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Utilization of fish viscera for protease production and used for digestion of waste in the pond

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Abstract
Mozambique Tilapia (*Tilapia mossambica*) viscera extracts were prepared using water, KCl solution and EDTA homogenization. The extract with the highest protease specific activities of 1687.5 and 2800 IU/mg protein, respectively. The enzyme was active at the optimum pH (4.0) and temperature (45 °C) when casein was used as a substrate. The enzyme showed the highest activity and purification when precipitated at 0-80% ammonium sulphate effectively improved specific activity of enzyme. Thus, the results revealed that active digestive enzymes could be prepared from tilapia viscera and can be used to digest the waste build-up in the pond and which greatly increase the clarity of the water by hydrolysing the proteinacious waste materials that build up. This research was carried out for the utilization of fish by-products for producing enzymes and also to the reduction of waste disposal problems.

Keywords: Protease, tilapia, ammonium sulphate precipitation, thermo stability, pH optima

1. Introduction
Proteases constitute the most important group of industrial enzymes used in the world today and it have several applications in the food industry [1]. Proteases may be extracted from any living organism. Very wide range of sources are used for commercial enzyme production [2]. Proteases are mainly derived from plant, animal and microbial sources, whereas their counterparts, derived from marine and other aquatic sources, have not been extensively used [3]. Fish processing generates large amounts of solid and liquid wastes. Fishery by-products are typically feeds and fertilizers that have a low dollar value. There is growing interest in obtaining higher value biochemical and pharmaceuticals from fishery wastes, notably enzyme [4]. There have been relatively few attempts to use fish proteases as industrial processing aids. In recent years, additional applications of proteases in the seafood industry have emerged. These include the selective removal of skin, hydrolysis of membranes and other supportive tissue and recovery of pigments and flavour extract [5]. Crude forms of both acidic and alkaline proteases extracted from bolti fish viscera convert 54% and 79% of fish protein wastes at a 50 h reaction time [6].
Tilapia is one of the most important fish species in aquaculture [7]. The mass industrial processing of tilapia, frozen fillets as a priority, generates large amount of waste especially viscera [7, 8]. Fish viscera have been reported to be a good source of digestive enzymes [9, 10, 11, 12] and their properties are highly valued in a wide range of industrial applications and processes since some proteases are stable and active under harsh conditions (high temperature and pH) and in the presence of oxidizing agents or surfactants [13]. Digestive proteases from fishes are hydrolytic in their action and catalyse the cleavage of peptide bonds with the participation of water molecule as reactants [11]. Proteases found in the viscera of fish include trypsin, chymotrypsin, collagenase, elastase, Carboxypeptidase and carboxyl esterase [3]. Until now, very few information has been reported on preparation of crude visceral extract containing protease from tilapia viscera. The viscera are a waste and are not used as a source of food and thus are rejected. This leads to a large accumulation of wastes in commercial places contributing to pollution of water and soil resources. Hence, the objectives of the study were to prepare the crude protease extract from visceral organ wastes from tilapia and to evaluate properties of this enzyme. That proteases, can be used to digest the waste build-up in the pond and which greatly increase the clarity of the water by hydrolysing the proteinaceous waste materials that build up.

This research was carried out as a contribution to the utilization of fish by-products for producing enzymes and also to the reduction of waste disposal problems.

2. Material and Methods

2.1 Fish viscera

Experimental fish in the present study were Tilapia. They were taken from unpolluted private fish farm located in Ras Al Khaimah, UAE. The initial body length and weight of fish were (11-14.5 cm) and (64-85 g), respectively. All Tilapia (four) were transported in plastic containers with continuous aeration to the lab. Fishes were dissected. Viscera were removed and weighed, then stored in sealed plastic bags at -36 °C until used for enzyme extraction.

2.2 Preparation of Homogenate of Viscera

Digestive tracts (viscera) were partially thawed. 10% Sample Solution (viscera) was prepared by using tissue homogenizer (Tissue homogenizer, REMI RQ-127A, India) mixed with 0.1 M Tris HCL buffer with EDTA (pH 7.4). Pipetted 0.75 mL of viscera solution (10%) made final volume of sample up to 1.0 mL with water. Add 0.25 mL of 20% v/v Trichloroacetic acid and sample kept for incubation in refrigerator about 45 min, after incubation add 0.8 mL of water centrifuged at 2000 rpm for 20 min at room temperature. In the supernatant add 0.3 mL of 2M Tris base, and 0.1 mL of 0.01 M DTNB (5,5 dithio bis 2- dinitrobenzoic acid, CAS No:422592J, VWR UK). Absorbance measured at 412 nm after 10 min by spectrophotometer (UV spectrophotometer, GENWAY 7315, UK).

2.3 Proteolytic Enzyme assay

Total protease activity was measured using a casein substrate by a modification of the Anson Method [14]. A 1 ml of the culture supernatant was mixed with 1 ml 0.05 M phosphate buffer-0.1 M NaOH (pH 7.0 adjusted with phosphoric acid) containing 2% casein, and incubated for 10 min at 37 °C. The reaction was stopped by adding 2 mL 0.4 M Trichloroacetic acid. After 30 min stand at room temperature, the precipitate was removed by centrifugation and the optical density of the assays was measured at 660 nm. A standard curve was generated using solutions of 0–60 µg/mL tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg/mL tyrosine under the experimental conditions used.

2.4 International units (IU)

One protease unit was defined as the amount of enzyme that released 1 µg of tyrosine per mL per minute under the above assay conditions.

2.5 Protein content

The protein content of the crude enzyme extract was determined by the method of Lowry *et al*. [15, 16] using bovine serum albumin as a standard.

2.6 Partial Purification

The crude extract was fractionated by using ammonium sulphate at saturation level of 0-20%, 20-40%, 40-60%, and 0-80%. Protein concentration of each fraction was determined by Lowry method using bovine serum albumin (BSA) solution as a standard [17, 18].

2.7 Enzyme kinetic method (Stability of crude enzyme extract)

2.7.1 pH optima

The pH optimum of the protease enzyme was determined by preparing the substrate in various buffer solutions (0.2 M HCl–KCl buffer of pH 2.0, 0.2 M citrate phosphate buffer of pH 3.0–7.0 and 0.2 M Tris–HCl buffer of pH 7.0–11.0) and applying the enzyme extract to the substrate to assay the enzyme activity.

2.7.2 pH stability

The influence of pH on the stability of the protease was determined by pre-incubating the enzyme in the above mentioned buffer solutions for 30 min at room temperature (25±1 °C) then determined the remaining activity.

2.7.3 Temperature optima

The influence of temperature on the activity of the acidic protease was determined at various temperature intervals (25–60 °C).

2.7.4 Thermo stability

The enzyme solution was incubated at various temperatures (25- 60 °C) for 3 hrs. Samples were removed at intervals of 30 min and residual activities of protease was examined.

2.7.5 Statistical analysis

Experimental error was determined for triplicate assays and expressed as standard deviation (SD).

2.7.6 Application of protease from Tilapia viscera to hydrolyse waste of fish aquarium

The partially purified protease was added in the fish aquarium that containing proteinaceous waste. 24-hour treatment was done with 1mL/L of enzyme in the aquarium.

3. Results

In the present investigation the viscera of Tilapia fish were used for protease enzyme extraction and characterization. The weight of fish and viscera was taken (Table 1).

Table 1: Weight of fish and viscera

S. No.	Sample	Weight of fish(g)	Weight of viscera(g)
1	TF1	85	2.359
2	TF2	65	1.264
3	TF3	71	1.018
4	TF4	69	1.002

The purification steps, protein concentration, specific activity and yield of protease are shown in Table. The specific activity and purification fold were 2800 IU/mg protein and 1.65, respectively, when 0-80% ammonium sulphate used (Table 2).

Table 2: Purification steps of protease from viscera of Tilapia

Purification step	Total protease units (IU)	Protein(mg)	Specific activity(IU/mg)	Purification fold	% yield
Crude enzyme	94500	56	1687.5	1	100
0-80% Ammonium sulphate fractionation	70000	25	2800.0	1.65	74.074

3.1 Enzyme kinetics

3.1.1 pH optima

The partially purified protease had the highest activity at pH 4.0 and it then decreased with increasing of pH (Fig 1.). Over pH 4.0, more than 50% of the relative activity was lost at pH 8.0. There was near complete loss of protease activity at pH values less than 3.0 and more than 9.0.

This result was very close to those reported for protease, the optimum pH for the hydrolysis of haemoglobin by partially purified Polar cod pepsin was found to be 3.2 at 30 °C and 2.5 at 5 °C [19], also crude pepsin had a broad optimum of pH 2.0–3.0 at 28 °C.

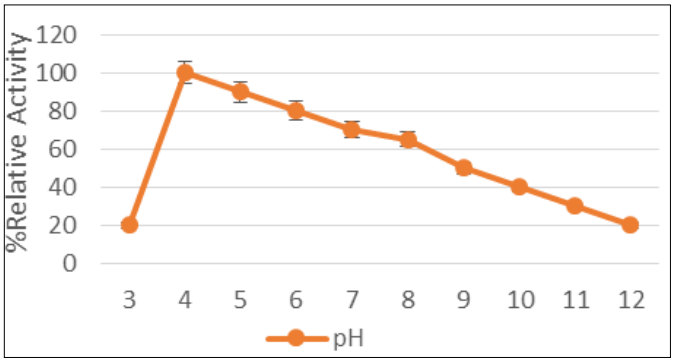

Fig 1: pH Optima of Protease

3.1.2 pH stability

Fig 2. Illustrates pH stability of the protease. The protease retained more than 90% of its original activity in the pH range 4.0-6.0 and then decreased with increasing pH and reached its lowest relative activity at pH 11.0. These data clearly indicate that the protease was most stable in the pH range 4.0–6.0 and least stable within the pH range 7.0–11.0. Generally, these data are similar with that reported by Squires *et al*. [20] for Green Land Cod gastric protease and Ibrahim [6] for bolti acidic proteases.

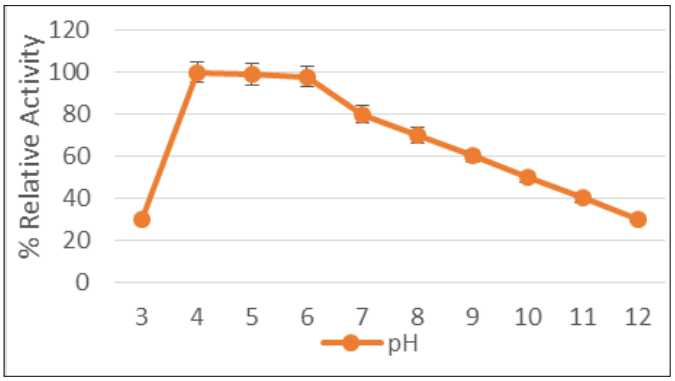

Fig 2: pH Stability of protease

3.1.3 Temperature optima

The temperature stability profile of protease activity revealed that the enzyme is maximally active at moderately high temperatures ranging from 37 °C to 50 °C (Fig 3) with highest activity at 45 °C (Fig 3) incubation temperature for 1h. The relative activity increased with increasing the temperature from 25 °C to 45 °C and then decreased; however very less activity was detected at 60 °C. Generally, these are similar with those reported by Simpson, Simpson, & Haard [21] and Dimes, Garcia-Carreno, & Haard [22].

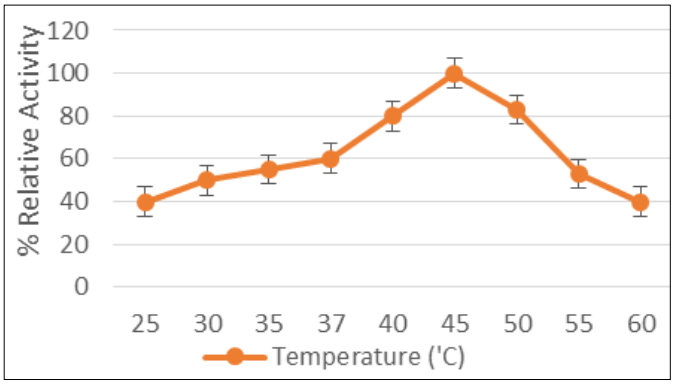

Fig 3: Temperature Optima of Protease

3.1.4 Thermo stability

Thermo stability of the protease is shown in Fig 4. The protease retained more than 50% of its activity after heating at 37 °C and 50 °C for 60 min, it lost 20% after heating at the same temperature for 120 min. A further increase in the reaction temperature caused significant drop in the protease activity. These results are in similar with those reported by Dimes *et al*. [22] and Garcia-Carreno and Haard [23].

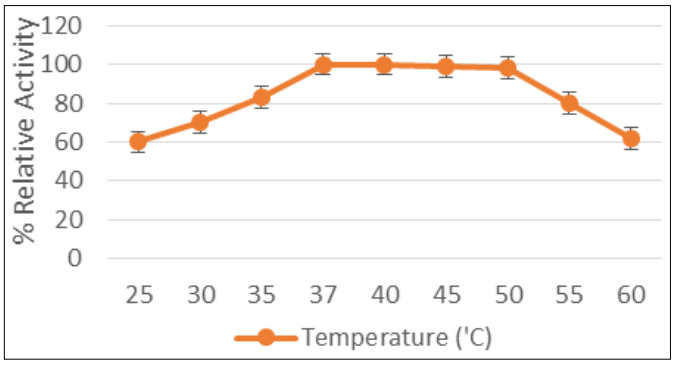

Fig 4: Temperature Stability of Protease

3.2 Application of Protease

The partially purified protease applied in the fish aquarium. The addition of protease to the fish aquarium not only results in the removal of haze and cloudiness but it also hydrolyzed proteinaceous material (Fig 5).

(a) (b)

Fig 5: (a) Control – waste build-up in Aquarium (b) Treated with Protease to hydrolyse waste build-up in aquarium

4. Conclusion

The viscera of Tilapia fish contained considerable amounts of proteases, which can be used to digest the waste build-up in the pond and which greatly increase the clarity of the water by hydrolysing the proteinaceous waste materials. This protease can be used in different food processing aids. The utilization of fish by-products for producing protease enzyme also led to

the reduction of waste disposal problems. The obtained enzyme had a pH optimum of 4.0, temperature optimum 45 °C. The obtained crude protease showed maximum activity at 0-80% ammonium sulphate fractionation.

5. Conflicts of interest

The authors declare no conflict of interest pertaining to the research report in this manuscript.

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