
**ISSN Print:** 2394-7500
**ISSN Online:** 2394-5869
**Impact Factor:** 5.2
IJAR 2019; 5(12): 36-40
www.allresearchjournal.com
Received: 22-10-2019
Accepted: 24-11-2019

**Saif Al Ghais**
Environment Sustainability Department Environment Protection & Development Authority (EPDA), UAE

**Vibha Bhardwaj**
Environment Sustainability Department Environment Protection & Development Authority (EPDA), UAE

**Pramod Kumbhar**
Environment Sustainability Department Environment Protection & Development Authority (EPDA), UAE

**Correspondence Author:**
**Vibha Bhardwaj**
Environment Sustainability Department Environment Protection & Development Authority (EPDA), UAE

# A new milk coagulating enzyme from *Thunnus tonggol* (Bleeker, 1851) tuna fish waste as rennet substitute

**Saif Al Ghais, Vibha Bhardwaj and Pramod Kumbhar**

**Abstract**
The growing demand for natural coagulants led to an increased necessity for rennet substitutes. Many attempts have been made to find new proteases. A novel protease with milk-clotting activity was isolated and purified from tuna fish stomach. The aim of this study was to investigate the protease enzyme as a novel milk-clotting in the development of cheese. The milk coagulating time for tuna protease was studied at pH (3.0–12.0) with the optimum at 4.0 and showed its maximum activity at 50 °C when casein was used as a substrate. The enzyme showed the highest activity and purification when precipitated at 20-40% ammonium sulphate and dialyzed effectively improved specific activity of enzyme. The cheese manufactured by using the purified tuna protease showed similar textural properties as compared to cheese produced using commercial rennet. This research was carried out for the utilization of tuna fish by-products for producing enzymes and also to the reduction of waste disposal problems. Considering the special characteristics, including high milk-clotting activity, considerable stability over wide ranges of pH and temperature, the results revealed that the tuna protease might be the promising candidate for the dairy industry as well as other food and biotechnological industries.

**Keywords:** Protease, tuna fish, ammonium sulphate precipitation, thermo stability, pH optima

## 1. Introduction
Milk clotting is the basic step in the manufacture of all types of cheeses and based on that all cheese varieties (>2000 types) are classified into three superfamilies those include rennet coagulated, acid coagulated, and a combination of heat and acid coagulated cheeses (Badgujar and Mahajan 2014 [5] Fox *et al*. 2015) [9]. However, in the last decades there has been increased demand for cheese production and consumption due to the population explosion (Elsamani *et al*. 2014 [8] Tajalsir *et al*. 2014) [26]. This combined with the elevated price of calf rennet and reduced quantity of natural calf rennet (Mohamed Ahmed *et al*. 2010). All the above reasons have demanded the search for a new enzyme with a high ratio of milk-clotting/ proteolytic activity and low preparation cost to be used as a rennet substitute and/or additive (Tajalsir *et al*. 2014) [26]. Consequently, much research interest has been focused on the discovering milk-clotting Enzymes from other sources, and, as a result, several enzyme preparations of animal, microbial, and plant origin have been discovered (Jacob *et al*. 2011) [12]. However, most of the enzyme preparations from the above sources were found unsuitable because they produced cheese with low yield and bitter taste due to the low ratio of milk-clotting/ proteolytic activities (Anusha *et al*. 2014 [2] Shah *et al*. 2014) [25]. Therefore, the search for a rennet substitute, having a high ratio of milk-clotting/proteolytic activity is extremely needed to be used for the production of cheese with better quality.
The processing of fishery products generates significant amounts of biological by-products including viscera, skin, fins, bone, heads, blood, among others. Commonly, these by-products are converted into meal and oil with low commercial value and, occasionally, are disposed of into the environment, where these become pollution sources (Arvanitoyannis and Kassaveti, 2008) [3] and foster the proliferation of pests. However, such biological wastes and by-products contain valuable protein and lipid fractions with high nutritional quality as well as are rich in bioactive chemicals such as vitamins, peptides, collagen, fatty acids,

hydrolytic enzymes (mainly proteases), and pigments, among other compounds, all of which are characterized by a high added value (Olsen *et al*., 2014) [20]. The main and most studied digestive proteolytic enzymes from fish viscera are the aspartic protease pepsin secreted by stomach (if it is present). To date, there are some reports about the purification and characterization of gastric proteases, mainly pepsins, from marine fish (Bkhairia *et al*., 2016 [6] Bougatef *et al*., 2008 [7] Klomklao *et al*., 2007 [13] Nalinanon *et al*., 2010) [18]. On the other hand, proteases from marine organisms might be excellent alternatives to replace conventional milk-coagulating enzymes such as chymosin. This is of great importance, taking in account the current demand for milk-coagulating enzymes (Jacob *et al*., 2011) [12]. Therefore, the aim of the present study was the isolation, biochemical characterization, purification and application of proteases isolated from the stomach of the tuna fish and the evaluation of its stability and its potential use as a new source of milk clotting proteases.

## 2. Material and Methods

### 2.1 Fish Stomach

Experimental fish in the present study were Tuna fish. The viscera (stomach) were taken from fish market located in Emirates of Ras Al Khaimah, UAE. All Tuna fish stomach (four) were transported in ice bag to the laboratory. The initial weight of tuna fish stomach was (55g -95g). Stomach were stored in sealed plastic bags at -36 °C until used for enzyme extraction.

### 2.2 Extraction of Enzyme crude extracts from tuna fish stomach

Enzyme Crude extract (ECE) were prepared from the whole stomach. The stomach received frozen in dry ice, were partially thawed, split, cleaned, and briefly rinsed in tap water. The inner mucosa linings were peeled away from the outer muscular layer, chopped in small pieces using a sharp knife and homogenized using a homogenizer (Tissue homogenizer, REMI RQ-127A, India) with cold distilled water (DW), using a mass/water ratio of 1:3 (w/w). The homogenate was centrifuged at 9000xg for 30 minutes at 4 °C. The top layer of fat was removed and supernatant was recovered and stored at -20 °C. This gastric extract was used for enzyme studies.

### 2.3 Purification of stomach proteolytic enzyme

The supernatant (ECE) was precipitated with ammonium sulfate using a method described by Saif Al Ghais *et al* (2019) [21, 22, 23]. Chilled ECE was placed in a beaker immersed in ice and solid ammonium sulfate $(NH_4)_2SO4$ was added to reach 20% saturation. After 30 min, the suspension was centrifuged at 8000 x g at 4 °C for 30 min. The supernatant was recovered and saturated to 40% with solid ammonium sulfate, mixed 30 min in ice, and centrifuged at 8000 x g at 4 °C for 30 min. The precipitate was recovered, resuspended with 10mM Gly-HCl buffer at pH 3.0, and dialyzed for 24 h against two changes of 1 L of resuspension buffer at 4 °C and using a dialysis tubing. Dialyzed protein fraction (semi-purified enzyme) was stored at – 20 °C until use for enzyme analysis Vibha Bhardwaj *et al* (2014) [28].

### 2.4 Proteolytic Enzyme assay

Total protease activity was measured using a casein substrate by a modification of the Anson Method (1938). 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 280 nm ((UV spectrophotometer, GENWAY 7315, UK). 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.

### 2.5 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.

### 2.6 Protein Quantification

The protein content of the crude enzyme extract was determined by the method of Lowry *et al* (1951) [14] using bovine serum albumin as a standard (Saif Al Ghais *et al*, 2018) [24].

## 3. Enzyme kinetic method (Stability of crude enzyme extract)

### 3.1 pH optima

The pH optimum of the protease enzyme was determined by preparing the substrate in various buffer solutions (0.2 M citrate phosphate buffer of pH 3.0–7.0 and 0.2 M Tris–HCl buffer of pH 8.0–12.0) and applying the enzyme extract to the substrate to assay the enzyme activity (Saif Al Ghais *et al*, 2019) [21, 22, 23].

### 3.2 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.

### 3.3 Temperature optima

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

### 3.4 Thermostability

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.

### 3.5 Milk coagulation activity of semi-purified extract enzyme

Milk-coagulating activity (MCA) was determined according to modified method described by Mazorra-Manzano *et al*. (2013) [15]. Briefly, 10 mL of pasteurized low-fat milk (< 1% total fat) containing 0.022% of $CaCl_2$ was mixed with 0.5mL of semi-purified proteases and incubated at different temperatures (25–60 °C). Milk-coagulating time (t) was registered as the time elapsed from the addition of enzymatic solution until milk clot formation was observed. A control treatment was assayed where 10mM Gly-HCl

buffer at pH 3.0 was used instead of enzymatic source. One milk-coagulating unit was defined as the amount of enzyme source (mL) required for clotting 100 mL of milk in 40 min (2400 s), under the assay conditions. The MCA was calculated by using the following equation and expressed in units per mL of coagulant (SU/mL). MCA (U/mL) = (2400/t) x (S/E).
Where t = clotting time (s); S = volume of used milk (mL); and, E = volume of enzymatic solution.

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

## 4. Results
In the present investigation the stomach of Tuna fish (TF) was used for protease enzyme extraction and characterization. The weight of fish stomach was taken (Table 1).

**Table 1:** Weight of fish stomach

| S. No. | Sample | Weight of fish stomach (g) |
|---|---|---|
| 1 | TF1 | 55 |
| 2 | TF2 | 69.3 |
| 3 | TF3 | 95.0 |
| 4 | TF4 | 69 |

The purification steps, protein concentration, specific activity and yield of protease are shown in Table 2. The specific activity and purification fold were 5.77 IU/mg protein and 4.80, respectively, when 20-40% ammonium sulphate used (Table 2). After dialysis, the specific activity was 30.3 IU/mg and purification fold was 25.25

**Table 2:** Purification steps of protease from stomach of Tuna

| Purification step | Total protease units (IU) | Protein (mg) | Specific activity (IU/mg) | Purification fold | % yield |
|---|---|---|---|---|---|
| Crude enzyme | 20.8 | 16.58 | 1.2 | 1 | 100 |
| 20-40% Ammonium sulphate fractionation | 10.91 | 1.89 | 5.77 | 4.80 | 52.45 |
| Dialysis | 16.362 | 0.54 | 30.3 | 25.25 | 78.66 |

### 4.1 Enzyme kinetics
#### 4.1.1 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 30% 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 semi purified protease were 2.0 and 3.0 at 65 °C (Idalia Osuna-Ruiz *et al* 2019) [11]. Also, our result is similar as reported by Assia Nasr *et al* 2015, that at pH 5.0 had highest milk clotting acticity.

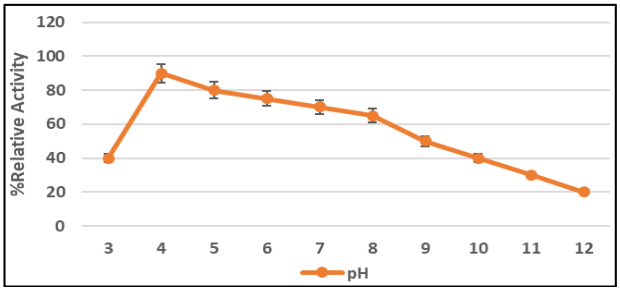


**Fig 1:** pH Optima of Protease

#### 4.1.2 pH stability
Fig 2. illustrates pH stability of the protease. The protease retained more than 90% of its original activity in the pH range 4.0-5.0 and then decreased with increasing pH and reached its lowest relative activity at pH 12.0. These data clearly indicate that the protease was most stable in the pH range 4.0–5.0 and least stable within the pH range 9.0–11.0. Generally, these data are similar with that reported by Gildberg (1988) [10] that the optimal pH lies between 3.0 and 4.0 for pepsin I, and between 2.0 and 3.0 for pepsin II. Similar to our result, the acid protease of B. panamensis showed the highest activity at pH of 2 and 3 (Idalia Osuna-Ruiz *et al* 2019) [11].

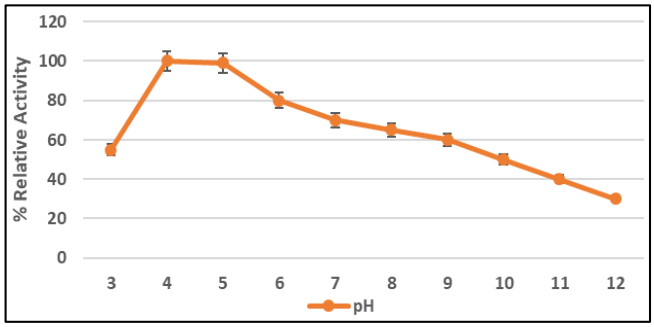


**Fig 2:** pH Stability of protease

#### 4.1.3 Temperature optima
The temperature stability profile of protease activity revealed that the enzyme is maximally active at moderately high temperatures ranging from 40 °C to 50 °C (Fig 3) with highest activity at 50 °C (Fig 3) incubation temperature for 1h. The relative activity increased with increasing the temperature from 25 °C to 50 °C and then decreased; however very less activity was detected at 60 °C. Generally, these are similar with those reported by Miura *et al*. (2015) [16] Nalinanon *et al*. (2010) [18] Noda and Murakami (1981) [19] that the optimum temperature for the pepsin studied and peak temperatures recorded (50–55 °C) for different fish species.

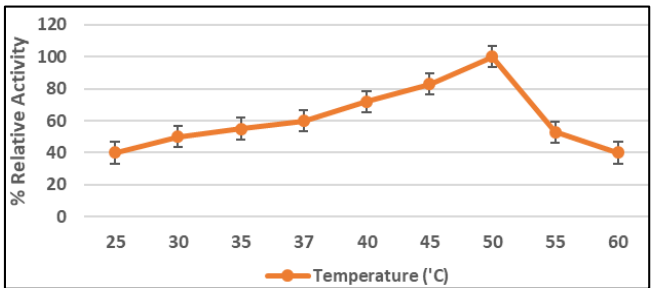


**Fig 3:** Temperature Optima of Protease

#### 4.1.4 Thermostability
Thermostability of the protease is shown in Fig 4. The protease retained more than 50% of its activity after heating at 40 °C to 50 °C for 120 min. A further increase in the reaction temperature caused significant drop in the protease activity. These results are in similar with our result that, fish species that produce pepsins with a similar thermostability profile are C. pectoralis (Klomklao *et al*., 2007) [13] and M. mustelus (Bougatef *et al*., 2008) [7] which retain more than 70% of baseline activity at an incubation temperature of 50 °C.

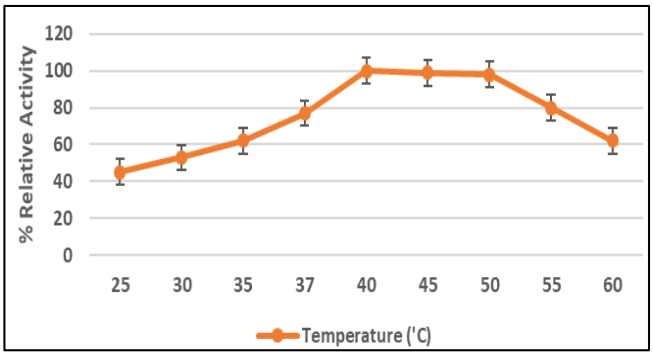


**Fig 4:** Temperature Stability of Protease

### 4.2 Milk coagulating activity

When tuna stomach proteases were applied to the substrate, clotting occurred. The effect of temperature on milk coagulating activity (MCA) is shown in Fig. 5. The peak MCA was recorded at 35 °C. MCA increased with temperature within 25–35 °C, but temperatures above 40 °C decreased the MCA, probably due to enzyme denaturation (Fig. 5). Moreover, coagulating was not observed when acid solution was used instead of enzyme (Control treatment). In line with our results, Tavares *et al*. (1997) [27] reported that the peak MCA of gastric proteases isolated from the stomach of bigeye tuna (Thunnus obesus) was observed at temperatures around 30–38 °C.

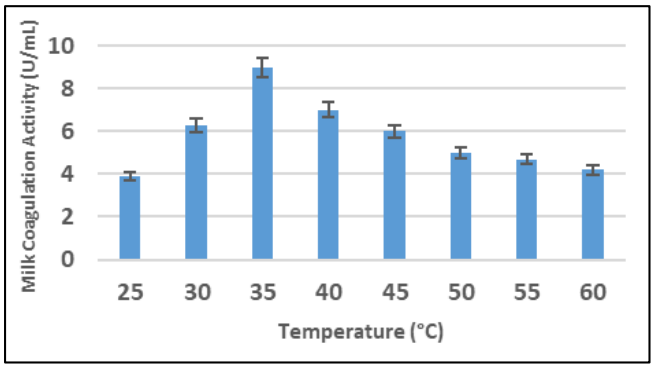


**Fig 5:** Milk coagulating activity

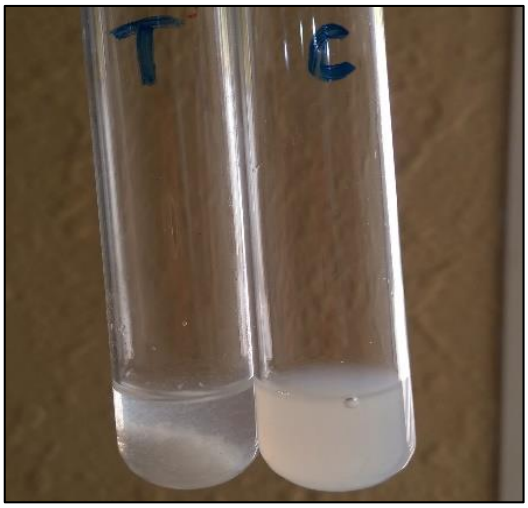


**Fig 6:** Control (C) Without Enzyme Treatment; Treated (T) Coagulation of milk by Tuna Stomach Protease

## 5. Conclusion

In the present study, a novel milk coagulating protease from stomach of tuna fish was extracted and purified using two step purification procedure and characterized and used as a cheap milk-clotting preparation for cheese making. High milk-clotting activity, the accessibility of raw materials and low susceptibility to autolysis can pave the way for its use in the dairy industry both for milk clotting as a replacement or in combination with calf rennet, and for the enhancement of cheese ripening procedure in order to save time and storing costs for cheese maturation. Besides, due to its high stability at a wide range of pH, temperature, this protease may turn out to be an effective choice in pharmaceutical and biotechnological industries as well as in detergent formulation.

## 6. Abbreviations

ECE: Enzyme Crude Extract; DW: Distilled water; mM: Millimolar; h: Hour; HCl: Hydrochloric acid; MCA: Milk Coagulating activity; Gly: Glycine; TF1: Tuna fish 1; TF: Tuna Fish.

## 7. Conflicts of interest

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

## 8. Availability of data and materials

The relevant data and materials are available in the present study.

## 9. Competing interests

The authors declare that they have no competing interests. All procedures followed were in accordance with the ethical standards (institutional and national). All institutional and national guidelines for the care and use of laboratory animals were followed.

## 10. Acknowledgement

This work is supported by EPDA, Ras Al Khaimah, UAE. Authors would like to thank all individuals who provided their efforts for this research**.**

## 11. Funding

Not applicable

## 12. Ethics approval and consent to participate

Not applicable.

## 13. Consent for publication

Not applicable.

## 14. References

1. Anson ML. The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. J Gen. Physiol. 1938; 22:79-89.
2. Anusha R, Singh MK, Bindhu OS. Characterization of potential milk coagulants from *Calotropis gigantean* plant parts and their hydrolytic pattern of bovine casein. Eur. Food Res. Technol. 2014; 238:997-1006.
3. Arvanitoyannis IS, Kassaveti A. Fish industry waste: treatments, environmental impacts, current and potential uses. Int. J Food Sci. Technol. 2008; 43:726-745.

4. Assia IAM Nasr, Isam A, Mohamed Ahmed, Omer IA Hamid. Characterization of partially purified milk-clotting enzyme from sunflower (*Helianthus annuus*) seeds. Food Science & Nutrition. 2016; 4(5):733-741.
5. Badgujar SB, Mahajan RT. Nivulian-II a new milk clotting cysteine protease of *Euphorbia nivulia* latex. Inter. J. Biol. Macromol. 2014; 70:391-398.
6. Bkhairia I, Mhamdi S, Jridi M, Nasri M. New acidic proteases from *Liza aurata* viscera: Characterization and application in gelatin production. Int. J Biol. Macromol. 2016; 92:533-542.
7. Bougatef A, Balti B, Zaied SB, Soussi N, Nasri M. Pepsinogen and pepsin from the stomach of smooth hound (*Mustelus mustelus*): Purification, characterization and amino acid terminal sequences. Food Chem. 2008; 107:777-784.
8. Elsamani MO, Habbani SS, Babiker EE, Mohamed Ahmed IA. Biochemical, microbial and sensory evaluation of white soft cheese made from cow and lupin milk. LWT –Food Sci. Technol. 2014; 59:553-559.
9. Fox PF, Uniacke-Lowe T, McSweeney PLH, O' Mahony JA. Chemistry and Biochemistry of Cheese, in Fox, Uniacke- Lowe, McSweeney, and O'Mahony, eds. Dairy chemistry and biochemistry, 2nd edn. Springer International Publishing. Switzerland, 2015, 499-544
10. Gildberg A. Aspartic proteinases in fishes and aquatic invertebrates. Comp. Biochem. Physiol. B. 1988; 91:425-435.
11. Idalia Osuna-Ruiz, Marı´a Fernanda Espinoza-Marroquin, Jesu´ s Aaro´n Salazar-Leyva, Emyr Pen˜a, Carlos Alfonso A ´ lvarez-Gonza´lez, Isaura Ban˜uelos-Vargas *et al*. Biochemical characterization of a semi-purified aspartic protease from sea catfish Bagre panamensis with milk-clotting activity. Food Sci Biotechnol, 2019. https://doi.org/10.1007/s10068-019-00614-8.
12. Jacob M, Jaros D, Rohm H. Recent advances in milk clotting enzymes. Inter. J Dairy Technol. 2011; 64:14-33.
13. Klomklao S, Kishimura H, Yabe M, Benjakul S. Purification and characterization of two pepsins from the stomach of pectoral rattail (*Coryphaenoides pectoralis*). Comp. Biochem. Physiol. B. 2007; 147:682-689.
14. Lowry OH, Rosenbrougj NJ, Farr AL, Randall RL. Protein measurement with the Folin Phenol Reagent. Journal of Biological Chemistry. 1951; 193(1):265-275.
15. Mazorra-Manzano MA, Moreno-Herna´ndez JM, Ramı´rez Sua´rez JC, Torres-Llanez MJ, Gonza´lez-Co´rdova AF, Vallejo-Co´rdoba B *et al*. Sour orange *Citrus aurantium* L. flowers: a new vegetable source of milk-clotting proteases. LWT Food Sci. Technol. 2013; 54:325-330.
16. Miura Y, Kageyama T, Moriyama A. Pepsinogens and pepsins from largemouth bass, *Micropterus salmoides*: Purifications and characterization with special reference to high proteolytic activities of bass enzymes. Comp. Biochem. Physiol. B. 2015; 183:42-48.
17. Mohamed Ahmed, Morishima IAI, Babiker EE, Mori N. Characterization of partially purified milk-clotting enzyme from *Solanum dubium* fresen seeds. Food Chem. 2009a; 116:395-400.
18. Nalinanon S, Benjakul S, Kishimura H. Biochemical properties of pepsinogen and pepsin from the stomach of albacore tuna (*Thunnus alalunga*). Food Chem. 2010; 121:49-55.
19. Noda M, Murakami K. Studies on proteinases from the digestive organs of sardine. II. Purification and characterization of two acid proteinases from the stomach. Biochim. Biophys. Acta. 1981; 658:27-34.
20. Olsen RL, Toppe J, Karunasagar I. Challenges and realistic opportunities in the use of by-products from processing of fish and shellfish. Trends Food Sci. Technol. 2014; 36:144-151.
21. Saif Al Ghais, Vibha Bhardwaj, Omar Al Shehhi. Phytase production from fish viscera and used as probable aqua feed ingredient. International Journal of Fisheries and Aquatic Research. 2019; 4(2):55-58.
22. Saif Al Ghais, Vibha Bhardwaj. Utilization of fish viscera for protease production and used for digestion of waste in the pond. International Journal of Fisheries and Aquatic Studies. 2019; 7(3):112-115.
23. Saif Al Ghais, Vibha Bhardwaj, Pramod Kumbhar, Omar Al Shehhi. Effect of copper nanoparticles and organometallic compounds (dibutyltin) on tilapia fish, The Journal of Basic and Applied Zoology, Springer Nature, 2019, 80:32, https://doi.org/10.1186/s41936-019-0101-7.
24. Saif Al Ghais, Vibha Bhardwaj. Nannochloropsis Protein as Potential Fish Feed. International Journal of Science and Research (IJSR) 2018; 7(12)278-282.
25. Shah MA, Mir SA, Paray MA. Plant proteases as milk clotting enzymes in cheese making: a review. Dairy Sci. Technol. 2014; 94:5-16.
26. Tajalsir AE, Ebraheem ASA, Abdallah AM, Khider FJ, Elsamani MO, Mohamed Ahmed IA *et al*. Partial purification of milk clotting enzyme from the seeds of *Moringa oleifera*. J Microbiol. Biotech. Food Sci, 2014; 4:58-62.
27. Tavares JFP, Baptista JAB, Marcone MF. Milk-coagulating enzymes of tuna fish waste as a rennet substitute. Int. J Food Sci. Nutr. 1997; 48:169-176.
28. Vibha Bhardwaj and Neelam Garg Production, Purification of Pectinase from Bacillus sp. MBRL 576 Isolate and its Application in Extraction of Juice. International Journal of Science and Research (IJSR) 2014; 3(6):648-652.