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Comparative Study on Precipitation Techniques for Protease Isolation and Purification from *Labeo Rohita* Viscera

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Abstract

Precipitation techniques play a vital role in the industrial extraction of enzymes. The present study aimed to extract the proteases from the viscera of *Labeo rohita* (commonly called Rohu) and to compare the precipitation techniques for the isolation and purification of the enzyme. The enzyme is usually discarded as tons of waste during processing. Hence, a trial has been carried out to isolate the protease enzymes from viscera of the fresh water fish *Labeo rohita*. The proteases were precipitated with ammonium sulfate, ethanol, and acetone. Acetone precipitation was found to be the best option for the recovery of enzymes (54%) from the viscera of Rohu, and two caseinolytic protease bands were shown in the Zymogram. The precipitates with highest proteolytic activity were further subjected to dialysis, and their molecular weight was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

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Keywords

- Labeo rohita ,
- Fish viscera,
- Precipitation techniques,
- Zymogram,
- SDS-PAGE

Abstract

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Introduction

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Proteases (EC 3.4.21–24 and 99) are enzymes that catalyze the hydrolysis of proteins (Murado et al., 2009**16.** Murado, MA., Gonzalez, MP. and Vazquez, JA. 2009. Recovery of proteolytic and collagenolytic activities from viscera by-products of Rayfish (*Raja clavata*). *Mar. Drugs* , 7: 803–815.
[CrossRef], [Web of Science ®]
View all references). They have an enormous field of research and usage in food, pharmaceutical, detergent, and agrochemical industries (Gupta et al., 2002**8.** Gupta, R., Beg, Q. and Lorenz, P. 2002. Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl Microbial Biotechnol* , 59: 15–32.
[CrossRef], [PubMed], [Web of Science ®], [CSA]
View all references). Proteases are mainly derived from plant, animal, and microbial sources, whereas their counterparts are derived from marine and other aquatic sources that have not been extensively used (Haard and Simpson, 1994**10.** Haard, N.F. and Simpson, B.K. 1994. *Fisheries processing: Biotechnological application* , London , , U.K: Chapman and Hall.

View all references; El-Beltagy et al., 2005**4.** El-beltagy, A.E., El-adawy, T.A., Rahma, E.H. and El- bedawey, A.A. 2005. Purification and characterization of an alkaline protease from the viscera of bolti fish (*Tilapia nilotica*). *Food Chem* , 29: 445–448.

View all references).
Fish processing in India generates over 300,000 tons of waste in the form of visceral mass. The digestive tract or the viscera constitutes 5 to 8% of the fish weight (Mahendrakar, 2000**14.** Mahendrakar, N.S. 2000. "Aqua feeds and meat quality of cultured fish". In *Aquaculture – feed health* , Edited by: John, G. and Ninawe, A.S. 26–30. Biotech , New Delhi : Consort. India Ltd.

View all references). This material has nutritional value equivalent to that of whole fish (Strom and Eggum, 1981**26.** Strom, T. and Eggum, B. O. 1981. Nutritional value of fish viscera silage. *J. Sci. Food Agric* , 32: 115–120.
[CrossRef], [PubMed], [Web of Science ®], [CSA]
View all references). Often, these wastes are not utilized further but disposed of in landfills or dumping at sea. Therefore, there is a need to find ecologically acceptable means for reutilization of these wastes. For instance, the enormous quantities of fish waste can be used as a source of proteins for all sorts of animals (Ennouali et al., 2006**5.** Ennouali, M., Elmoualdi, L., Labioui, H., Ouhsine, M. and Elyachioui, M. 2006. Biotransformation of the fish waste by fermentation. *Afr. J. Biotechnol* , 5: 1733–1737.
[Web of Science ®]
View all references). There is a growing interest in obtaining higher value biochemicals and pharmaceuticals from fishery waste, notably enzymes (Haard, 1998**9.** Haard, N.F. 1998. Specialty enzymes from marine organisms. *Food technol* , 52: 64–67.
[Web of Science ®]
View all references). Various digestive proteolytic enzymes have been isolated from the internal organs of fish. The most important digestive enzymes from fish viscera are the aspartic protease, pepsin, and the serine proteases, trypsin, chymotrypsin, and elastase (Barkia et al., 2010**2.** Barkia, A., Bougatef, A., Nasri, R., Fetoui, E., Balti, R. and Nasri, M. 2010. Trypsin from the viscera of Bogue (Boops boops): Isolation and characterisation. *Fish physiol. Biochem* , 39: 893–902.
[CrossRef]
View all references). Digestive enzyme activities in fish vary among species, which can be influenced by the age as well as by the quantity and composition of diet (Peres et al., 1998**21.** Peres, A., Zambonino Infante, J.L. and cahu, C.L. 1998. Dietary regulation of activities and mRNA levels of trypsin and amylase in sea bass (*Dicentrarchus labrax*) larvae. *Fish physiol.Biochem* , 19: 145–152.
[CrossRef], [Web of Science ®], [CSA]
View all references).
The present research was aimed at partial purification and identification of proteolytic enzymes from *Labeo rohita* (commonly called Rohu), the most important Indian Major Carp (IMC) cultivated commercially across the country (Debnath et al., 2007**3.** Debnath, D., Pal, A.K., Sahu, N.P., Yengkokpam, S., Baruah, K., Choudhury, D. and Venkateshwarlu, G. 2007. Digestive enzymes and metabolic profile of *Labeo rohita* fingerlings fed diets with different crude protein levels. *Comp.Biochem.Physiol. PartB* , 146: 107–114.
[CrossRef], [Web of Science ®]
View all references). The viscera of Rohu contained nearly 45% fat (Pathak et al., 1953**20.** Pathak, S.P., Pande, G.D. and Mathur, S.S. 1953. The component acids of the fats of some indian fresh-water fishes. *Biochem* , 57: 449–453.

View all references) which can make homogenization difficult, and the extract contains an enormous quantity of finely dispersed lipid which complicates the subsequent purification steps. Therefore, the study was focused to compare and select precipitation methods which will give high protein recovery from Rohu visceral extract that might be further used by the food and pharmaceutical industries.

MATERIALS AND METHODS

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Analytical reagents

Bovine serum albumin, casein, trichloroacetic acid, Folin ciocalteu's reagent, sodium carbonate, tris (hydroxylmethyl) aminomethane, coomassie brilliant blue R-250, acrylamide, bisacrylamide, N, N, N′, N′-tetramethyl ethylenediamine (TEMED), sodium dodecyl sulfate, ammonium persulfate, ethylene diamine tetraacetic acid (EDTA), and glycine were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Protein standard markers (14.4–116.0KDa) were purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada). Dialysis tubing was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). All other reagents were of analytical grade.

Sample Collection

Rohu were purchased from the local market in Mettur Dam, Tamilnadu, India. The fish were kept in ice and transported to the research laboratory within 1 hr. After washing the fish with distilled water, visceral organs were separated and then stored in sealed plastic bags at -20 °C until used for enzyme extraction.

Preparation of crude protease extract

Viscera from Rohu (80g) were thawed for about 2 hrs at room temperature and homogenized with 250 ml of 10 mM Tris- HCl buffer (pH 8.0) (Barkia et al., 20102. Barkia, A., Bougatef, A., Nasri, R., Fetoui, E., Balti, R. and Nasri, M. 2010. Trypsin from the viscera of Bogue (Boops boops): Isolation and characterisation. *Fish physiol. Biochem*, 39: 893–902.
[CrossRef]
View all references). The homogenate was centrifuged at 8500 × g for 30 min at 4°C. The pellet was discarded, and the supernatant (200 ml) was collected and used as crude protease extract. This 200ml protease was further fractioned into 4 parts of 50 ml each. Each 50ml sample was subjected to various precipitation techniques to identify the highest recovery percentage and purification fold of crude protease extract. All the precipitation methods were performed with the same crude enzyme extract with the protein concentration of 3.2 mg/ml.

Acetone Precipitation

In the 50 ml of crude protease extract, an equal volume of ice-cold acetone (50 ml) was added in a drop wise manner with continuous stirring on ice. After the addition of the organic solvent was completed, the stirring was continued on ice for 10–20 min. The mixture was transferred to the chilled screw-cap polycarbonate centrifuge tubes, and the precipitated proteins were recovered by centrifugation at 10,000 × g for 10 min at 4°C. The supernatants were discarded, and the centrifuge tubes were inverted over filter paper for air drying (Simpson, 200425. Simpson, R.J. 2004. *Purifying proteins for proteomics, a laboratory manual*, 719–722. I.K International Pvt. Ltd.

View all references). Then, the pellets were suspended in 15 ml of 25 mM Tris-HCl buffer (pH 8.0).

Ammonium sulfate fractional precipitation

To the 50 ml of crude protease extract, ammonium sulfate (5.35g) was added to reach 0–20% saturation. After gentle vortexing for 10 min, the samples were left at ice cold condition for 1 hr and centrifuged at 10,000 × g at 4°C for 10 min. The supernatant was transferred to another tube, and 11.45 g of ammonium sulfate was added to reach 20–40% saturation; then the mixture was treated as above. The proteins in the supernatant were further fractionated to 40–60%, 60–80%, and 80–100% with ammonium sulfate (Simpson, 200425. Simpson, R.J. 2004. *Purifying proteins for proteomics, a laboratory manual*, 719–722. I.K International Pvt. Ltd.

View all references). Each of the five pellets was separately suspended in 15 ml of 25 mM Tris-HCl buffer (pH 8.0).

Ethanol precipitation

In the 50 ml of crude protease extract, an equal volume of ice cold ethanol was added. The sample mixture was kept for 10–20 min on ice, and the precipitate was collected by centrifugation at 10,000 × g at 4°C for 10 min (Simpson, 200425. Simpson, R.J. 2004. *Purifying proteins for proteomics, a laboratory manual*, 719–722. I.K International Pvt. Ltd.

View all references). Then, the pellets were suspended as above.

Protein Determination

Protein concentration was estimated by Lowry et al. (1951)13. Lowry, Q.H., Rosenbrough, N.J., Farr, L.A. and Randall, R.J. 1951. Protein measurement with the Folin Phenol reagent. *J. Biol Chem*, 193: 256–275.

View all references using Bovine Serum Albumin as standard.

Assay of protease activity

Protease activity was assayed by the Anson method (19381. Anson, M.L. 1938. The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. *J. Gen. Physiol*, 22: 79–89.
[CrossRef], [PubMed], [Web of Science ®]
View all references) with some modifications. The enzyme solution (1 ml) was mixed with 5.0 ml of substrate (0.65% casein in 25 m M Tris-HCl buffer, pH 8.0) at room temperature for 30 min. After incubation, TCA (110 mM) was added to attenuate the reaction. This mixture was allowed to incubate for

30 min at room temperature and centrifuged at 10,000 × g at 4°C for 5 min. Release of amino acids were measured as tyrosine by the method of Folin and Ciocalteu (19297. Folin, O. and Ciocalteau, V.J. 1929. On tyrosine and tryptophan determinations in proteins. *J. Biol. Chem* , 73: 627–650.

View all references).
The precipitates were further assayed by the Anson method (19381. Anson, M.L. 1938. The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. *J. Gen. Physiol* , 22: 79–89.
[CrossRef], [PubMed], [Web of Science ®]
View all references), and the precipitate which showed good protease activity was subjected to dialysis.

Dialysis

The precipitate obtained from the ice cold acetone precipitation was dissolved in 25 mM Tris-HCl buffer at pH 8.0 and dialyzed against the same buffer for 24 h at 4°C. The buffer was changed twice (after 8 and 16 hours) (Nedra et al., 200918. Nedra El Hadj Ali, Hmidet, Noomen, Bougatef, Ali, Nasri, Rim and Moncef, Nasri. 2009. A laudry detergent- stable alkalin trypsin from striped seabream (*Lithognathus mormyrus*) Viscera: Purification and characterization. *J. Agric. Food Chem* , 57: 10943–10950.
[CrossRef], [Web of Science ®]
View all references).

SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to determine the molecular weight of partially purified enzyme by the method of Laemmli (1970)11. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* , 277: 680–685.
[CrossRef], [Web of Science ®]
View all references using 4% stacking gel and 10% (W/V) separating gel. The samples were prepared by mixing the partially purified enzyme extract with distilled water containing 0.062 M Tris, 4% SDS, 10% glycerol, 1.5% β-mercaptoethanol, and 0.002% bromophenol blue (pH 6.8). The polyacryamide (monomer 8.33% concentration) vertical gel tubes contained 0.15% SDS and 0.375 M Tris, 6.1 g glycine, and 0.25 g SDS per 250 ml. Then, 10 µl (20µg) of sample was applied on the gel surface and fractionated for 4 hrs at 50 volt. The gel was stained at half an hour (methanol-50 ml, acetic acid -7 ml, CBG250-250 mg, distilled water to make up to 100 ml). Then, the gel was destained using distilled water, methanol, acetic acid, and water in the ratio of 50:7:43. Standard protein markers used for calibration were β galactosidase (116.0 KDa), bovine serum albumin (66.2 KDa), ovalbumin (45.0 KDa), lactate dehydrogenase (35.0 KDa), REase BSP981 (25.0 KDa), β-lactoglobulin (18.4 KDa), and lysozyme (14.4 KDa).

Detection of protease activity by zymography

Proteases obtained from Rohu viscera were resolved in 10% SDS polyacrylamide gels containing the following substrates: 0.1% gelatin, 0.1% casein. Samples (10 µl) were mixed with sample buffer (25 mM Tris HCl). Samples were not boiled. The protein sample was electrophoresed under standard conditions. After electrophoresis, SDS was removed from the gel by immersing in a solution containing 2.5% Triton X 100, and then the gels were incubated overnight at room temperature in 50 mM Tris HCl (pH-8.0) buffer supplemented with $CaCl_2$ (1 mM), $ZnCl_2$ (0.001 mM), and NaCl (150 mM).

The protease activity was visualized by staining the gels with coomassie brilliant blue (Twinig et al., 199328. Twining, S.S., Kirschner, S.E., Mahnke, L.A. and Frank, D.F. 1993. Effect of *Pseudomonas aeruginosa elastase*, alkaline protease and exotoxin A on Corneal proteinases and protein. *Invest. Opthalmol. Vis. Sci* , 34: 2699–2712.
[Web of Science ®]
View all references).

RESULTS AND DISCUSSION

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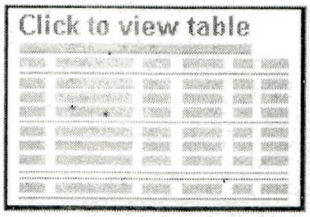
Partial purification of protease enzymes from *L. rohita* visceral organs sample through ammonium sulfate fractionation, ethanol, and acetone precipitation is shown in Table 1. Ammonium sulfate fractionation of the sample showed an increase in total protease, total protein, and specific activity up to 40% in comparison with the crude protein. High recovery of protease (18%) was observed at 20–40% pellet, but the purification fold was high at 0–20% with 7 fold. An earlier study on protease purification from fish samples showed high recovery at 70% through precipitation (Subash et al., 201127. Subash, A., Devi, D. M. and Shobana, A. 21–23 March 2011. "Isolation, purification and characterization of protease from visceral organ wastes and head and tail wastes of grey mullet fish". In *World Congress on Biotechnology* , 21–23 March, India: HICC Hyderabad.

View all references); however, a study on porcine pancreas showed high protease activity at both 20 and 40% pellets with alanine and phenylalanine peptidase. Therefore, the result of the present study through ammonium sulfate fractionation showed high protease recovery at 20–40% pellet which may be rich in alanine and pheylalanine peptidase.

Table 1. Partial Purification of Proteases of *Labeo Rohita* Viscera with Ammonium Sulfate, Ethanol, and Acetone Fractionation



Among the acetone and ethanol used in the solvent precipitation technique in extraction of protease, acetone showed highest total protease, total protein, and recovery, above 50%. In the solvent precipitation technique, solubility of proteins in solvent depends on dielectric constant, boiling point, and dipole movement. The characteristics of acetone with its 20.7 dielectric constant, which is moderately low with low boiling point of 56°C and high dipole movement of 2.88, facilitates the effective precipitation of proteases. Compared to other solvents, acetone was found to have less dielectric constant, which discourages the dispersion of protein molecules in the media (Nam Sun Wang, 2010**17.** Nam sun wang. 2010. *Enzyme purification by acetone precipitation* , 1–3. Department of Chemical and Biomolecular Engineering, University of Maryland.

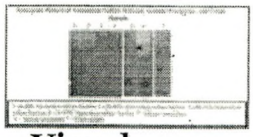
View all references). The advantage of acetone precipitation is that it is a very feasible procedure to perform (Fic et al., 2010**6.** Fic, E., Kedracka-krok, S., Jankowska, U., Pirog, A. and Dziedzicka-wasylewska, D. 2010. Comparison of protein precipitation methods for various rat brain structures prior to proteomic analysis. *Electrophoresis* , 31: 3573–3579.
[CrossRef], [Web of Science ®]
View all references). Moreover, acetone precipitation may have a lesser tendency to cause denaturation than ethanol, and it is also more volatile, which enables it to be removed easily from re-dissolved precipitates under reduced pressure (Scopes, 2004**24.** Scopes, R.K. 2004. *Protein purification principles and practice. Springer* , 3rd 89

View all references).
The precipitate which was obtained in the ice cold acetone fraction was found to be higher (54.4%) than the other precipitation techniques used. It is noted that the recovery obtained in ethanol precipitation was 12%. In the ammonium sulfate fractionation, the higher yield was found at 20–40% and 18.7%, respectively. This result indicates that the ice cold acetone precipitation was found to be the most effective technique in the isolation of protease when compared to ammonium sulfate and ethanol precipitations (Table 1). The effectiveness of cold acetone as a purification agent for proteolytic enzymes was reported by Popova and Pishtiyski (2001)**22.** Popova, V. and Pishtiyski, I. 2001. Isolation of cyclodextrine glucanotransferase preparations of different purities. Eur. *Food Res. Technol* , 213: 67–71.
[CrossRef], [Web of Science ®]
View all references. Also, insufficient partial purification by ammonium sulfate was observed by Wang et al. (2002)**29.** Wang, S., Hsaio, W. and Ghang, W. 2002. Purification and characterization of an antimicrobial chitinase extracellularly produced by Monascus purpureus CCRC31499 in a shrimp and cramb shell powder medium. *J. Agric. Food Chem* , 50: 2249–2255.
[CrossRef], [PubMed], [Web of Science ®], [CSA]
View all references, Olivas–Burrola et al. (2001**19.** Olivas-Burrola, H., Ezquera-Brauer, M.J., Rouzaud-Sandez, O. and Pacheo-Aguilar, R. 2001. Protease activity and partial characterization of the trypsin like enzyme in the digestive tract of the tropical sierra *Scomberomorus concolor* . *J. Aqua. Food Prod. Technol* , 10: 51–63.
[Taylor & Francis Online], [CSA]
View all references), and Michail et al. (2006)**15.** Michail, M., Vasiliadou, M. and Zotos, A. 2006. Partial purification and comparison of precipitation techniques of proteolytic enzymes from trout (*Salmo gairdnerii*) heads. *Food Chem* , : 50–55.
[CrossRef], [Web of Science ®]
View all references. Moreover, this method has proved efficient in removing the characteristic smell of fish viscera and in clarifying the crude extract. The proteolytic activity of different precipitates on casein were shown (SDS-zymography) in Plate 1, where lanes 1 to 4 display ammonium sulfate fractions, lane 5 displays ethanol precipitate, lane 6 displays acetone precipitate, and lane 7 displays crude sample. The zymogram of ammonium sulfate fractions showed one prominent band up to 0–40% and no prominent band until 40–80%. However, many non-prominent bands were also seen in all the fractions. The zymogram of ethanol showed bands which are not prominently seen, whereas the acetone zymogram showed two prominent caseinolytic bands. The crude protein zymogram showed no prominent band, but fully hazy bands were seen. Zymography is a sensitive and rapid assay method for analyzing protease activity (Barkia et al., 2010**2.** Barkia, A., Bougatef, A., Nasri, R., Fetoui, E., Balti, R. and Nasri, M. 2010. Trypsin from the viscera of Bogue (Boops boops): Isolation and characterisation. *Fish physiol. Biochem* , 39: 893–902.
[CrossRef]
View all references).
The above zymogram results perfectly synchronized with our earlier results of partial purification of protease enzyme in this study (Table 1). Non-prominent bands seen in the ammonium sulfate zymogram may be due to the presence of other isoenzymes resulting from high fat content of the fish. Due to fat interference, it may not have the efficiency to precipitate the majority of proteins with proteolytic activity; therefore, the bands are lighter than the acetone fraction. Also, the zymogram of the ammonium sulfate fractionations (0–80%) showed better recovery of proteolytic enzymes than ethanol. The single band in the zymogram of ethanol and acetone precipitates further confirms the fat interference since fat dissolves in these solvents. The SDS-PAGE of the crude, acetone precipitate, and dialysate are presented in Plate 2, which shows proteins of the crude extract in lane 1, acetone precipitated fraction in lane 2, dialysate in lane 3, and standard protein markers in lane 4. Results of this SDS-PAGE also synchronized with the above SDS-Zymogram and partial purification table. Whereas the crude enzyme extract showed the presence of various protein bands, acetone precipitated fraction and dialysate showed two major dark bands. However, prominent bands in the zymogram of acetone precipitate and SDS-PAGE show that acetone is able to partially purify and concentrate the majority of proteins with proteolytic activity from the crude extract. The molecular weight of these activity zones ranged from 48.0–90.0 kDa.

Dialysis

The precipitate obtained from the ice cold acetone precipitation was dissolved in 25 mM Tris-HCl buffer at pH 8.0 and dialyzed against the same buffer for 24 h at 4°C. The dialysis increased the specific activity from 1.04 to 1.2. The purification fold also increased after dialysis from 1.6 to 1.9 (not shown). El-Beltagy et al. (2005)**4.** El-beltagy, A.E., El-adawy, T.A., Rahma, E.H. and El- bedawey, A.A. 2005. Purification and characterization of an alkaline protease from the viscera of bolti fish (*Tilapia nilotica*). *Food Chem* , 29: 445–448.

View all references stated that the dialysis of alkaline protease crude extract (*Tilapia Nilotica*) increased the purification fold. Similar results found by Raksakulthai and Haard (1999)**23.** Raksakulthai, R. and Haard, N.F. 1999. Purification and characterization of amino peptidase fractions from squid (*Illex illecebrosus*) hepatopancreas. *Food Biochem* , 23: 123–144.
[CrossRef], [Web of Science ®]
View all references showed that the dialysis of amino peptidase crude extracts (*Suid hepatopancreas*) increased the purification fold.



View larger version(29K)

Figure 1 Zymogram Pattern of Ammonium Sulfate, Ethanol, Acetone Precipitate, and Crude Sample.

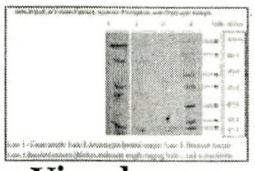


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Figure 2 SDS-PAGE of Crude Extract, Acetone Precipitate and Dialysate Sample

Conclusions

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Precipitation techniques play a vital role in the industrial extraction of enzymes. Thus, the aim of this research was to partially purify and identify proteolytic enzymes from *Labeo rohita* viscera. A trial has been carried out to compare the three precipitation techniques, namely ammonium sulfate, ethanol, and acetone precipitation, for the isolation and precipitation of proteins with proteolytic activity. Among these three, acetone precipitation showed the highest protease activity with 54.4% recovery. Due to the high activity detected, further research is underway to determine if *Labeo rohita* viscera can be used to obtain a high value-added product (proteolytic enzymes) that could be used for the reduction of waste disposal problems in the fishing industry.

References

- **1.** Anson, M.L. 1938. The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. *J. Gen. Physiol*, 22: 79–89. [CrossRef], [PubMed], [Web of Science ®]
- **2.** Barkia, A., Bougatef, A., Nasri, R., Fetoui, E., Balti, R. and Nasri, M. 2010. Trypsin from the viscera of Bogue (Boops boops): Isolation and characterisation. *Fish physiol. Biochem*, 39: 893–902. [CrossRef]
- **3.** Debnath, D., Pal, A.K., Sahu, N.P., Yengkokpam, S., Baruah, K., Choudhury, D. and Venkateshwarlu, G. 2007. Digestive enzymes and metabolic profile of *Labeo rohita* fingerlings fed diets with different crude protein levels. *Comp.Biochem.Physiol. PartB*, 146: 107–114. [CrossRef], [Web of Science ®]
- **4.** El-beltagy, A.E., El-adawy, T.A., Rahma, E.H. and El- bedawey, A.A. 2005. Purification and characterization of an alkaline protease from the viscera of bolti fish (*Tilapia nilotica*). *Food Chem*, 29: 445–448.
- **5.** Ennouali, M., Elmoualdi, L., Labioui, H., Ouhsine, M. and Elyachioui, M. 2006. Biotransformation of the fish waste by fermentation. *Afr. J. Biotechnol*, 5: 1733–1737. [Web of Science ®]
- **6.** Fic, E., Kedracka-krok, S., Jankowska, U., Pirog, A. and Dziedzicka-wasylewska, D. 2010. Comparison of protein precipitation methods for various rat brain structures prior to proteomic analysis. *Electrophoresis*, 31: 3573–3579. [CrossRef], [Web of Science ®]
- **7.** Folin, O. and Ciocalteau, V.J. 1929. On tyrosine and tryptophan determinations in proteins. *J. Biol. Chem*, 73: 627–650.
- **8.** Gupta, R., Beg, Q. and Lorenz, P. 2002. Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl Microbial Biotechnol*, 59: 15–32. [CrossRef], [PubMed], [Web of Science ®], [CSA]
- **9.** Haard, N.F. 1998. Specialty enzymes from marine organisms. *Food technol*, 52: 64–67. [Web of Science ®]
- **10.** Haard, N.F. and Simpson, B.K. 1994. *Fisheries processing: Biotechnological application*, London, , U.K: Chapman and Hall.
- **11.** Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature*, 277: 680–685. [CrossRef], [Web of Science ®]
- **12.** Little, S.E., and Little, J.T. Rapid protease screening using ammonium sulfate precipitations and the Avanti™ J–25 High performance centrifuge http://www.beckmancoulter.com/
- **13.** Lowry, Q.H., Rosenbrough, N.J., Farr, L.A. and Randall, R.J. 1951. Protein measurement with the Folin Phenol reagent. *J. Biol Chem*, 193: 256–275.

- **14.** Mahendrakar, N.S. 2000. "Aqua feeds and meat quality of cultured fish". In *Aquaculture – feed health*, Edited by: John, G. and Ninawe, A.S. 26–30. Biotech, New Delhi: Consort. India Ltd.
- **15.** Michail, M., Vasiliadou, M. and Zotos, A. 2006. Partial purification and comparison of precipitation techniques of proteolytic enzymes from trout (*Salmo gairdnerii*) heads. *Food Chem*, : 50–55. [CrossRef], [Web of Science ®]
- **16.** Murado, MA., Gonzalez, MP. and Vazquez, JA. 2009. Recovery of proteolytic and collagenolytic activities from viscera by-products of Rayfish (*Raja clavata*). *Mar. Drugs*, 7: 803–815. [CrossRef], [Web of Science ®]
- **17.** Nam sun wang. 2010. *Enzyme purification by acetone precipitation*, 1–3. Department of Chemical and Biomolecular Engineering, University of Maryland.
- **18.** Nedra El Hadj Ali, Hmidet, Noomen, Bougatef, Ali, Nasri, Rim and Moncef, Nasri. 2009. A laudry detergent- stable alkalin trypsin from striped seabream (*Lithognathus mormyrus*) Viscera: Purification and characterization. *J. Agric. Food Chem*, 57: 10943–10950. [CrossRef], [Web of Science ®]
- **19.** Olivas-Burrola, H., Ezquera-Brauer, M.J., Rouzaud-Sandez, O. and Pacheo-Aguilar, R. 2001. Protease activity and partial characterization of the trypsin like enzyme in the digestive tract of the tropical sierra *Scomberomorus concolor*. *J. Aqua. Food Prod. Technol*, 10: 51–63. [Taylor & Francis Online], [CSA]
- **20.** Pathak, S.P., Pande, G.D. and Mathur, S.S. 1953. The component acids of the fats of some indian fresh-water fishes. *Biochem*, 57: 449–453.
- **21.** Peres, A., Zambonino Infante, J.L. and cahu, C.L. 1998. Dietary regulation of activities and mRNA levels of trypsin and amylase in sea bass (*Dicentrarchus labrax*) larvae. *Fish physiol.Biochem*. 19: 145–152. [CrossRef], [Web of Science ®], [CSA]
- **22.** Popova, V. and Pishtiyski, I. 2001. Isolation of cyclodextrine glucanotransferase preparations of different purities. Eur. *Food Res. Technol*, 213: 67–71. [CrossRef], [Web of Science ®]
- **23.** Raksakulthai, R. and Haard, N.F. 1999. Purification and characterization of amino peptidase fractions from squid (*Illex illecebrosus*) hepatopancreas. *Food Biochem*, 23: 123–144. [CrossRef], [Web of Science ®]
- **24.** Scopes, R.K. 2004. *Protein purification principles and practice. Springer*, 3rd 89
- **25.** Simpson, R.J. 2004. *Purifying proteins for proteomics, a laboratory manual*, 719–722. I.K International Pvt. Ltd.
- **26.** Strom, T. and Eggum, B. O. 1981. Nutritional value of fish viscera silage. *J. Sci. Food Agric*, 32: 115–120. [CrossRef], [PubMed], [Web of Science ®], [CSA]
- **27.** Subash, A., Devi, D. M. and Shobana, A. 21–23 March 2011. "Isolation, purification and characterization of protease from visceral organ wastes and head and tail wastes of grey mullet fish". In *World Congress on Biotechnology*, 21–23 March, India: HICC Hyderabad.
- **28.** Twining, S.S., Kirschner, S.E., Mahnke, L.A. and Frank, D.F. 1993. Effect of *Pseudomonas aeruginosa elastase*, alkaline protease and exotoxin A on Corneal proteinases and protein. *Invest. Opthalmol. Vis. Sci*, 34: 2699–2712. [Web of Science ®]
- **29.** Wang, S., Hsaio, W. and Ghang, W. 2002. Purification and characterization of an antimicrobial chitinase extracellularly produced by Monascus purpureus CCRC31499 in a shrimp and cramb shell powder medium. *J. Agric. Food Chem*, 50: 2249–2255. [CrossRef], [PubMed], [Web of Science ®], [CSA]