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ISOLATION, PURIFICATION AND CHARACTERISATION OF PROTEASES FROM FISH WASTE

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ABSTRACT

Proteases are found to have wide application in biotechnology, research, food and detergent industries. The aim of the present investigation was to isolate proteases from the intestines of three fishes namely *Scomberomorus commerson, Rastrelliger kanagurta and Eleutheronema tetradactylum.* The enzyme was extracted from fish wastes using Tris-HCl buffer, purified by ammonium sulphate precipitation and dialysis method. Purified enzymes were characterised by determining its specific activity, optimum temperature, optimum pH, effect of inhibitor and metal salts on its activity. Molecular weight of the enzyme was determined using SDS-PAGE analysis. The optimum temperature and pH of the enzyme was found to be 55^0C and 10. The results depict that the proteases isolated from the intestines of the three fishes were alkaline proteases and metal salts had enhanced the activity of the enzyme. The present investigation suggests that proteases isolated from fish wastes exhibited high enzymatic activity and consequently may alleviate the demand for proteases commercially.

INTRODUCTION

Proteases (EC 3.4.21-24 and 99) are enzymes that catalyse the hydrolysis of proteins [1]. Protease forms a large group of enzymes belonging to the class of hydrolases, ubiquitous in nature and performs a major role with respect to their applications in both physiological and commercial fields. These enzymes are widely distributed nearly in all plants, animals and microorganisms. In higher organisms about 2% of the genes codes are formed by these enzymes. Traditionally the proteinases have been regarded as degradative enzymes which are capable of cleaving protein foods. They liberate small peptides and amino acids needed by the body. Also they participate in the turnover of cellular protein. Indeed, this is one of the best characteristic of the proteinases, such as the mammalian digestive enzymes trypsin, chymotrypsin, and pepsin and the lysosome enzymes cathepsin B and cathepsin D. Proteolytic enzymes have the ability to carry out selective modification of proteins by limited cleavage such as activation of zymogenic forms of enzymes, blood clotting and lysis of fibrin clots, and processing and transport of secretory proteins across the membranes. These properties add considerable interest to an already important group of enzymes. Additionally proteolytic enzymes have been used for a long time in various forms of therapy. Their use in medicine is gaining more and more attention because several clinical studies are indicating their applications in oncology, inflammatory conditions, blood rheology control and immune regulation. These are also used in crucial biological processes such as regulation of metabolism, enzyme modification, photogenecity, complement system, apoptosis pathways, invertebrate prophenoloxidase activating cascade etc. Furthermore, a study of proteolytic enzymes is valued because of their importance as reagents in laboratory, clinical, and industrial processes. Proteinases from both microbial and non-microbial sources, are extensively used in the food industry (baking, brewing, cheese manufacturing, meat tenderizing), in the tanning industry, and in the manufacture of biological detergents. Thus, there is an increasing interest in the proteinases and peptidases of both eukaryotic and prokaryotic microorganisms [2]. Proteases execute a large variety of pharmaceutical functions; particularly their involvement in the life cycle of disease causing organisms has led them to become a potential target for developing therapeutic agents against fatal diseases such as cancer and AIDS. The vast diversity of proteases, in contrast to the specificity of their action, has attracted worldwide attention in an attempt to exploit their physiological and biotechnological applications. The present estimated value of the worldwide sales of industrial enzymes is $1billion. Hydrolytic enzymes contribute 75 % of total enzyme sale including protease, carbohydrase and lipase. Proteases represent one of the three largest groups of industrial enzymes and account for about 59% of the total worldwide sales of enzymes. Proteases are widely distributed in each part of biological source. Acid proteases in the stomach and alkaline proteases in the intestine are the most important proteases of fish viscera, accounting for about 5 % of the total body mass [3].

Proteases from tropical fish have thermal stability and long shelf life and high activity over a wide range of pH levels [4], hence for the present investigation, intestines of three fishes namely *Scomberomorus commerson, Rastrelliger kanagurta and Eleutheronema tetradactylum* were taken for the isolation of proteases. The enzyme thus isolated was purified using dialysis and precipitation techniques. Characterization of the enzyme was carried out by determining its optimum temperature, optimum pH, effect of inhibitors and metal salts on its proteolytic activity. The molecular weight of the protease was determined using SDS-PAGE.

MATERIALS & METHODS

Materials

The intestines of *Scomberomorus commerson (Indian Mackerel), Rastrelliger kanagurta (Salmon), Eleutheronema tetradactylum (Seer fish)* were collected freshly from the local market. They were kept at -20^{0}C soon after they were collected. The samples were then used for extraction.

Enzyme Extraction

The intestines of the three fishes were ground seperately in the homogenizer (40 mg (w/v) of tissue/ ml in 0.9% (w/v) NaCl). The resulting preparation was centrifuged at 10,000 g for 10 minutes at 10^{0}C to remove cell debris and nuclei. The supernatant (crude extract) was frozen at -20^{0}C, and used for purification steps [5]. After centrifugation the supernatant was collected in a separate beaker and the beakers were marked as S (for *Scomberomorus commerson*), R (for *Rastrelliger kanagurta*) and E (for *Eleutheronema tetradactylum).*

Enzyme Partial Purification

For ammonium sulphate precipitation, the supernatant obtained from enzyme extraction was added to ammonium sulphate (60%) to precipitate the total protein at 4ºC. After 1 h, the precipitate was recovered by centrifugation (30,000 rpm for 45 min), dissolved in Tris buffer (pH 8.5), and dialysed twice (for 16 h the first time and 4 h the second time) against Tris buffer (pH 8.5) [6]. Enzyme subjected to ammonium sulphate precipitation and dialysis was further used for enzymatic assay.

Enzymatic Assay

Protease activity was determined by measuring the release of trichloroacetic-acid soluble peptides from 1 % casein in Tris buffer (pH 10) at 55ºC for 30 min. One unit enzyme activity was defined as the amount of enzyme that releases 1 µg of tyrosine per ml per min under the above assay conditions. Specific enzyme activity was expressed as units/mg protein. Protein was measured using bovine serum albumin as the standard [7].

Molecular Weight Determination

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 10 % acrylamide gel [8]. Proteins were stained with Coomasie brilliant blue R-250 (Sigma). The molecular weight of the protease was determined by comparison of the migration distances of standard marker proteins consisting of serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; lysozyme, 14.4 kDa.

Characterisation of the Enzyme

Optimal pH of Enzyme Activity

Optimum pH of protease enzymes isolated from the intestines of *Scomberomorus commerson, Rastrelliger kanagurta and Eleutheronema tetradactylum* were determined by using 1 % casein as a substrate dissolved in different pH of sodium acetate buffer (1.0-5.5), sodium phosphate buffer (pH 5.6-7.0)and Tris-HCl buffer(pH 7.0-12)at 55 ºC [9].

Optimal Temperature for Enzyme Activity

Optimum temperature of protease enzymes isolated from the intestines of *Scomberomorus commerson, Rastrelliger kanagurta and Eleutheronema tetradactylum* were determined by using 1 % casein as a substrate dissolved in Tris-HCl buffer (pH 10) and incubated at varied temperatures ranging from 10-100 ºC [9].

Effect of Inhibitors on Enzyme Activity

The substrate inhibitor [ethylenediaminetetraacetic acid (EDTA) and trypsin inhibitor was added to the protease enzymes isolated from the intestines of *Scomberomorus commerson, Rastrelliger kanagurta and Eleutheronema tetradactylum* at a concentration of 1 mM before analysing the enzyme activity. The enzyme activity was then determined by using 1% casein as a substrate dissolved in Tris-HCl buffer (pH 10) at 55ºC [9].

Effect of Metal Salts on Enzyme Activity

The metal salt ($CaCl^2$, $MgCl^2$, $MnCl^2$) at a concentration of 1 mM was was added to the purified protease isolated from the intestines of *Scomberomorus commerson, Rastrelliger kanagurta and Eleutheronema tetradactylum* before analysing the enzyme activity. The enzyme activity was then determined by using 1% casein as a substrate dissolved in Tris-HCl buffer (pH 10) at 55ºC[9].

RESULTS

The intestines of *Scomberomorus commerson***,** *Rastrelliger kanagurta***,** *Eleutheronema tetradactylum* were collected freshly from the local market. They were kept at -20^{0}C

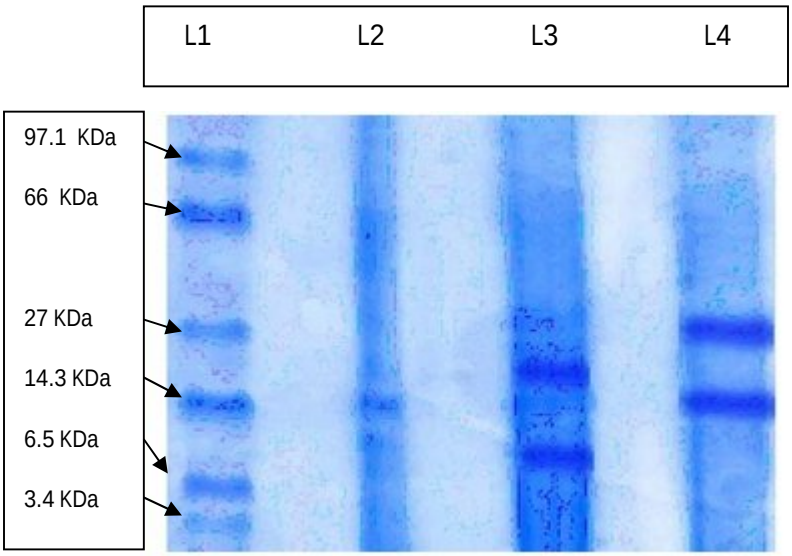
soon after they were collected and were taken separately, homogenised and centrifuged. After centrifugation the supernatant was collected in a separate beaker and the beakers were marked as S (for *Scomberomorus commerson*), R (for *Rastrelliger kanagurta*) and E (for *Eleutheronema tetradactylum).* The enzymes extracted were then purified by ammonium sulphate precipitation. The precipitated enzyme was further purified by dialysis. The dialysed enzyme was used for the assay of its protease activity.

Table 1: Enzyme activity of proteases isolated from the intestines of *Scomberomorus commerson, Rastrelliger kanagurta, Eleutheronema tetradactylum*

Proteases	**Total Activity (U)**	**Total Protein (mg)**	**Specific Activity (U/mg Protein)**
Scomberomorus commerson	10.5	530	0.0198
Rastrelliger kanagurta	6.8	340	0.02
Eleutheronema tetradactylum	18.6	940	0.0197

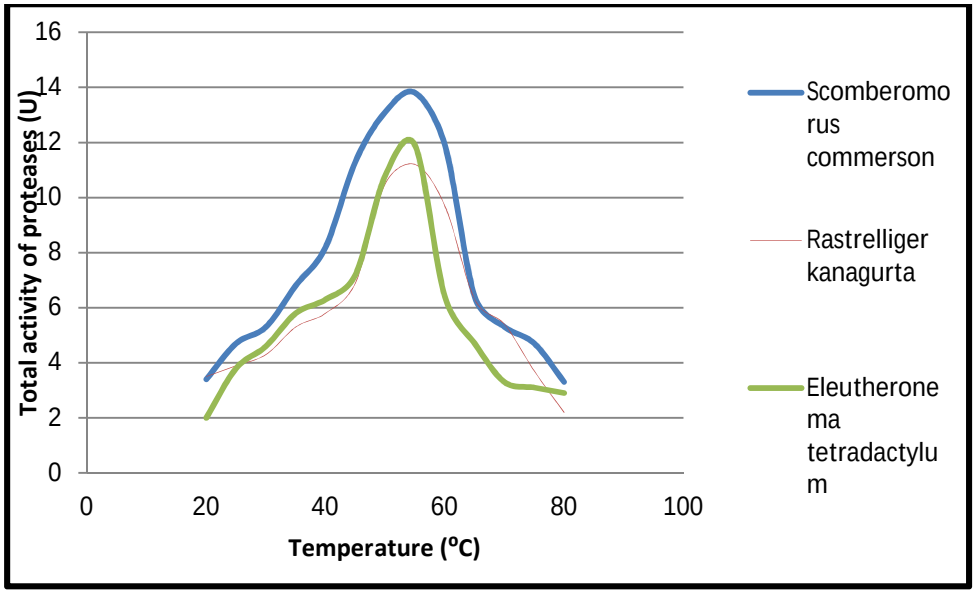
Proteases isolated from the intestine of *Rastrelliger kanagurta* showed a higher specific activity than proteases isolated from the intestines of *Scomberomorus commerson* and *Eleutheronema tetradactylum* (Table 1). It is also evident from table 1 that the specific activities of proteases isolated from the intestines of *Scomberomorus commerson* and *Eleutheronema tetradactylum* were found to be similar. Molecular weight of the protease enzyme isolated from the intestines of *Scomberomorus commerson, Rastrelliger kanagurta, Eleutheronema tetradactylum* was determined by SDS-PAGE (Fig.1). Proteases isolated from *Scomberomorus commerson* depicted a single band of molecular weight around 15 KDa, whereas proteases isolated from *Rastrelliger kanagurta* and *Eleutheronema tetradactylum* showed two bands. Molecular weight of one band from *Rastrelliger kanagurta* was found to be 20 KDa and that of the other band was around 10 KDa, whereas in *Eleutheronema tetradactylum* one band had molecular weight around 28 KDa and that of the other band was found to be around 15 KDa, thus proteases from *Rastrelliger kanagurta* and *Eleutheronema tetradactylum* were a dimer comprising of two subunits.

Figure1. SDS-PAGE of proteases isolated from the intestines of *Scomberomorus commerson, Rastrelliger kanagurta, Eleutheronema tetradactylum* Lane 1: molecular weight of standard protein markers : Phosphorylase B 97.1 KDa; Bovine serum albumin 66 KDa; Triose phosphate isomerise 27 KDa; Lysozyme 14.3 kDa, Aprotinin 6.5 KDa; Insulin A 3.4 KDa Lane 2: *Scomberomorus commerson* protease, Lane 3: *Rastrelliger kanagurta* protease, Lane 4: *Eleutheronema tetradactylum* protease



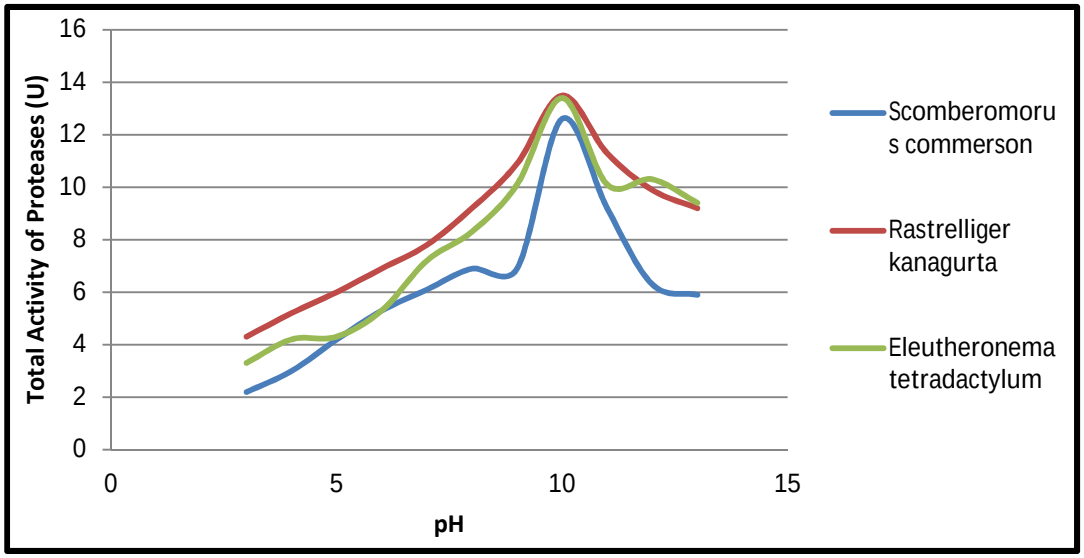
The optimum temperature for protease activity was assayed at different temperature from 10-100 ºC using casein as a substrate dissolved in Tris-HCl buffer (pH 10). Proteases isolated from the intestines of the three fishes showed maximum activity at the same temperature. The optimum temperature of proteases isolated from *Scomberomorus commerson, Eleutheronema tetradactylum* and *Rastrelliger kanagurta* was found to be 55°C (Fig. 2).

Figure2. Effect of temperature on proteases isolated from the intestines of *Scomberomorus commerson, Rastrelliger kanagurta, Eleutheronema tetradactylum*



The optimum pH for protease activity was assayed at different pH from 1-10 using casein as a substrate at 55 ºC. The optimum pH of protease activity was found to be 10 for the proteases isolated from the intestine of the three fishes (Fig. 3).

Figure3. E Effect of pH on proteases isolated from the intestines of *Scomberomorus commerson, Rastrelliger kanagurta, Eleutheronema tetradactylum*



The effect of two inhibitors on protease activity isolated from the intestines of *Scomberomorus commerson, Rastrelliger kanagurta* and *Eleutheronema tetradactylum* is shown in Table 2. In all the three samples (proteases isolated from the intestines of *Scomberomorus commerson, Rastrelliger kanagurta* and *Eleutheronema tetradactylum*) EDTA inhibited the enzyme more. trypsin inhibitor was found to be a weak inhibitor when compared to EDTA.

Table 2. Effect of inhibitors on proteases isolated from the intestines of *Scomberomorus commerson, Rastrelliger kanagurta* and *Eleutheronema tetradactylum*

Inhibitors	**Activity of Proteases (U)**		
	Scomberomorus commerson	***Rastrelliger kanagurta***	***Eleutheronema tetradactylum***
EDTA	1.01	1.23	0.75
Trypsin Inhibitor	1.8	2.0	1.5

The protease activity was enhanced with an addition of Mn^{2+}, $Ca^{2+}$and $^{Mg2+}$ resulting in an increased enzyme activity of proteases isolated from *Scomberomorus commerson, Rastrelliger kanagurta* and *Eleutheronema tetradactylum.* The metal salts served as an

enhancer of protease enzyme activity. Of the three metal salts, Mn^{2+} had increased the activity of proteases isolated from from the intestines of *Scomberomorus commerson*, *Rastrelliger kanagurta* and *Eleutheronema tetradactylum* (Table 3).

Table 3. Effect of metal salts on proteases isolated from the intestines of *Scomberomorus commerson*, *Rastrelliger kanagurta* and *Eleutheronema tetradactylum*

Enhancers	**Activity of Proteases (U)**		
	Scomberomorus commerson	***Rastrelliger kanagurta***	***Eleutheronema tetradactylum***
$CaCl_2$	10.0	10.0	9.0
$MgCl_2$	10.01	10.2	9.3
$MnCl_2$	11.2	11.0	11.5

DISCUSSION

Proteases are a unique class of enzymes; they have immense physiological as well as commercial importance. They possess both degradative and synthetic properties. They occur ubiquitously in plants, animals and microbes. Crude preparation of the enzyme has a wide specificity due to the presence of various proteinase and peptidase isozymes. The activity of the enzyme depends on the source, and the methods used in its extraction and purification [10]. Proteases from *Scomberomorus commerson*, *Rastrelliger kanagurta* and *Eleutheronema tetradactylum* were purified by ammonium sulphate precipitation and dialysis. Specific activity of proteases isolated from *Rastrelliger kanagurta* showed a higher specific activity than proteases isolated from *Scomberomorus commerson* and *Eleutheronema tetradactylum* suggesting that ammonium sulphate precipitation had concentrated the enzyme; a similar observation was made on the specific activity of alkaline protease isolated from the viscera of *Tilapia nilotica* (bolti fish)[11] after ammonium sulphate precipitation. The SDS-PAGE analysis of proteases isolated from *Scomberomorus commerson* depicted a single band of molecular weight around 15 KDa, whereas proteases isolated from Rastrelliger *kanagurta* and *Eleutheronema tetradactylum* showed two bands. Molecular weight of one band from *Rastrelliger kanagurta* was found to be 20 KDa and that of the other band was around 10 KDa, whereas in *Eleutheronema tetradactylum* one band had molecular weight around 28 KDa and that of the other band was found to be around 15 KDa, thus proteases from *Rastrelliger kanagurta* and *Eleutheronema tetradactylum* were a dimer comprising of two subunits. In an earlier study, the molecular masses of alkaline proteases upon SDS-PAGE analysis were in the ranges of 15 to 30 KDa [12]; this is in accordance with the present study. The optimum temperature of proteases isolated from the intestines of three fishes was found to be 55°C,

this is in accordance with an earlier report that the optimum temperature of alkaline proteases ranged from 50-70°C [13]. The optimum pH was found to be 10 for the proteases isolated from the three fishes, indicating that the proteases isolated from the intestines of *Scomberomorus commerson***,** *Rastrelliger kanagurta* and *Eleutheronema tetradactylum* were alkaline proteases, similarly allkaline proteases from the digestive tract of four tropical fishes [14] were isolated with the optimum pH between 7 and 10 for each of the four fishes. EDTA is found to be the most important inhibitor which is used in most of the characterization studies of proteases. In accordance with the present study, in an earlier report [15] inhibitors decreased the activity of alkaline proteases. The protease activity isolated from intestines of *Scomberomorus commerson***,** *Rastrelliger kanagurta* and *Eleutheronema tetradactylum* was enhanced with an addition of Mn^{2+}, Ca^{2+} and Mg^{2+} suggesting that metal ions had a capability to protect enzyme against denaturation and maintained its active conformation, a similar report was observed in proteases isolated from *Bacillus* species[16].

CONCLUSION

The results of the present investigation indicate that the proteases isolated from the intestines of *Scomberomorus commerson***,** *Rastrelliger kanagurta* and *Eleutheronema tetradactylum* is alkaline proteases. The specific activity of proteases isolated from *Rastrelliger kanagurta* showed a higher specific activity than proteases isolated from *Scomberomorus commerson* and *Eleutheronema tetradactylum.* Proteases isolated from the intestines of the three fishes exhibited maximum activity at 55 °C temperature and 10 pH. The activity of the protease enzyme isolated from *Scomberomorus commerson***,** *Rastrelliger kanagurta* and *Eleutheronema tetradactylum* was decreased in the presence of inhibitors where as the activity of the enzymes was enhanced with an addition of Mn^{2+}, Ca^{2+} and Mg^{2+} salt. Thus, these proteases may turn out to be an efficient choice for the pharmaceutical, medicinal, food and biotechnology industry.

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REFERENCES

1. Murado MA, Gonzalez MP, Vazquez JA., Recovery of proteolytic and collagenolytic activities from by products of rayfish (*Raja clavata*). Marine Drugs, 7:803-815,(2009)
2. Raghunath T, Mahajan, Badgujar SB., Biological aspects of proteolytic enzymes: a review. J Phar Res, 3(9): 2048-2068, (2010)
3. Simpson BK., Digestive proteases from marine animal. In NF Haard & BK Simpson (Eds.), Seafood enzymes, New York: Marcel Dekker: 191-213, (2005).

4. Souza AAG, Amaral IPG, Espirito Santo AR, Carvalho LB, Bezerra RS, Trypsin-like enzyme from intestine and pyloric caeca of spotted goatfish(*Pseudupeneus maculatus*). Food Chemistry, 100, 1429-1434,(2007).
5. Bezerra RS, Lins EJF, Alencar RB, Paiva PMG, Chaves MEC, Coelho LC, Alkaline proteinase from intestine of Nile tilapia (*Oreochromis niloticus*). Process Biochemistry, 40, 1829-1834, (2005).
6. Secades P, Guijarro JA., Purification and characterisation of an extracellular protease from the fish pathogen *Yersinia ruckeri* and effect of culture conditions on production. Applied and Environmental Microbiology : 3969-3975, (1999)
7. Aoyama M, Yasuda M, Nakachi K, Kobamoto N, Oku H, Kato F., Soybean milk coagulating activity of *Bacillus pumilus* derives from a serine protease. Appl Microbiol Biotech, 53: 390-395, (2000)
8. Laemmili UK., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 277:680-685, (1970)
9. Yosan S, Reungsang A,Yasuda M., Purification and characterisation of alkaline protease from *Bacillus megaterium* isolated from Thai fish sauce fermentation process. Science Asia, 32:377-383, (2006)
10. Rao MB, Tanksale AM, Ghatge MS, Deshpande VV., Molecular and Biotechnological protease. Microbiol & Molecular Biol Reviews, 62 (3):597- 635, (1998)
11. El-Beltagy AE, El-Adawy TA, Rahma EH, El-Bedawey AA, Purification and characterisation of the viscera of Bolti Fish (*Tilapia Nilotica*). Journal of Food Chemistry, 29: 445-448, (2005)
12. Fogarty WM, Griffin PJ, Joyce AM., Enzymes of *Bacillus* species. Proc Biochem, 9:27-35,(1974).
13. Kumar CG, Takagi H, Microbial alkaline proteases: From a bioindustrial viewpoint. Biotechnol Adv,17: 561-594, (1999)
14. Alencar RB, Biondi MM, Paiva PMG, Viera VLA., Alkaline proteases from the digestive tract of four tropical fishes. Brazilian Journal of food technology, 6:279-284, (2003).
15. Rajeshwari J, Ramakrishna V, Rao SK, Rao SP., Purification and characterisation of a cysteine protease from germinating cotyledons of horse gram. BMC Biochem, 10:28-39, (2009)
16. Nascimento WCA, Martins MLL., Production and properties of an extracellular protease from thermophilic *Bacillus* species. Brazilian Journal of Microbiology, 35: 91-96, (2004)