respectively.

Similarly, the optimal temperature of alkaline protease from species such as *Raja clavata* [15] and *Salaria basilica* [16] was found to be 50°C. Furthermore, the optimum temperature stability obtained for the visceral protease of *Saurida undosquamis* and *Scorpaena scrofa* was 55°C [17-18].

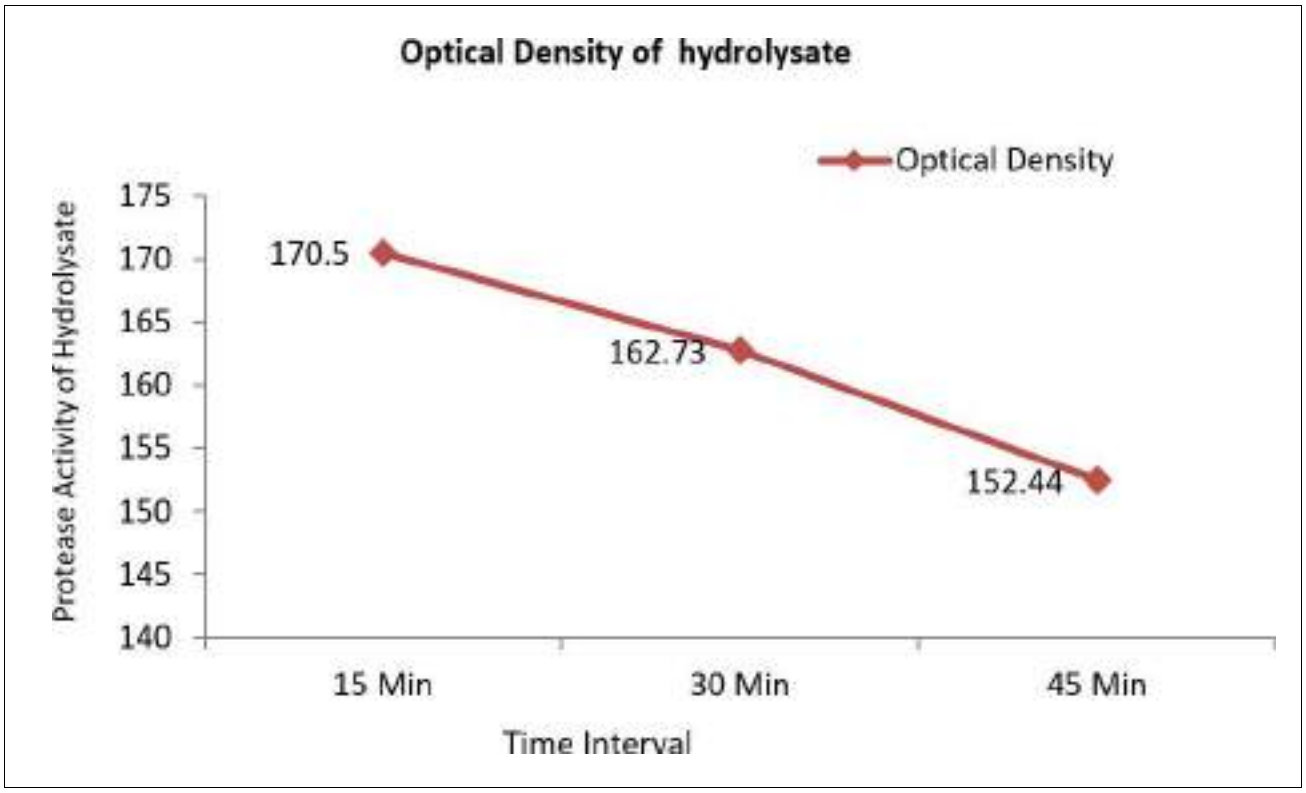


Fig 4 Graph showing protease activity of hydrolysate (Crude fish visceral protease + waste X-Ray film)

In accordance, the crude visceral protease from *Labeo rohita* show high efficiency in removing gelatin layer and there was a loss of weight in waste X-ray film was confirmed, before and after the treatment with crude fish visceral protease [19]. Likewise, Alkaline protease from *Conidiobolus coronatus* investigated for enzymatic hydrolysis of gelatin from waste X-ray films, showed the complete removal of gelatin and silver leaving the polyester film clean and the silver recovered in the hydrolysate [20] and *Bacillus subtilis* isolated from slaughter house soil could degrade the gelatinous coating of X-ray films [21]. 50°C as the stripping temperature for the enzyme from *Bacillus subtilis* as reported by [8].

CONCLUSION

The fish visceral waste is contemplated as one of the wealthiest sources of proteolytic enzymes. The fish viscera are not being used as a food item and considered as a waste material, which generally dumped or hauled into ocean. The large accumulation of such waste shows the way to pollution of water and other resources. The present study authenticated the ability of fish visceral protease to hydrolyze gelatin on waste X-ray film. This study also confirmed that Fish Visceral protease has the potential of being reused for extracting silver from used X-ray films in a recyclable way. Since the emulsion layer on X-ray film contains silver and gelatin, it is feasible to break down the gelatin layer using proteases and release the silver without damaging the polyester film base. In the present study, it was observed that treatment of X-ray films with protease enzyme obtained from *Sphyraena obtusata* resulted in the sliver bound with gelatin being stripped off into the reaction mixture and the clean plastic film being recovered. The optimum range of temperature on protease activity and stability were obtained as 50°C and optimum pH obtained was 8. The gelatin layer was stripped entirely within 45 minutes under optimum condition of pH 8 and temperature 50°C. The silver was recovered from the hydrolysate and the presence was confirmed using qualitative test.

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