THE MAJOR DIGESTIVE ENZYMES IN *ETROPLUS SURATENSIS* AND *OREOCHROMIS MOSSAMBICUS*: DISTRIBUTION AND CHARACTERISTICS

Thesis Submitted to the

Cochin University of Science and Technology

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in

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Under the Faculty of Marine Sciences

By

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Dedicated to my Family, Teachers and God almighty....

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Certificate

This is to certify that the thesis entitled ***"The Major Digestive Enzymes in Etroplus suratensis and Oreochromis mossambicus: Distribution and Characteristics"*** *is an authentic record of the research work carried out by* ***Mr. Hari Sankar. H.S*** *under my supervision and guidance in the Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, in partial fulfilment of the requirements for the award of the degree of* ***Doctor of Philosophy in Biochemistry*** *and that no part of this work has previously formed the basis for the award of any degree, diploma or associateship in any University.*

Kochi-682016
30th December 2013

Dr. Babu Philip

Declaration

*I hereby do declare that the thesis entitled "**The Major Digestive Enzymes in Etroplus suratensis and Oreochromis mossambicus: Distribution and Characteristics**" is a genuine record of research work done by me under the supervision and guidance of Dr. Babu Philip, Ret. Professor, Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, in partial fulfilment of the requirements for the award of the degree of **Doctor of Philosophy in Biochemistry** and that no part of this work has previously formed the basis for the award of any degree, diploma or associateship in any University.*

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LIST OF NOTATIONS AND ABBREVIATIONS

%	:	Percent
µg	:	Microgram
µM	:	Micro molar
µMol	:	Micro mole
^{0}C	:	Degree centigrade
ANOVA	:	Analysis of Variance
Asp	:	Aspartic acid
ATP	:	Adenosine tri phosphate
ATPase	:	Adenosine tri phosphatase
Ba	:	Barium
BApNA	:	Benzoyl-L-Arginyl-p-nitro anilide
Ca	:	Calcium
cDNA	:	Complementary DNA
cm	:	Centimeter
Cu	:	Copper
DNSA	:	3, 5-Dinitro salicylic acid
DSI	:	Digestive somatic index
EC	:	Enzyme Commission
FAO	:	Food and Agriculture Organization
FCR	:	Feed conversion ratio
Fe	:	Iron
FOS	:	Fructo oligosaccharides
g	:	Gram
Gln	:	Glutamine
Glu	:	Glutamic acid
Gly	:	Glycine
GSI	:	Gastro somatic index
H	:	Hydrogen
HCl	:	Hydrochloric acid

Hg	:	Mercury
HIS	:	Hepato somatic index
hr	:	Hour
Ile	:	Isoleucine
IQ	:	Intestinal Quatient
K	:	Potassium
Km	:	Michaelis-Menten Constant
Leu	:	Leucine
M	:	Molar
Mg	:	Magnesium
mM	:	Milli molar
Mn	:	Manganese
Mol	:	Mole
mRNA	:	Messenger RNA
N	:	Normal
Na	:	Sodium
NaOH	:	Sodium hydroxide
nm	:	Nano meter
O.D	:	Optical density
pH	:	Pondus Hydrogenii
pNPA	:	p-nitrophenyl acetate
pNPB	:	p -nitrophenyl butyrate
pNPP	:	p-nitrophenyl palmitate
PUFA	:	Poly unsaturated fatty acid
RNA	:	Ribonucleic acid
RPM	:	Revolutions per minute
SGR	:	Specific growth rate
TCA	:	Trichloro acetic acid
TGs	:	Tri aclylglycerols
Thr	:	Threonine
TLCK	:	Tosyl-L-lysyl chloromethyl ketone

TOS	:	Transgalacto oligosaccharide
TPCK	:	Tosyl phenylalanyl chloromethyl ketone
Tyr	:	Tyrosine
U	:	Units
UV	:	Ultra violet
UVCD	:	Ultra violet circular dichroism
Vmax	:	Maximum Velocity
WHO	:	World Health Organization
Zn	:	Zinc

Contents

Chapter 1
INTRODUCTION

Contents

Enzymes are molecules that are evolved to integrate life with nutrients, metabolism and their regulations in accordance with environment to maintain life in harmony. Living beings relay on nutrients for their development, growth and maintenance as well as for energy purposes. Health and fitness of every organism has its base on the digestive capabilities and its nutrient expenditure. Growth is generally considered as the mass gain which is accomplished through the synthesis of protein. Enzymes are proteins which execute and regulate all physiological reactions in living beings. They are catalysts that initiate and control all biochemical process in the body. The digestion of food and absorption of nutrients depends on the availability and efficiency of digestive enzymes (Furne *et al*., 2005). Various environmental factors influence the digestive enzyme activity in different ways. Knowledge about the biochemical characteristics of digestive enzymes and their functional zonation in different gastro intestinal sections has many applications. In sustainable culture practice, feeding strategies are very important. Knowledge on nutrient hydrolysis in the digestive tract is a key factor in optimizing fish feeding procedures (Suarez *et al*., 1995). Compared to mammals, digestive processes in fish have less extensively been examined. Variations in nature, source and quantity of nutrients can alter the enzyme profile and/or the amount of digestive enzyme secretion. Such adaptive attributes of digestive enzymes

can be successfully used as a tool for studying the intake of nutrient content from the diets (Moraes and Bidinotto, 2000). The best use of dietary nutrients can be reflected in the concentration of various metabolites in different body compartments leading to alterations in metabolic profile. Thus, metabolic profile can be considered as a possible tool to analyze the inter-relationship between the digestive biochemical processes and the organismal outcome (Correa *et al*., 1998; Moraes and Bidinotto, 2000). Obviously the digestive enzymes play an important role in the development and growth of fishes. Fish viscera, one of the most important by-products of fishing industry are recognized as a potential source of digestive enzymes, especially its high activity over a wide range of pH and temperatures (Shahidi and Kamil, 2001). The biological diversity of fish species provides a wide array of enzymes with unique properties. The recovery of enzymes from fishery by-products is of great importance since low-cost enzyme preparations could promote new industrial applications. The maximum utilization of marine resources can be achieved by the application of enzymes from fish viscera in the food industry as processing aids. The most common clinical application of digestive enzymes is in curing the digestive disorders related to insufficient enzyme production. An approach in supporting the patients with digestive enzyme inadequacy is orally supplementing the deficient enzymes to maintain adequate digestive capacity. Studies in aquaculture have shown that the rate of digestion and absorption of essential nutrients can be determined by knowing functional properties of enzymes in the digestive tract and it can be used for the formulation of good artificial diet. The use of probiotics in aquaculture has been receiving significant attention thanks to their eco-friendly nature. This ensures minimization or elimination of the use of antibiotics in the aquaculture practices.

In the present study, *Etroplus suratensis* and *Oreochromis mossambicus* are subjected for detailed biochemical analysis for understanding more of their major digestive enzymes. The digestive physiology and enzymology of *E. suratensis* has not been examined thoroughly even when they are commercial and economical important. The *O. mossambicus* commonly known as Tilapia was first introduced in 1952 for culture practice in India. Its culture in Indian waters was later discouraged as it was found to be a serious threat to native species. This is due to their adaptive nature in different environmental conditions and such adaptive ability could be the reflection of their capacity to explore different feeds available in their habitat. A comparison between the digestive capabilities of native *E. suratensis* and alien *O. mossambicus* would be invaluable in understanding their competitive adaptive behavior.

1.1. *Etroplus suratensis*

Etroplus suratensis (Pearlspot, in Malayalam: *Karimeen*) is one of the most popular and precious fish among the cichlids indigenous to peninsular India and Srilanka. *E. suratensis* is the largest among the three indigenous cichlids namely *Etroplus suratensis*, *Etroplus maculatus and Etroplus canarensis* (Bindu and Padmakumar, 2012). *E. suratensis* is widely distributed in most of the brackish and freshwater systems (Bhaskaran, 1946; Hora and Pillay, 1962). The high market demand and large size of this fish makes it one of the most popular and promising species for aquaculture throughout India. This species can tolerate a wide range of salinity. Commercial culture practice of pearlspot is reported to be highly successful in various agro-climatic conditions prevailing in India (Jhingran and Natarajan, 1972; Thampy 1980; Sumitra *et al*., 1981; Anon, 1983). Mono culture or mixed culture practices with fishes belonging to *Cyprinidae* or *Channa* in freshwater and *Mugil*

cephalus, *Chanos chanos* or shrimps in brackish water were adopted for the production of *E. suratensis* by aquaculturists. With the flourishing of backwater tourism in the state of Kerala in India, the demand for Pearlspot has been on the rise. Overexploitation of this valuable species increased its vulnerability to its maximum. Pearlspot constituted almost 10% of the total fish landings in the backwaters of the state of Kerala during the sixties (George and Sebastain, 1970), during 1991-1992 it has been reduced to 9.7% (CIBA Bulletin, 1995). Since economically valuable species are generally exploited to the maximum, the fishery of this species is further subject to increasing pressures (Padmakumar *et al*., 2002). *E. suratensis* feeds on detritus, plankton and small aquatic insects whereas *E. maculatus* is carnivorous in nature, feeding on a wide range of available food. Both of the species are available for catch almost throughout the year, but the peak seasons are January to April and September to November. *E. suratensis* matures on attaining a length of 10-12 cm and grows to a maximum length of about 25 cm. This species contribute a significant percentage of the landings in Chilka Lake, Pulicat Lake and Kerala backwaters. Nevertheless, this species is facing severe threat in its natural habitats due to uncontrolled exploitation and challenges from invasive species like tilapia.

Kingdom:	Animalia
Phylum:	Chordata
Infra phylum:	Gnathostomata
Super Class:	Osteichthyes
Class:	Actinopterygii
Infra class:	Teleostei
Super order:	Acanthopterygii
Order:	Perciforms
Suborder:	Labroidei
Family:	Cichlidae
Subfamily:	Etroplinae
Genus:	Etroplus
Species:	*Etroplus suratensis*

1.2. *Oreochromis mossambicus*

Tilapia, having immense adaptability in a wide range of physical and environmental conditions, ability to reproduce in captivity, relative resistance to various stresses and disease-causing agents compared to other cultured finfish species, good flesh quality, feeding over a low trophic level and excellent growth rate on a wide variety of natural and artificial diets, is the most abundantly cultured species worldwide (Welker and Lim, 2011). Even though it is endemic to tropical freshwaters of Africa, Jordan and Israel, distribution of this species has been established following introductions into other regions of the world in the early part and after the middle of the 20th century. Tilapias are presently cultured in virtually all types of production systems, in both fresh and salt waters, and in tropical, subtropical and temperate climates (Lim and Webster, 2006). Tilapia is one of the most important euryhaline finfishes cultured all over the world and they contribute to approximately 6% of total farmed fish production (FAO, 2004). Tilapia is the second most widely cultured fish worldwide, first being the carps (Welker and Lim, 2011). These varieties of fish had been transplanted to many countries and the first consignment was brought to India in August 1952 by the Central Marine Fisheries Research Institute (CMFRI), Mandapam from Bangkok. The second consignment was brought in the same year from Sri Lanka. Thereafter, it was introduced in the ponds and dams of other South Indian States by the respective State fisheries departments. The damage caused by tilapia on the native fauna has not been assessed thoroughly so far. Being a precocious and juvenile breeder, it overpopulates in the habitat resulting in filling the water bodies with profusion of small fishes. Due to its high fecundity, aggressive behavior and ecological plasticity it has the potential for rapid and explosive invasion and has become a significant pest in those

habitations. Mozambique tilapia (*Oreochromis mossambicus*) has been listed in 100 of the world's worst invasive alien species (Lowe *et al*., 2004).

International organisations such as World watch Institute consider bio-invasion as the second most threat to biological diversity, the first being habitat degradation. *Oreochromis mossambicus* is one of the hardiest fishes in aquaculture farms. Once introduced into a habitat, they generally establish themselves very quickly. Tilapias tolerate a variety of environmental conditions and can adapt to wide ranges of salinity, oxygen tension and overcrowding. Tilapias exhibit relatively short reproductive cycles, breed prolifically under culture conditions, are strongly resistant to disease and infection and are amenable to handling. Most importantly, tilapias are highly valued by peoples as a food source.

Kingdom:	Animalia
Phylum:	Chordata
Infra phylum:	Gnathostomata
Super Class:	Osteichthyes
Class:	Actinopterygii
Subclass:	Neopterygii
Infra class:	Teleostei
Super order:	Acanthopterygii
Order:	Perciforms
Suborder:	Labroidei
Family:	Cichlidae
Subfamily:	Pseudocrenilabrinae
Tribe:	Tilapiini
Genus:	Oreochromis
Species:	*Oreochromis mossambicus*

1.3. Objectives of the study

1. To characterize the pepsin-like aspartic acid protease, alpha-amylase, total alkaline protease and lipase from *E. suratensis* and *O.*

mossambicus biochemically by analysing their optimum temperature, pH and substrate concentration for maximum activity.

2. To study the effect of different metal ions on the activity of different digestive enzymes in the selected species.
3. To determine the pH stability and temperature denaturation kinetics of the major digestive enzymes.
4. To identify efficient alpha-amylase inhibitors from plants and to determine the mechanism of inhibition by kinetic studies.
5. To understand the functional zonation of the major digestive enzymes along the gastro-intestinal tract of *E. suratensis* and *O. mossambicus*
6. To examine the effect of different probiotics on the digestive enzyme activity and their influence on growth of *E. suratensis* and *O. mossambicus*

1.4. Review of Literature

The strength and survivability of almost every living organism is based on its nutrient expenditure and digestion. If either of them is defective, difficulty is certain. Enzymes are protein molecules responsible for most of the physiological reactions in the living system. They are catalysts that initiate and regulate millions of biochemical process in the body. The digestion of food into subunits appropriate for absorption in the digestive tract of the animal depends on the availablility and efficiency of enzymes secreted into their gastro intestinal system. Various environmental factors influence the digestive enzyme activity in different ways. The biochemical capacity of the fish for transforming feed is concerned with the role of many other factors like age (Kuzmina, 1996), type of feeding (Jones, 1986), diet composition (Deguara *et*

al., 2003), season and temperature of acclimation (Kuzmina, 1991). The studies on the effect of aerobic exercise training revealed an increase in the activity of digestive enzymes and thereby maximum digestive metabolism could be attained in juvenile fishes (Pang *et al*., 2013). Such factors have a cumulative action on the digestive enzymes. The digestive enzyme pattern reflects the feeding habit of the fish (herbivore, detritivore, omnivore and carnivore) and reflects its digestive capacity (Smith, 1980). Results of studies on the responses of digestive enzymes to feeding can be used to derive nutritionally effective diet formulations (Lundstedt *et al*., 2004). Studies on the digestive secretions in fish can reveal certain aspects of their nutritive physiology and help to resolve some nutritional requirements like matching of an artificial diet to the nutritional needs of the fish (Furne *et al.*, 2005). During the development of aquaculture, the art of fish farming, many researchers started to enrich their knowledge about the fate of feed ingested and the resultant changes in the fish.

1.4.1. Digestive organs in the Fish

Digestion is the process of hydrolysis and solubilization of ingested nutrient polymers into molecules and elements suitable for transport across the intestinal wall. Fish digestive system consists of the alimentary canal and associated glands. The anatomy of the gastrointestinal tract of fishes follows the same basic pattern as in other vertebrates with slight variations reflected in phylogeny and ontogeny, diet, and environment. The intramural glands present in the digestive tract provide lubricating mucus, enzymes, water etc. Liver, pancreas and gall bladder are external glands. In elasmobranches pancreas is relatively compact and usually well developed as a separate organ, often two lobed. In teleost, the pancreas is diffused in the liver to form hepatopancreas

(Bond, 1979). The gall bladder is vestigial in deep sea fishes but it is prominent in other fishes. The alimentary canal consists of mouth, which opens into buccopharynx, which in turn opens in to the oesophagus. The oesophagus opens into the stomach or intestine. The mouth, buccal cavity and pharynx are considered as non tubular while other parts of the alimentary canal are considered as tubular. The gastrointestinal tract of fishes can be subdivided into four topographical regions: namely headgut, foregut, midgut and hindgut (Harder, 1975). The headgut is composed of the mouth and pharynx, and its function is to acquire food and mechanically process it (Horn, 1997; Clements and Raubenheimer, 2006). The foregut follows and is comprised of the esophagus and stomach, where chemical digestion of food begins. In some fishes, the mechanical breakdown of food may also occur partially or fully in the stomach. The midgut or intestine accounts for the greatest proportion of the gut length and is where enzymatic digestion continues and absorption mainly takesplace. The hindgut is the final section, in some cases there is no clear morphological distinction between mid gut and hindgut. This includes the rectum. The foregut epithelium is of ectodermal and the mid gut of endodermal in origin.

The ingested food is broken down physically by peristaltic movement and chemically by the enzymatic hydrolysis. The digestive enzymes secreted from the stomach and exocrine pancreas is of major importance for enzymatic hydrolysis of complex dietary proteins, fats and carbohydrates into smaller fragments. The fragments are further digested by the enzymes located in the brush border membrane of the enterocytes, making them small enough for absorption. The detritus, mucus, microorganisms, desquamated cells, the bile pigments and the undigested food are excreted as feces. Some amount of water is taken up by the fish along with food during feeding was driven out through

operculum. The feeding behavior of fish is very complex. The visual, chemical, taste and lateral line system controls the momentary feeding act. The feeding behavior is affected by season, light and dark periods, light intensity, time of last feeding temperature and internal rhythm of the fish. De Groot (1971) has found out that all family of flat fishes (*Pleuronectiformes*) has visual, chemical and mechanical sense organs.

1.4.2. Digestive enzymes in Fish

As in other animals, species-specific isoforms of the various enzymes exist in fishes with differences in temperature and pH optima, molecular mass, specific activities and several various specificities (Pivnenko *et al*., 1997; Asgeirsson and Cekan, 2006; Ogiwara and Takahashi, 2007). Proteases are enzymes that catalyse the hydrolysis of protein molecules to peptides or to amino acids. Fishes have the capability to modulate digestive protease secretion when the concentration and/or source of dietary protein are modified (Rodiles *et al*., 2012). Pepsinogen and pepsin from several fish species have been characterized (Wu *et al*., 2009). In fishes, more than one form of pepsin is present and the different forms show different activation rates, pH optima (varying between 1 and 5), specific activities and substrate specificities. Generally only one secretory cell type is observed within fish gastric glands without differentiation into distinct pepsinogen-secreting chief (peptic) cells and acid-secreting parietal (oxyntic) cells as observed in mammals (Barrington, 1957; Hirschowitz, 1957; Michelangeli *et al*., 1988). This cell type in fishes possesses certain structural characteristics common to both the peptic and oxyntic cells of mammals (Rebolledo and Vial, 1979; Mattisson and Holstein, 1980; Stroband and Kroon, 1981; Murray *et al*., 1994). These oxynticopeptic or oxyntopeptic cells thus contain the cellular machinery for

both functions and it has been shown to secrete pepsinogens in some fish species (Reifel *et al*., 1985; Yasugi *et al*., 1988; Inui *et al*., 1995; Huang *et al*., 1998) and hydrochloric acid in others (Smolka *et al*., 1994). Gawlicka *et al* (2001) proved that these cells synthesize pepsinogens and secrete hydrochloric acid simultaneously and confirmed that they are of the oxynticopeptic type. Morphologically, acid secretion is associated with a well-developed intracytoplasmic membrane system consisting of a tubulovesicular network of smooth membranes from which the HCl is secreted by active transport into the stomach (Noaillac- Depeyre and Gas, 1978; Ezeasor, 1981). The gastric proton pump has been identified as an H^+/K^+-ATPase, a heterodimer consisting of an 'a' and 'b' subunit belonging to the same P-type ATPase family as Na^+/K^+-ATPase (Yao and Forte 2003). The enzyme hydrogen potassium ATPase (H^+/K^+ ATPase) is unique to the parietal cells and transports the H^+ against a concentration gradient of about 3 million to 1, which is the steepest ion gradient observed in mammals. The expression of the gastric proton pump and pepsinogen has been confirmed by in situ hybridization in winter flounder (Gawlicka *et al*., 2001), in porgy (Darias *et al*., 2007) and by immunohistochemistry (Yasugi 1987; Yasugi *et al*., 1988). In tilapia (*O. mossambicus, O. niloticus, O. zilii*), the oxyntic cells have the characteristics of an acid-secreting cell and no pepsinogen production (Gargiulo *et al*., 1997). However, these results contrast with the finding of eosinophilic granules by Osman and Caceti (1991) in wild *O. niloticus*, and activity measurements in stomach extracts and mRNA expression and partial cloning of a pepsinogen (A2) by Lo and Weng (2006) in *O. mossambicus* (AY513876.1). In fish species with stomachs, the low pH denatures most of the proteins and helps pepsin to access the peptide linkages. Pepsins are endopeptidases with a high affinity to hydrophobic bonds involving amino acids like tyrosine and

phenylalanine. The chyme formed by this partial hydrolysis of food enters into the intestine through the pyloric sphincter. The primary function of the intestine is the completion of the digestive processes started in the stomach and the absorption of nutrients. Intestinal surface area is increased in all fish by folding (primary, secondary or tertiary) of the mucosa and by apical plasma membrane amplification through brush border microvilli. Proteins and peptides entering the intestine are diluted and dissolved in alkaline secretions from the liver, pancreas or gut wall. The actions of the pancreatic endopeptidases trypsin, chymotrypsin and elastases I and II as well as the exopeptidases, carboxypeptidase A and B result in a mixture of free amino acids and smaller peptides.

Although not well characterized functionally, the exocrine pancreas of various fish species, irrespective of whether discrete or diffused in its anatomical structure and location, contains acinar cells with zymogen granules which produce and store digestive enzymes (Kurokawa and Suzuki, 1995). The zymogen granules or intestinal content of at least some fish species contain the pancreatic enzymes or enzymatic activities corresponding to lipase, co-lipase, phospholipase, α-amylase, proteolytic enzymes trypsin, chymotrypsin, elastase, carboxypeptidases A and B, as well as DNAase and RNAase (Kurokawa and Suzuki, 1995; Pivnenko *et al*., 1997; Krogdahl and Sundby, 1999; Kurtovic *et al*., 2009). Rothman *et al* (2002) proved that instead of being completely degraded in the small bowel with the food they help to digest, a large fraction of the digestive enzymes secreted by the pancreas are absorbed and recycled in an enteropancreatic circulation.

It is reported that due to the low level of carbohydrates in the natural diet, gluconeogenesis is considered to be the main process involved in meeting the glucose requirements in carnivorous fishes under natural conditions

(Cowey *et al*., 1977). Starch-converting enzymes are basically divided into four groups namely; endoamylases, exoamylases, debranching enzymes and transferases. Fishes have two categories of endogenous enzymes involved in carbohydrate digestion namely pancreatic α-amylase and disaccharidases in the brush border membrane of the intestinal epithelial cells. Pancreatic α-amylase hydrolyzes α (1-4) glycosidic bonds producing maltose and branched oligosaccharides from poly saccharides such as glycogen and starch. The term 'alpha' in alpha-amylase indicates the retention of α-anomeric configuration in the hydrolytic products (Sivaramakrishnan *et al*., 2006). The ability of fish to produce amylase has a great benefit for the aquaculture practices because starch is used not only as a cheap energy source but also as an efficient binder in formulated diets. Amylase activity varies significantly among fish species. Usually it is highest in herbivorous species. The salinity may also play a part in the digestion of dietary starch. Krogdahl *et al* (2004) observed that, in freshwater, plasma glucose in rainbow trout was increased nearly four-fold during feeding trials with high carbohydrate diet compared to a low carbohydrate feed. On the contrary, in saltwater, the increase was less than two-fold in fish fed on same two diets. In addition, the plasma glucose levels were significantly lower in fish from saltwater in comparison with plasma of fish fed on high carbohydrate diet in freshwater. However, in the same trial they observed that in Atlantic salmon the plasma glucose was only marginally increased in response to the high dietary starch diet and no effect of water salinity have been found (Krogdahl *et al*., 2004).

The digestive enzymes from the pancreatic tissues are released into ductules which converge into pancreatic ducts. These ducts are finely structured and numerous in fish with diffusely located pancreatic tissue. The ducts release the enzymes into the lumen of the pyloric caeca or proximal

intestine or into the bile ducts (Kurokawa and Suzuki, 1995; Krogdahl and Sundby, 1999; Morrison *et al*., 2004). The proteolytic enzymes and co-lipase are secreted as pro-enzymes that are activated in the intestinal lumen, whereas most lipases and the α-amylase are released in active forms. The final steps of hydrolysis take place at the brush border of the enterocytes. The events that lead to activation of the pro-enzymes is initiated by enterokinase (enteropeptidase), EC.3.4.21.9, a heterodimeric glycoprotein present in the duodenal and jejunal mucosa. Enterokinase activates trypsinogen to form trypsin (Ogiwara and Takahashi, 2007). The activation of trypsin initiates a cascade of proteolytic reactions leading to the activation of many pancreatic zymogens including chymotrypsinogen, proelastase, procarboxypeptidase and some prolipases (Ogiwara and Takahashi, 2007). It has been suggested that lipolytic enzymes predominate in fishes. Fish digestive lipases are either of the co-lipase-dependent pancreatic lipase or carboxyl ester lipase type, and pancreatic lipase may be present mainly in freshwater fish and carboxyl ester lipase in marine species (Kurtovic *et al*., 2009). Colipase activity has been observed in *Oncorhynchus mykiss* (Leger *et al*., 1979) and in *Squalus acanthius* (Sternby *et al*., 1984). Studies on digestive glands of various marine animals by Smichi *et al* (2012) suggested that only pancreas contained the classically known colipase in cartilaginous fish. Cartilaginous fishes, represented by shark and rays, could be considered as the oldest vertebrates possessing a complex digestive system similar to that of mammals. Lipases from *Pagrus major* and *Pseudopleuronectes americanus* exhibit a bile-salt dependency (Iijima *et al*., 1998; Murray *et al*., 2003) indicating that bile salt-dependent carboxyl ester lipase is secreted from pancreatic tissue in some species. In some fish species the wall of the digestive tract from the foregut to the most distal regions may also be a source of lipases (Tocher, 2003) since

highest lipase activity is observed in the proximal intestinal region in most of the fishes and in the distal region in some others. The lipolytic activity observed in the pancreases of cartilaginous fishes was found to be greater than those in bony fishes, mollusks and crustaceans (Smichi *et al.*, 2012). The nutrient composition and digestibility of the diet also differentially influences secretion of specific enzymes and other factors. A diet containing a high level of protein, proteins with low digestibility, and the anti-nutritional factors like trypsin inhibitors in plant feedstuffs, have been shown to affect the pancreatic secretions (Olli *et al*. 1994; Krogdahl *et al*. 1999, 2003, Peres *et al*., 1998). The specific activity and the quantity of various pancreatic enzyme secretions appear to be species specific and differ in their dietary habits. The diffusely located pancreatic tissue of many species makes such studies challenging.

Fish enzymes typically show higher specific activity and substrate affinities than the enzymes in homoeothermic animals, presumably representing an evolutionary adaptation to function at lower temperatures (Asgeirsson and Cekan, 2006; Klomklao *et al*., 2006; Ogiwara and Takahashi, 2007; Desrosiers *et al*., 2008; Jellouli *et al*., 2009). Trypsin from Atlantic cod, for example, has 17 times higher catalytic efficiency than bovine trypsin when measured at the same temperature range (Asgeirsson and Cekan, 2006). The contribution of exogenous digestive enzymes present in the natural diet to total digestive capacity is likely to be underestimated and poorly examined.

Fishes can be classified according to trophic level feeding as (1) herbivores, 2) carnivores, (3) omnivores and (4) detritivores although the designation of these categories is problematic (Clements and Raubenheimer, 2005). Kramer and Bryant (1995) indicate that gut length as a reflection of diet should be applied only to identifying broad categories. Harder (1975) suggested that there are no clear relationships between intestinal morphology

and feeding type and it is not possible to draw conclusions on one from the other. However, Zeng *et al* (2012) proposed that feeding commonly triggers a series of related physiological and morphological responses. The feeding and fasting regimes and seasonal foraging patterns in many carnivorous and herbivorous species can be altered by temporal and spatial fluctuations in food availability and quality (Starck, 1999; German *et al*., 2010). The length and relative mass of the intestine is generally greater in herbivores compared to carnivores, which might be to allow more duration for processing of relatively difficult-to-digest materials (Horn, 1997; Clements and Raubenheimer, 2005). Within omnivorous fishes there is no clear relationship with degree of herbivory or carnivory (Kramer and Bryant, 1995). *Etroplus suratensis*, in general, is a bottom feeding scavenger with a tendency for herbivory (Kesava *et al*., 1988). *O. mossambicus* is omnivorous and feeds on almost anything from algae to insects (Mert and Cicek, 2010).

Using digestive enzyme assays, we measured the activity levels of amylase, trypsin and lipase, which hydrolyse starches, proteins and lipids respectively. These enzymes are synthesised in the pancreas and secreted into the gut lumen via the hepatopancreatic duct. Based on the adaptive modulation hypothesis (Karasov and Hume, 1997; Karasov and Martinez del Rio, 2007) it is expected that the digestive enzyme activities may match with dietary biochemistry and this reflects the relative importance of ingested nutrients. The microflora associated with the epidermis, gill and gastrointestinal tract of several fishes has been the subject of qualitative and quantitative studies (Cahill, 1990; McDonald *et al*., 2012). Application of probiotics is another important tool in disease control and in enhancing growth and development in animals. The biological studies on fish nutrition are particularly relevant when

they focused on commercially meaningful species like *E. suratensis* and *o. mossambicus*.

1.5. Scheme of the Thesis

The thesis is divided in to eight chapters. The first chapter discusses the theme of the thesis and includes introduction, general review of literature, objectives and importance of the study.

Pepsin belongs to the group of enzymes which participates first in the process of digestion. The second chapter deals with the major digestive enzyme, pepsin-like aspartic acid proteases. The chapter is divided in to five sections. The first section includes introduction to the specific study on pepsin-like aspartic acid protease. Review of literature has been presented in the second section. The materials and methods are included in the third section of this chapter. The fourth section consists of the results and major findings. The discussion is presented in the final section followed by a summary.

Amylase is one of the major carbohydrases which participate in carbohydrate metabolism. The third chapter deals with the major digestive enzyme amylase. Apart from the general biochemical characterization, screening of phytochemical inhibitors for alpha- amylase has also been done. The chapter is divided in to five sections. The first section includes introduction of the specific study on amylase. Review of literature has been presented in the second section. The materials and methods are included in the third section of this chapter. The fourth section consists of the results and major findings. The discussion on the results has been presented in the final section. It is divided into two subsections; the first section consists of general biochemical characteristics of alpha-amylase from *E. suratensis* and *O.*

mossambicus, and the second section deals with the phytochemical inhibitors. It is followed by a summary.

After the gastric proteolysis, protein digestion is aided by the alkaline proteases present in the intestine. The fourth chapter deals with the major digestive enzyme - total alkaline proteases. The chapter is divided in to five sections. The first section includes introduction to the specific study on total alkaline proteases. Review of literature has been presented in the second section. The materials and methods are included in the third section of this chapter. The fourth section consists of the results and major findings. The discussion of the results has been provided in the final section followed by summary.

Lipids are the most energy rich molecules in the diet of living beings and they are metabolized by the enzyme lipase. The fifth chapter deals with the major digestive enzyme lipase. The chapter is divided in to five sections. The first section includes introduction of the specific study on lipase. Review of literature has been given in the second section. The materials and methods are included in the third section of this chapter. The fourth section consists of the results and major findings of the present study. The findings are interpreted in the discussion section and then concluded.

The digestion of various food materials takes place in distinct sections of the gastro intestinal tract of animals. It depends on the presence of different digestive enzymes in the specific regions of gastro intestinal tract. The sixth chapter deals with the functional zonation of major digestive enzymes in *Etroplus suratensis* and *Oreochromis mossambicus*. The chapter is divided in to five sections. First section includes introduction of the specific study on zonation of major digestive enzymes in *Etroplus suratensis* and *Oreochromis*

mossambicus. Review of literature is provided in the second section. The materials and methods are included in the third section of this chapter. The fourth section consists of the results and major findings. The results are discussed in the final section followed by a summary.

The digestion process is affected by various external and internal factors among which the intestinal micro flora plays a major role. The seventh chapter deals with the effect of probiotics on digestive enzymes. The chapter is divided into five sections. First section includes introduction to the specific study on the effect of probiotics on digestive enzymes. Review of literature is given in the second section. The materials and methods are included in the third section of this chapter. The fourth section consists of the results and major findings of the present study. The discussion of the results is presented in the final section. It is followed by a summary.

The eighth chapter is the summary and conclusion of the research work. The references are presented in the reference section of the thesis.

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Chapter 2

PEPSIN-LIKE ASPARTIC ACID PROTEASES

Contents

The chapter deals with the major digestive enzyme pepsin-like aspartic acid proteases. The chapter is divided in to five sections. First section includes introduction of the specific study on pepsin-like aspartic acid protease. Review of literature on the relevant studies conducted so far has been discussed in the second section. The materials and methods are included in the third section of this chapter. Methods to analyse effect of pH, temperature, metal ions and determination of optimum substrate concentration for enzyme action are included in this section. The fourth section consists of the results and major findings of the present study. The results have been discussed in the final section. It is followed by a summary.

2.1. Introduction

Aspartic proteinases are found in all eukaryotes, either as secreted digestive proteinases like mammalian pepsin, plant cyprosin and fungal endothiapepsin or as lysosomal proteinases like cathepsins D and E. The acid proteases are of varying functions in the control of key processes in living organisms; for example, renin in kidney plays a key role in the cascade regulating the formation of a hypertensive agent, angiotensin II (Guruprasad *et al*., 1994). Pepsins constitute a widely distributed protein super family among

proteases, to attain protein degradation at low pH and are found in a multitude of organisms such as vertebrates, plants, fungi and retroviruses (Davies, 1990). In fungi, acid proteases play an important role in sporulation, the retroviral proteases cleave retroviruses during the activation of the virus and in vertebrates like masu salmon (*Oncorhynchus masou*), a pepstatin A-sensitive enzyme involved in yolk formation (Brik and Wong, 2003; Hiramatsu *et al*., 2002). Pepsin-like aspartyl proteases constitute the largest family of acid proteases. In terms of evolutionary history, pepsin is regarded as the first enzyme in the aspartic protease family. They perform a variety of functions in different forms of living organisms. Among the metabolic functions of these aspartic proteases, the most intensily investigated enzymes are digestive proteases such as pepsin, gastricsin, chymosin, etc. and lysosomal proteases like cathepsins. Pepsin is the principal proteolytic enzyme of vertebrate gastric fluid. During the 17^{th} century, van Helmont pointed out that something except acid was concerned with the digestive action of stomach, but early studies were mainly concentrated on the peristaltic movements of stomach muscle. After that, Spallanzani (1785) observed the presence of protein digestive power in gastric juice in vitro (David and Soybel, 2005). The name pepsin was given by Theodor Schwann in 1836, and it was arrived from 'pepsis', the term for digestion in Hippocratic writings (Kousoulis *et al*., 2012). Pepsin has been the subject of more intensive studies than any of the other proteinases. The protein nature of enzymes was not established until John H. Northrop crystallized pepsin in 1930, an attainment for which he shared the Nobel Prize in 1946 with Sumner, and Stanley. Fruton and Bergmann (1938) defined this enzyme as one which is acting on the protein that is dissociated into a base in acid media, and hydrolyses specific peptide bonds of it. The development of new separation techniques like chromatography and protein crystallization

helped in the determination of pepsin and pepsinogen. In 1959, Ryle and Porter isolated two different forms of pepsin, namely, Pepsin B and C from porcine stomach. Recently, the pepsin-like enzymes and their inhibitors received increased attention since HIV-proteases are recognised as a member of this aspartic protease family (Campos, 2003). All the vertebrates possessing a stomach have pepsin without exception. At a pH below 6.0, pepsinogen in the stomach mucosa of animals is converted to active pepsin from the zymogen automatically but some species of fishes which have no stomach (e.g. carps), do not secrete it. Pepsin is secreted mainly from the chief cells of the fundic glands in the stomach, partly from the cells of the cardiac and pyloric glands. Glands in the mucous-membrane lining of the stomach make and store an inactive protein called pepsinogen. Impulses from the vagus nerve and the hormonal secretions of gastrin and secretin stimulate the release of pepsinogen into the stomach, where it is mixed with hydrochloric acid and rapidly converted to the active enzyme pepsin. Generally the digestive power of pepsin is greatest at the acidity of normal gastric juice (pH 1.5–2.5). In the intestine the gastric acids are neutralized by the bile secretions and pepsin is no longer active. Only partial degradation of proteins into shorter peptides is achieved in the stomach. These peptides are either absorbed from the intestine into the bloodstream or are broken down further by pancreatic enzymes. Small amounts of pepsin pass from the stomach into the bloodstream, where it breaks down some of the larger, or still partially undigested, fragments of protein that may have been absorbed from the small intestine (*Britannica*).

Many physiological functions attained by these aspartic proteinases have been explained, such as intra-lumen proteolysis by chymosin (Foltmann *et al*., 1979) and pepsin (Chen *et al*., 1975), pro-protein processing by cathepsin D (Nishimura *et al*., 1989), poly-protein processing by the human

acquired immunodeficiency syndrome (AIDS) virus aspartic proteinase (von der Helm, 1977) and blood pressure control by renin (Haber *et al*., 1969). In general, these aspartic proteinases show significant similarity (usually with 25 to 80% similarity to one another) in their primary structure (Foltmann, 1981; Cooper *et al*., 1990; Davies, 1990). Based on their different molecular characteristics and tissue or cellular localization, aspartic proteinases have been classified into several subgroups, including the cathepsins (Tang and Wang, 1987), chymosins (Inagami *et al*., 1977) and pepsins (Roberts and Taylor, 1987). Their activity is generally restricted to acidic environments within cells and tissues. The milk-clotting enzyme was called chymosin 50 years before the name rennin was coined (Lea and Dickinson, 1890). Deschamps (1840) suggested the name chymosin for rennin, derived from the Greek word for gastric liquid 'chyme'. Deschamps discovered that chymosine is rather different from pepsin. Chymosin is mainly milk-clotting and poorly active on proteolysis of meat proteins; the opposite can be observed with pepsin (Foltmann, 1993). This designation was later used in continental European languages, whereas in English the name rennin, derived from rennet, was used (Lea and Dickinson, 1890). Misinterpretations often occurred between rennin and renin from the kidneys, and therefore the designation chymosin was recently adopted in English (Foltmann, 1970) and it is now used in the recommended international enzyme nomenclature (IUB, 1986).

Pepsins belong to a family of aspartic proteinases accomplishing important digestive functions in both invertebrates and vertebrates (Kageyama, 2002). These enzymes are active at extremely low pH values (Fruton, 1970; Dunn *et al*., 1987) in both the stomach and the duodenum, and are quickly denatured when the pH exceeds 5.5 (Foltmann, 1981), thus preventing continuous proteolytic action that may damage these organs. Pepsin C has a

slightly higher optimal pH than pepsin A, but substrate specificity is nearly the same for the two enzymes (Fusek and Vetvicka, 1995). Most of our knowledge of pepsin is derived from studies on mammalian enzymes, especially the human enzymes (Abad-Zapatero *et al*., 1990; Gildberg *et al*., 1990; Sanchez *et al*., 1992; Fujinaga *et al*., 1995), while the information available on enzymes from other vertebrates, including fish is scanty (Gildberg, 1988; Tanji *et al*., 1988; Gildberg *et al*., 1990).

2.1.2. Historical importance of Pepsin

Studies on pepsin have resulted in many important events in the history of science. Pepsin is considered as the first enzyme in the aspartic protease family formed through evolution. It is the first enzyme to get recognition as having activity in digestive processes and the first to be given a name. Porcine pepsin was one of the first proteins extracted and purified (Gillespie, 1898). It is one of the proteins first to be crystallized (Northrop, 1930). It was the first enzyme that was studied using x-ray crystallographic patterns from its crystals (Bernal and Crowfoot, 1934). The extracted enzyme needs to be acidified in order to express its full activity. It was noted that different concentration of hydrochloric acid yielded different levels of pepsin activity. The activities were plotted against hydrogen ion concentration by Sorenson (1909). The plot had a scaling issue. Sorenson resolved it by using a logarithmic abscissa, thus inventing the pH scale.

2.2. Objective of the study

The objective of the present study is to characterize the pepsin-like aspartic acid protease from *E. suratensis* and *O. mossambicus* biochemically by analysing their optimum temperature, pH and substrate concentration. The study also investigates the effect of different metal ions on the activity of

selected enzymes. The temperature denaturation kinetics of these enzymes is another aim of this research. pH stability is one of the important aspects with respect to enzymes and this chapter reports the results of estimation of pH stability of pepsin-like aspartic acid proteases.

2.3. Review of Literature

Pepsins (EC 3. 4. 23. 1-4) are normally synthesized and secreted from the gastric mucosa into the gastric lumen as pepsinogens. They are autocatalytically converted into their corresponding active forms (pepsins) under harsh acidic environments, releasing the activation segments from their NH_2 terminal region by a one-step pathway or stepwise pathway (Dykes and Kay, 1976; Kageyama, 2002). As a major digestive proteinase, pepsin plays a vital physiological role in animal feed digestion. Pepsins are one of the proteases that have been studied comprehensively and their structures have been determined at high resolutions (Fujinaga *et al*., 1995). In addition, other types of aspartic proteinases have been isolated from invertebrates (Kay *et al*., 1996; Francis *et al*., 1997), plants (Guruprasad *et al*., 1994; Brodelius *et al*., 1995), retroviruses (Phylip *et al*., 1992; von der Helm, 1996), a number of microbial sources (Toogood *et al*., 1995; Hill and Phylip, 1997) and mammals such as the Japanese monkey (Kageyama and Takahashi, 1976), bovine sources (Harboe *et al*., 1974), man (Sogawa *et al*., 1983), pig (Nielsen and Foltmann, 1995), and goat (Suzuki *et al*., 1999). The gastric aspartic proteinases are mainly classified into five groups in mammals: pepsinogens A, B and F, progastricsin (pepsinogen C), and prochymosin (Kageyama, 2002; Tanji *et al*., 2007). The sequence of protein and the coding nucleic acids of more than 50 pepsinogens other than pepsinogen B have been determined to date. In fishes, one to four different gastric pepsinogen isoforms have been

described in different species (Zhao *et al*., 2011) and their nomenclature varies among researchers. Some studies classify the pepsinogen as pepsinogens A, B, and C, while in other studies, pepsinogens are named from I to IV. Phylogenetic analyses based on nucleotide sequences of more than 50 pepsinogens other than pepsinogen B revealed that progastricsin diverged first followed by prochymosin, and that pepsinogens A and F are most closely related (Kageyama, 2002). Their primary structures and enzymatic properties are significantly different from each other. The two major groups of immunogenetically and biochemically distinct enzymes are pepsins A and pepsin C, the latter also known as gastricsin. Pepsin C has an optimal pH slightly higher than pepsin A, but substrate specificity is quite the same for the two enzymes (Fusek and Vetvicka, 1995). Pepsin is most efficient in cleaving peptide bonds. The cleavage occurs on the amino (N) side of peptide bonds formed by aromatic amino acids such as phenylalanine, tryptophan, and tyrosine. Chymosins (pepsins Y) and foetal pepsins F are expressed at high levels in newborn to become gradually substituted by pepsin A during post-natal growth. These forms have been found in mammals and aves and not in other vertebrate groups. It has been supposed that they are riveted in the process of milk digestion or in the cleavage of egg yolk (Kageyama, 2002). Two major clades, one of pepsins C and another comprising pepsins A and pepsins F and Y together were identified by the analysis of the DNA sequence coding for various pepsins in 30 vertebrate species, specifically seven from fish, four from amphibians, two from chicken and the remaining from mammals (Carginale *et al*., 2004). Piscine pepsins A formed a well-separated group with respect to mammalian pepsins A and foetal pepsins. This relationship strongly supports the view that diversification of amniote pepsins occurred after the separation from the fish group. Comparative biochemical

studies established that pepsins show broad functional and chemical differences, based on which particular enzymes can be assigned as members of different gene families (Carginale *et al*., 2004). In contrast, the lowest animal with gastric pepsinogens is fish, which includes the marine fish African coelacanth (*Latimeria chalumnae*) (Tanji *et al*., 2007), Atlantic cod (*Gadus morhua*) (Gildberg *et al*., 1990; Gildberg, 2004), shark (Nguyen *et al*., 1998), Antarctic rock cod (*Trematomus bernacchii*) (Brier *et al*., 2007), arctic fish capelin (*Mallotus villosus*) (Gildberg, 1983), bolti fish (*Tilapia nilotica*) (El-Beltagy *et al*., 2004), Pacific bluefin tuna (Tanji *et al*., 1988, 1996, 2009), sea bream (*Sparuslatus Houttuyn*) (Zhou *et al*., 2007), rainbow trout (*Salmo gairdneri*) (Twining *et al*., 1983), orange-spotted grouper (*Epinephelus coioides*) (Feng *et al*., 2008), albacore tuna (*Thunnus alalunga*) (Nalinanon *et al*., 2009), European eel (*Anguilla anguilla*) (Wu *et al*., 2009) and smooth hound (*Mustelus mustelus*) (Bougatef *et al*., 2008). Information is available on pepsinogens and pepsins from freshwater fish such as the mandarin fish (*Siniperca chuatsi*) (Zhou *et al*., 2008), European eel (*Anguilla anguilla*) (Wu *et al*., 2009) and snakehead (*Channa argus*) (Chen *et al*., 2009). These include most significant families such as cathepsin D, cathepsin E, pepsin, renin, napsin and a new member termed nothepsin. The name 'Nothepsin' was chosen for the aspartic proteases identified for the first time in the *Antarctic Notothenioidei* (Capasso *et al*., 1998), specifically expressed in fish liver under oestrogen control (Riggio *et al*., 2002). Nevertheless, only two types of pro-enzymes, pepsinogens A and C were identified in fishes (Feng *et al*., 2008; Tanji *et al*., 2009).

The isoelectric pH (pI) of pepsinogens obtained from marine and fresh water fishes varies significantly. The pI values of three pepsinogens of Japanese seabass were 5.3, 5.1 and 4.7 (Cao *et al*., 2010) and of Pacific bluefin

tuna were 5.69, 4.95, and 4.66 (Tanji *et al*., 2009). These pI values are higher than those of the pepsinogens of the freshwater fish snakehead (4.8, 4.4, and 4.0) (Chen *et al*., 2009). Pepsins C (gastricsin) and pepsin A usually display maximal activity at pH 2–3 against haemoglobin, but the former has a specific activity twofold higher (Kageyama, 2000) with preference for Tyr at the P1 position (Tang, 1970). The existence of multiple forms of pepsin A in certain species correlated to the type of food or to the feeding habit. Plaice and rockcod have two and three forms of pepsin A, respectively. As both fishes live in cold waters, it can be explained as the increase in enzyme production to aid digestion at low temperatures. These enzymes display quite a high level of conservation. Chymosins, on the other hand, have optimal pH around 4 and a difference at the level of subsite S1 with respect to both the pepsins A and C. The cleavage specificity of chymosin is apparently due to the presence of a negatively charged residue located near the edge of the active site cleft (Kageyama, 2002). Very little is known about the properties and the specificity of pepsins F.

As important commercial enzymes, pepsins have several applications in food industries, pharmaceutical industry, biotechnology and other fields. They are recognized as significant markers of gastric cell development (Yasugi, 1994) and gastric diseases in clinical diagnosis (Karita *et al*., 2004; Yamada *et al*., 2006). Pepsin is prepared commercially from swine stomachs. Crude pepsin is used in the leather industry to remove hair and residual tissue from animal hides prior tanning. It is also used to treat effluent waste water to remove protein products. Another use is in the enzymatic dechromation of chrome-tanned wastes (Choudhary *et al*., 2004). It is also used in the recovery of silver from discarded photographic films by digesting the gelatin layer that holds the silver compound (Pethick and Money Limited (US6335042) 2002).

The pepsins and other gastric enzymes are synthesized in the chief and mucous neck cells of the fundic mucosa. The enzyme is synthesized as a pre-proenzyme with the signal sequence being removed in the usual fashion in the endoplasmic reticulum. The proenzyme is packaged into granules and stored inside the cells. Under the control of several signals from the vagus nerve and the hormonal secretions of gastrin and secretin, the granules fuse with the cell surface membrane and release their contents into the stomach. The drop in pH leads to protonation of groups within the active site and self-processing to the mature enzyme. Pepsinogens are autocatalytically converted into their corresponding active forms (pepsins) under harsh acidic environments, releasing the activation segments from their NH_2-terminal region (Dykes and Kay, 1976; Kageyama, 2002). There are two different mechanisms by which pepsinogen are converted into pepsin. One is a bimolecular reaction (an intermolecular reaction), in which a pepsin molecule converts pepsinogen into pepsin, and the other is a unimolecular reaction (self-activation by intra molecular cleavage that itself converts pepsinogen to pepsin). Al-Janabi *et al* (1972) in a study of pepsinogen activation observed that intramolecular reaction is predominant at a pH lower than 3.0. At pH 3.0, the bimolecular activation was 6.5 times faster than self-activation while at pH 2.0; the self-activation was 2 times faster.

All aspartyl proteases belong to the class of "β-proteins". As the name reveals, pepsin is made up mostly of β-sheets with only 6 observed helical sections, none consisting of more than 10 amino acids. Pepsin has fewer basic amino acid residues than any other proteins. This explains the stability of pepsins at extremely low pH because positive charges in acid media decrease the stability of polymeric structures. The complex tertiary hydrogen bonding of the molecule between the β sheets and other elements further contributes to

its stability at low pH. Pepsin also has 3 disulfide bridges. Pepsin, as depicted in figure below (**Fig.2.3.1**) has a crescent moon shape with a large, obvious active site.

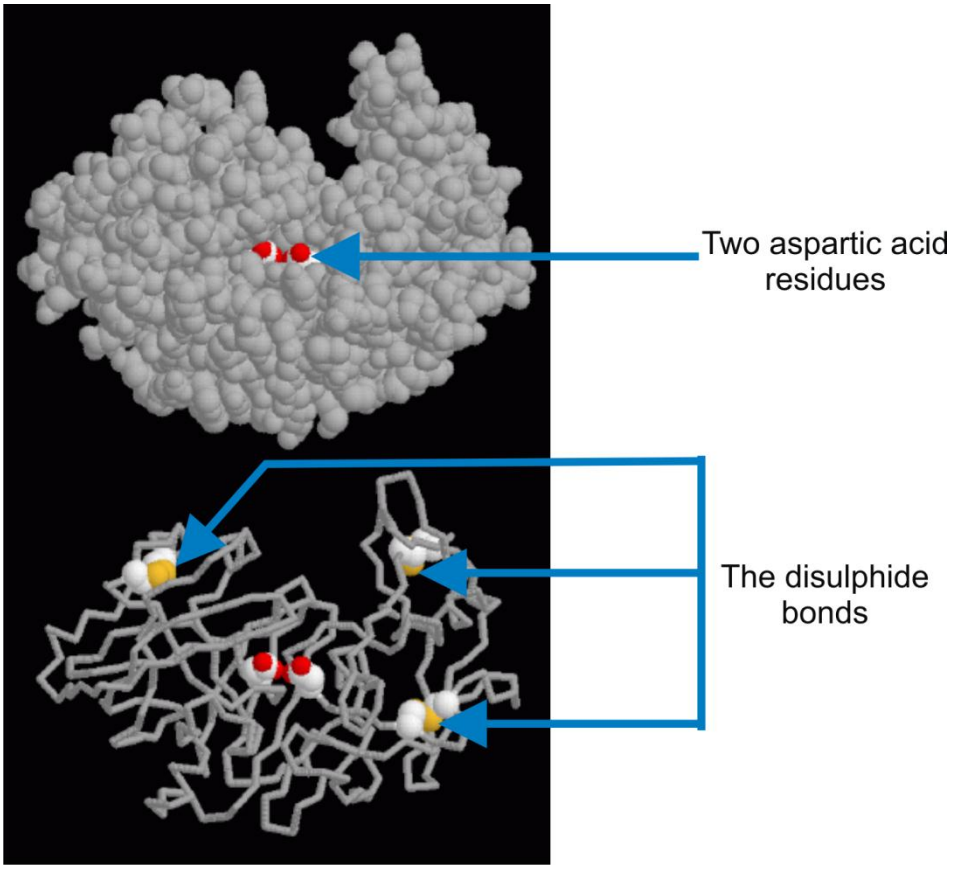


Fig. 2.3.1. Structure of pepsin adopted from Goodsell (2000)

The aspartic peptidase family of enzymes has been identified by the occurrence of the sequence Asp-Thr-Gly (Davies, 1990; Barrett *et al*., 1998). This is a critical amino acid sequence. The aspartic acid in this sequence provides one-half of the catalytic machinery of these enzymes. The Thr-Gly sequence following the Asp is essential for the formation of a unique catalytic conformation that also defines an enzyme of this class. In single-chain enzymes, two separate Asp-Thr-Gly sequences separated by 170 to 190 amino

acids are required to form the complete catalytic site. In the homodimeric enzymes of the retroviral aspartic proteinase subfamily, each monomeric unit has one Asp-Thr-Gly sequence. In either type of aspartic peptidase, the folding of the chains brings the two aspartates together to create the catalytic apparatus. Individual members of the superfamily differ in the topography of their active sites and in their cellular localization. In turn, these features determine physiological function (Takeda-Ezaki and Yamamoto, 1993; Sato *et al*., 1995). The primary structure of the zymogens of these enzymes includes a signal peptide (or presequence), its autocatalytic cleavage leads to the formation of the active enzyme (**Fig.2.3.2**) (Kageyama *et al*., 1989).

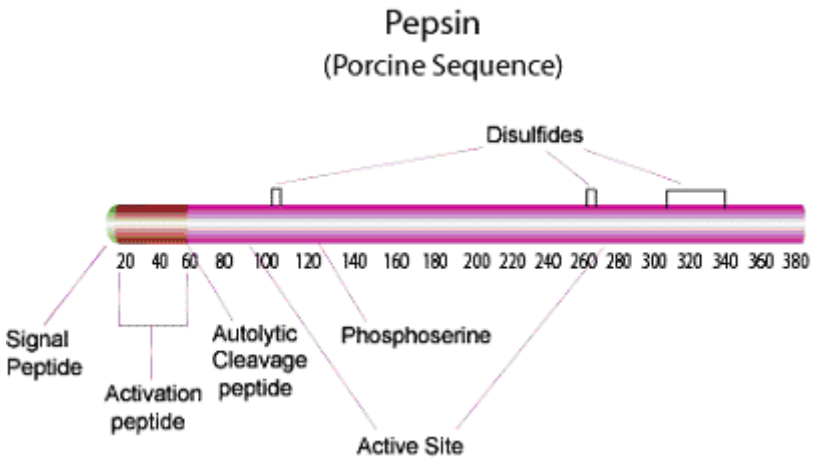


Fig.2.3.2. Porcine pepsin sequence adopted from *Sigma-Aldrich* product information sheet

The catalytic mechanism depends on the presence of two aspartic acids positioned roughly in the centre of a deep cleft forming the active site and covered by a hairpin loop (flap) protruding from the N-terminal lobe of the molecule.

The peptide bond that is cleaved by a protease is called the scissile bond**.** On the N-terminal side of the scissile bond, the residues of the substrate are denoted as P1, P2, P3... etc., in the C-to-N direction. On the C-terminal

side of the scissile bond, the residues of the substrate are denoted P1′, P2′, P3′... etc., in the N-to-C direction. The active site cleft of an aspartic acid protease can accommodate about seven residues of a substrate. These residues are usually designated as P4-P3-P2-P1*P1′-P2′-P3′ and the scissile peptide bond between P1 and P1′ indicated by an asterisk. The P1 and P1′ residues normally are of hydrophobic in nature. The corresponding subsites that constitute the topography of the active site cleft in each enzyme are designated accordingly as S4-S3-S2-S1-S1′-S2′-S3′ (Fusek and Vetvicka, 1995). The single chain enzymes can also be described as two-domain proteins (Todd *et al*., 2001), with mainly beta structure. Both the Asp-Thr-Gly sequences are always lead by two hydrophobic amino acids. In porcine pepsin A1, a conserved tyrosine appears 43 residues after the first Asp. In the family A2 of retroviral peptidases, this Tyr is absent. The tyrosine residue plays an important role in defining the active site pockets that bind substrate side-chain residues, separating the S1 binding pocket from the S2′ pocket. A conserved glycine residue in the sequence Leu-Gly-Ile occur 47 residues after the tyrosine. It passes through a wide loop containing the Asp-Thr-Gly sequence to form the "Psi-loop." The Leu and Ile residues fill up space above and below the Gly so that the Psi-loop is "locked" in place. The Psi-loop motif is repeated in the second half of the protein.

2.3.1. Mechanism of Catalysis

On the basis of the available kinetic and X-ray crystallographic data, it is proposed that two catalytically competent carboxyl groups of aspartic proteases constitute a functional unit which mediates the proton from the attacking water molecule to the leaving nitrogen atom of the substrate peptide bond. The aspartate residue in the second domain (Asp 215) serves as the

general base to abstract a proton from a water molecule. The water molecule simultaneously attacks the carbonyl carbon while the first aspartate residue (Asp 32) provides electrophilic assistance to the carbonyl oxygen. The pH-dependence studies indicated that the two carboxyl groups must react in different forms, one in the ionized, and the other in the unionized form. The resulting tetrahedral intermediate (Fraser *et al*., 1992; Veerapandian *et al*., 1992) can break down if the nitrogen of the peptide bond acquires a proton from solvent or through inversion of the nitrogen so that Asp 215 can give back the proton it accepted from the attacking water molecule. In either case, the amino group would then be a suitable leaving group, resulting in the formation of the amine and carboxyl products of the reaction. The rate limiting final step would be the departure of products from the active site.

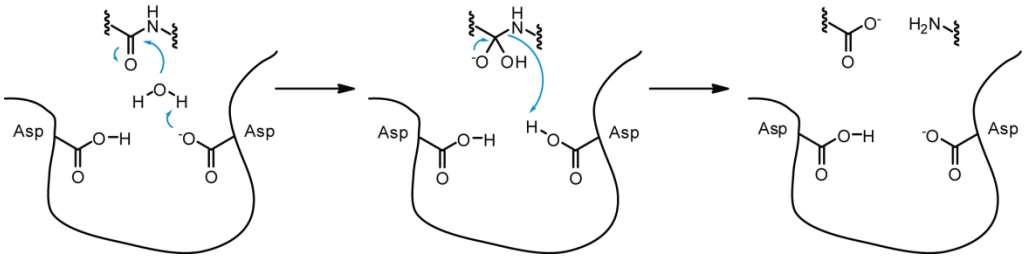


Fig.2.3.3. Mechanism of peptide hydrolysis by pepsin proposed by Suguna *et al* 1987

2.3.2. Secretion and Activation of Aspartic acid Proteases

In vertebrates, two different modes for the regulation of acid secretion in stomach have been identified. Some species exhibit a continuous acid secretion maintaining a low gastric pH during fasting. Others, as some teleosts, maintain a neutral gastric pH during fasting while the hydrochloric acid is released only after the ingestion of a meal. Those different patterns seem to be closely related to specific feeding habits (Yufera *et al*., 2012). The pepsins and other gastric enzymes are synthesized in the chief and mucous neck cells of fundic mucosa.

These enzymes are synthesized as pre-proenzymes with a signal sequence being removed in the usual fashion in the endoplasmic reticulum (ER). The proenzyme is packaged into granules and stored inside the cells. Under the control of several signals, the granules fuse with cell surface membrane and release their contents into the stomach. The drop in pH leads to protonation of groups within the active site and self-processing to mature enzyme. In contrast, cathepsin D and E are intracellular enzymes (Saku *et al*., 1991). The former is also made as a proenzyme and targeted to the lysosomes of cells by the mannose-6-phosphate receptor mediated pathway (Rijnboutt *et al*., 1991). There it is activated to mature form by the action of unknown cysteine proteinases within the organelle. The biological niche occupied by aspartic peptidases is extremely broad, although their presence is generally restricted to acidic environments within cells and tissues.

2.3.3. Specificity of Pepsin like aspartic acid proteases

Aspartic proteinases exhibit a range of substrate specificity, from the extremely broad to absolutely specific. Generally, a rate assay based on the protein substrates haemoglobin (Anson, 1938), casein (Kunitz, 1947) or serum albumin (Gripon and Hofmann, 1981) has been used for the evaluation of enzyme activities. Alternatively, the activation of trypsinogen has provided a very sensitive assay for many of the aspartic proteinases (Nakanishi, 1959; Sodek and Hofmann, 1970; Morihara and Oka, 1973). A milkclotting assay has been used for the determination of activity of enzymes from *Endothia parasitica* (Whitaker, 1968) and from *Mucor pusillus* (Arima *et al*., 1968). Like most proteolytic enzymes, the aspartic proteinases bind substrates and most inhibitors within an extended active site cleft. Bound ligands typically adopt an L-strand conformation. Interactions with groups on both sides of the cleft determine the

primary as well as secondary specificity of the enzymes (Dunn and Hung, 2000). Pepsin has broad specificity with a preference for peptides containing linkages with aromatic or acidic L-amino acids. It preferentially cleaves C-terminal to Phe and Leu and to a lesser extent Glu linkages. Pepsin's main role in protein proteolysis is to cleave aromatic amino acids, such as phenylalanine and tyrosine, from the N-terminus of proteins (Raufman, 2004). The enzyme does not cleave at Val, Ala, or Gly. The major sites of pepsin C attack were reported as the Ala-14-Leu-15 and Tyr-16-Leu-17 bonds (Ryle and Porter, 1959).The gastric pepsins and the secreted fungal enzymes are charged with a general role in degradation of nutrient proteins and tend to have powerful broad specificity. The acidic milieu aids in the unfolding of substrate proteins, so that the enzymes can readily attack the exposed peptide bonds. At the other extreme, human renin is very selective, cleaving only one bond within the sequence of human angiotensinogen (Green *et al*., 1990). In addition, animal renins are unable to cleave human angiotensinogen (Ganten *et al*., 1992). It may be that the structure of renin has evolved to recognize the cleavage sequence within a partly unfolded substrate protein. While the overall structure of renin is very similar to that of other members of the family, the active site is more restricted, leading to the suggestion that there is less adaptability in the fit between substrate and enzyme.

The aspartic proteinases from the Antarctic fish, *Notothenioidei* are in many ways different from the mammalian aspartic proteinases. Life in the harsh conditions of Antarctic waters has resulted in alterations to the enzymatic properties and expression of these proteases **(**Luca *et al*., 2009**)**. Secondary structure calculations from the far-ultra violet circular dichroism (far-UVCD) spectral data showed that both Antarctic enzymes contained a high proportion of β-sheets (56.1% for A1 and 52.2% for A2), as reported for mammalian pepsins (Cooper *et al*., 1990). The confirmation of fish pepsins A1

and A2 indicating no substantial secondary structural differences **(**Luca *et al*., 2009**).** Zymography analysis indicated that pepsins of Japanese seabass revealed three pepsins that were quite different in mobility and enzymatic activity under native conditions suggesting that they are different from each other (Cao *et al*., 2011).

2.3.4. Structure of Pepsin

Aspartic proteinases comprise a group of bilobal enzymes, characterised by the presence of a deep active-site cleft containing two aspartic acid residues that are essential for catalytic activity. In 1978, Tang *et al* predicted that monomeric aspartic proteinases are formed by gene duplication and fusion of an ancestral proteinase that exists as a dimer. The active site itself is formed at the interface on the N- and C-terminal domains and exhibits approximate two-fold symmetry. Since the retroviral proteases are only about one-third the size of the two-domain eukaryotic enzymes, they were hypothesized to function as dimers in which each monomer contributes a single aspartic acid to the active site (Pearl and Taylor, 1987). Subsequently, it has become apparent that retroviruses contain a similar dimeric proteinase, in which each subunit contains one aspartic acid and where the subunit corresponds to one half of the bilobal pepsin-like enzymes (Miller *et al*., 1989). Pepsin is a monomeric, two domain, mainly beta protein with a high percentage of acidic residues. Porcine pepsin has 4 basic residues and 42 acidic residues and is *O*-phosphorylated at S68 (Tang *et al*., 1973). For the protein to be active, one of the two aspartate residues in the catalytic site has to be protonated, and the other deprotonated. This occurs between pH 1 and 5, and above pH-7 pepsin is irreversibly denatured. The amino acid sequence of porcine pepsin was determined by Tang *et al* (1973) and Moravek and Kostka (1974), and later confirmed through cDNA analysis by Tsukagoshi *et al* (1988) and Lin *et al*

(1989). The pepsinogen-A gene is divided among nine exons (Sogawa, 1983). There are multiple versions of the pepsinogen- A genes found in human and chimp populations, but the activities of these various gene products are indistinguishable (Taggart *et al* 1985; Zelle, 1988). In contrast, Southern blot analysis of rhesus monkey found a single pepsinogen-A gene (Evers *et al*., 1988).

Pepsinogen A production is mainly controlled at the transcription level (Sogawa *et al*., 1983; Ichinose *et al*., 1988). In both humans and rats, it has been found that the pepsinogen A gene is under tissue-specific transcriptional control, with mRNA detected only in gastric fundic mucosa (Ichinose *et al*., 1990; Ichinose *et al*., 1991; Meijerink *et al*., 1993). Transcription of the pepsinogen A gene is regulated by transcription-activating proteins acting at 3 major regions in the promoter and initiation regions of the pepsinogen-A gene (Meijerink *et al*., 1993).

There are four reported pepsin proteins: pepsin A, pepsin B (parapepsin I), pepsin C (gastricsin), and pepsin D (an unphosphorylated version of pepsin A) (Lee and Ryle, 1967). Pepsin A is the predominant gastric protease with minor amounts of other pepsins. Pepsins B and C share a higher degree of homology with each other. In dog, B and C share 89% identity, A and B share 44% identity, and A and C share 45% identity (*Worthington Enzyme Manual*)

2.3.5. Pepsin mediated protein digestion in Teleosts

Two basic types of digestive tract can be observed in the different fish species, with and without stomach. In species with a stomach, the adult-mode of food processing implies an acid digestion phase and consequently a highly efficient extracellular digestion of proteins (Kapoor *et al*., 1975; Segner *et al*., 1994). This step marks the definitive change in feeding patterns, from a relatively continuous foraging on planktonic prey to a wide range of different species-specific feeding habits. In juvenile and adult fish, the gastric glands

covering the inner layer of the stomach follow a species-specific pattern and produce both pepsinogen and proton pump H^+/K^+-ATPase. This enzyme is responsible for the secretion of the hydrochloric acid that decreases gastric pH and induces the conversion of pepsinogen into pepsin (Darias *et al*., 2005; Darias *et al*., 2007; Wu *et al*., 2011).

Two special modes for regulation of stomach acid secretion have been described in vertebrates. First one, characterized by a continuous acid secretion and the maintenance of a low pH during fasting has been described in humans (McLauchlan *et al*., 1989; Kalantzi *et al*., 2006), dogs (Sagawa *et al*., 2009), some elasmobranchids (Papastamiou and Lowe., 2004) and some teleost fish (Montgomery and Pollak., 1988; Sugiura *et al*., 2006; Bucking and Wood, 2009). Second one, characterized by maintenance of a neutral gastric pH during fasting, on which hydrochloric acid is released followed by the ingestion of a meal, has been reported in some snakes (Secor, 2003; Cox and Secor, 2010) and in few teleosts (Deguara *et al*., 2003; Yufera *et al*., 2004; Nikolopoulou *et al*., 2011). These different patterns seem to be closely related to specific feeding habits. Although frequent feeders and species with random food availability have a propensity to maintain a low gastric pH (Papastamiou and Low, 2004; Papastamiou, 2007), less frequent feeders may recover a neutral gastric pH between meals (Papastamiou and Lowe, 2005; Cox and Secor, 2010). However, Montoya *et al* (2010) suggested that, at least in some species, gastric acidification could be modified by alterations in daily feeding patterns and feeding frequency. This adaptive capacity in the digestive function may be related to the ability to await some physiological responses exhibited by many vertebrates in order to optimize the digestion process.

Generally two types of pepsins, Pepsin I and Pepsin II, are present in fish. Pepsin I is reported to be most abundant in species like sardine (Sardinops

melanostica) and capelin (*Mallotus villosus*) (Noda and Murakami, 1981; Gildberg, 1983), while pepsin II seems to be most abundant in species like cod (*Gadus morhua*) and salmon (*Oncorhynchus keta*) (Gildberg, 1988; Sanchez-Chiang *et al*, 1987). Fish pepsins have very low activity on small peptide substrates, and like other aspartic proteases, exhibit high activity on hemoglobin (Sanchez- Chiang *et al*., 1987; Squires *et al*., 1986). Pepsin I hydrolyzes hemoglobin maximally between pH 3 and 4, and pepsin II between pH 2 and 3. Pepsins of cold and temperate water fishes have maximal stability between pH 2 and 5, while pepsins from warm water species are stable even at pH 7 (Kubota and Ohnuma, 1970). The optimal temperature for these enzymes is between 37 and 55°C, and they have 40 to 60% relative activity at 5 to 10°C, but lower thermostability than mammalian pepsins. Active pepsins have molecular weights close to 35 kDa (Gildberg, 1988; Gildberg, 1983). Isoelectric points (pI) of pepsin I and pepsin II are from 6.5 to 7, and 4 to 4.5, respectively. The acidic proteolytic enzymes isolated from Monterey sardine viscera belong to the aspartic protease class and are similar to pepsin II reported in other fish species (Castillo-Yanez, 2004). Generally, the isoelectric points of mammalian pepsins are lower than those of fish pepsins, may be because of the higher content of basic amino acids in fish pepsins (Herriot, 1962).

2.3.6. Applications

Conventional fish waste disposal techniques are inadequate for a more reasonable utilization approach of fish wastes as well as effective recovery of valuable ingredients. Fish wastes can be utilized as animal feed ingredients as well as organic fertilizers (Gildberg and Raa, 1977; El-Beltagy *et al*., 2004). The recovery of valuable biomolecules such as ω-3 fatty acids (Yoshida *et al*., 1999), trypsin (Genicot *et al*., 1996; Klomklao *et al*., 2006), chymotrypsin

(Castillo-Yáñez *et al*., 2006), elastase (Gildberg and Overbo, 1990), collagen (Nalinanon *et al*., 2007) from fish viscera have been reported. The use of such products, especially purified digestive enzymes in gelatin extraction (Nalinanon *et al*., 2008), in cheese making (Aehle, 2007) and as aids in digestion have also been reported. Along with these valuable products that can be recovered from fish, pepsin is one of the plenteous and valuable biomolecules that can be recovered from fish visceral wastes. Pepsins recovered from fish viscera not only reduce the capital costs of enzyme production significantly, but also partially reduce the cost of disposal of fish wastes and minimize environmental pressures associated with it.

Fish pepsin is considered as a promising enzyme in applications such as collagen extraction (Benjakul *et al*., 2009). Usually, collagen is extracted by the acid-solubilization process, in which pepsin assistance brings higher collagen yield (Jongjareonrak *et al*., 2005; Nalinanon *et al*., 2007; Benjakul *et al*., 2009). By using abundant fish stomach as a source of fish pepsin to produce collagen, the cost of pepsin and collagen production can be reduced significantly. Pepsin can be applied as a rennet ingredient for the production of good quality cheese. There are two types of commercial coagulants available in cheese processing: animal rennet and microbial coagulants (Aehle, 2007). Pepsin mixed with chymosin in a standardized ratio forms the animal rennet. Cod pepsin and tuna pepsin have been proved to be practicable in cheese production (Han, 1993; Tavares *et al*., 1997). Cheese production based on fish pepsin has not yet been commercialized (Gildberg, 1992; Aehle, 2007). Fish pepsin can help in the production of fish silage and fish sauce. Both fish silage and fish sauce are highly nutritious protein hydrolysates made from whole fish or fish viscera by fermentation (Hariono *et al*., 2005; Murado *et al*., 2009). Pepsins in cod viscera have been proven to function well under acidic

conditions in the aqueous phase of silage processing (Gildberg and Almas, 1986). Minced fish material can be easily degraded and fermented because pepsin is naturally present in fish stomach. Pepsin is utilized in the regulation of digestion, as a dental antiseptic and in the treatment of ailments including dyspepsia, gastralgia, obstinate vomiting, infantile diarrhea, apepsia and some cancers (Gorgas, 1884). Combined with HCl, many pepsin tablets and capsules are developed to support the digestibility of the gastrointestinal tract as well as to enhance patients' appetite (Murado *et al*., 2009). Apart from this function, pepsin from porcine stomach is used for the treatment of gastric ulcers with bismuth complexes added (Almas, 1990). Pepsin was also added for better digestibility of proteins in animal feed. It is used also in subculture of viable mammary epithelial cells (Riser, 1983).

2.4. Materials and Methods

2.4.1. Fish and Preparation of Crude Enzyme Extract

Experimental fishes of almost similar size (10-12cm) were collected from the Fisheries station, Kerala University of Fisheries and Ocean Studies, Puthuvyppu. The fishes were acclimated to laboratory condition for a week. A commercial diet with known proximate composition was given *ad libitum.* The fishes were starved for approximately 12 h prior to sampling, subsequently killed by cold shock, and dissected immediately. The stomach contents were squeezed out and rinsed with cold distilled water to remove feed remnants. Ten percent (w/v) tissues homogenate was prepared in cold Tris–HCl 50 mM buffer pH 7.2 using an electric homogenizer (KEMI Model No: KHH 1), in ice-cold condition. The homogenate was then centrifuged at 4°C at 10,000*g* for 10 min. The supernatant containing the enzymes was stored at -20^{0}C until the analysis.

2.4.2. Methods

The tissue homogenates were purified by Trichloro acetic acid (TCA) precipitation and the precipitate of soluble protein was dissolved in 0.1M NaOH. The soluble protein content of enzyme extract was measured according to Lowry *et al* (1951) by using Hitachi-2900 UV-Visible spectrophotometer.

2.4.2.1. Determination of Specific Activity of Pepsin-like Acid Protease

Pepsin like acid protease was determined by Anson's method (1938). Briefly, 50µl of tissue homogenate was mixed with 950µl of 0.2M HCl-KCl buffer of pH-2 and incubated at 37^0C for 5 minutes. To this 1ml of 2% Haemoglobin in 60mM HCl (pH-2) is added and mixed well. The reaction mixture was then incubated for 10 minutes at 37^0C in a water bath. The hydrolysis is stopped by adding 1ml of 12% ice cold trichloro acetic acid (TCA) and kept it for 15 minutes at 4^0C to complete protein precipitation after vigorous mixing of the mixture. The mixture was then centrifuged at 10000rpm for 15 minutes and the optical density of aromatic amino acids, specifically tyrosine (Tyr), in the resulting supernatant was determined at 280nm by using corresponding reagent blank. A standard tyrosine curve has been prepared and the specific enzyme activity is expressed in Anson Unit (mM of Tyr/ min/ mg protein).

2.4.2.2. Characterization of Enzyme Properties

The optimum temperature for the pepsin catalyzed protein hydrolysis has been estimated by incubating the reaction mixture at different temperatures ranging from 20^0C to 80^0C. A curve is plotted connecting specific activity and temperature and the optimum temperature is determined as the temperature at which the enzyme shows its maximum specific activity.

Different non- reactive buffer systems which are generally used to obtain specific pH values have been used in the determination of optimum pH for pepsin activity. 0.2M HCl-KCl buffer is used to obtain pH of 1, 1.5 and 2. A Glycine-HCl (0.2 M) buffer system is used to achieve pH of 2.5, 3 and 3.5. The Citrate-Phosphate buffer system (0.2M) is selected to get pH values 4, 4.5, 5, 5.5, 6, 6.5 and 7. 0.2M Tris-HCl is used to get pH 7.5, 8, 8.5 and 9. pH 9.5, 10 and 10.5 are obtained by using Glycine-NaOH buffer system. Since pepsin completely loses its activity beyond a pH of 7.0, alkaline pH values are not selected for the study. A graph of specific activity against pH has been plotted to obtain the optimum pH.

The optimum substrate concentration has been estimated by preparing a series of Haemoglobin concentrations ranging from 0.5% to 3% in 60mM HCl having pH of 2. A substrate saturation graph was constructed using the experimental data.

2.4.2.3. Determination of the Effect of Metal Ions

The effect of different metal ions, specifically Lithium (Li), Sodium (Na), Potassium (K), Magnesium (Mg), Calcium (Ca), Barium (Ba), Manganese (Mn), Iron (Fe), Copper (Cu), Zinc (Zn) and Mercury (Hg) has been determined. The specific metal chlorides (LiCl, NaCl, KCl, $MgCl_2.6H_2O$, $CaCl_2$, $BaCl_2.H_2O$, $MnCl_2$, $FeCl_3$, $CuCl_2.2H_2O$, $ZnCl_2$ and $HgCl_2$) were dissolved in double distilled water to obtain a concentration of 15mM, 20mM and 25mM solutions. The tissue homogenate after overnight dialysis in 50mM Tris buffer pH 7.2 (buffer is replaced in every 1 hour) was mixed with equal volumes of specific metal ion solutions and incubated for 15 minutes prior to the start of experiment and proceeded as described in the determination of specific activity.

Estimation of Metals in crude enzyme sample

The crude extract of different tissue samples (protein content 1g) after dialysis was repeatedly digested using 1:5 conc.$HClO_4$ and conc.HNO_3, and then evaporated to dryness (Loring and Rantala, 1992). The dry residue was dissolved in 0.1N HNO_3 and made up to 50ml. Further analysis was conducted by Thermo iCAP Duo ICP (Inductively Coupled Plasma) spectrophotometer at the Inter University Center for Development of Marine Biotechnology, CUSAT. The accuracy of the metal analyses was checked using triplicate analysis of a certified reference material (BCSS-1, National Research Council of Canada). The triplicate analysis of BCSS-1 showed a good accuracy and recovery rate.

2.4.2.4. Determination of Temperature Stability

The thermal inactivation kinetics of peptic hydrolysis has been obtained by incubating the tissue homogenate at different temperature for an hour with subsequent sampling of enzymes at specific time interval of 5 minutes (samples from the incubated enzyme were taken at time 0, 5, 10…and 60 minutes). Immediately after sampling the enzyme was cooled by placing ice cold water and the specific activity was estimated as described above.

2.4.2.5. Determination of pH Stability

The pH stability of the hydrolase has been estimated by mixing equal volumes of tissue homogenate and buffer with specific pH (1 to 7) and after incubation for an hour the specific activity has been determined. The stability is expressed as Relative activity which is obtained by dividing the actual

activity at specific pH with the highest activity obtained. A plot of relative activity Vs pH reveals the stability of enzymes at different pH values.

2.5. Results

Oreochromis mossambicus pepsin showed maximum hydrolytic activity in 2% acidified haemoglobin at pH 2.0 and 3.5, while *Etroplus suratensis* pepsin showed its maximum activity at pH 2.5 and 4-4.5 (**Fig.2.5.1** and **Fig. 2.5.4**). The stability of pepsins at different pH values shows dissimilar characteristics. The *O. mossambicus* pepsins are highly stable at pH 2.5 and pH 4 (**Fig.2.5.11**) and in the case of *E. suratensis*, at pH 2.5-3 and at pH 4, the pepsins showed significant stability (**Fig.2.5.12**).

O. mossambicus and *E. suratensis* showed different optima in substrate saturation concentration with respect to haemoglobin, 1% and 2% haemoglobin for *O. mossambicus* and *Etroplus suratensis* (**Fig. 2.5.2** and **Fig. 2.5.6**) respectively.

The pepsin activity of *E. suratensis* shows a broad temperature spectrum with optimum range 40-45^{0}C (**Fig.2.5.5**). The *O. mossambicus* has its optimum pepsin activity at 40^{0}C (**Fig.2.5.3**). This pattern was similar to other marine fish like Pacific bluefin tuna (*Thunnus orientalis)* (de la Parra *et al*., 2007). The thermal inactivation patterns of both *O. mossambicus* and *E. suratensis* pepsins show similar characteristics. They are very stable up to 45^{0}C and the activity diminishes rapidly at 55^{0}C (**Fig.2.5.9** and **Fig.2.5.10**).

The crude extracts of the various tissues, specifically stomach, hepatopancreas and intestine of the selected fishes, *Etroplus suratensis* and *Oreochromis mossambicus* showed below detectable levels (BDL) for the eleven selected metals namely Lithium (Li), Sodium (Na), Potassium (K),

Magnesium (Mg), Calcium (Ca), Barium (Ba), Manganese (Mn), Iron (Fe), Copper (Cu), Zinc (Zn) and Mercury (Hg). The lowest detection limit of each metal is given below.

Table 2.1 Lowest detection limit of different metals by ICP

Metal	Wave length (nm)	Lowest detection limit (μg/L)
Lithium (Li)	670.7	0.83
Sodium (Na)	589.5	1.80
Potassium (K)	766.4	5.10
Magnesium (Mg)	279.5	0.04
Calcium (Ca)	393.0	0.05
Barium (Ba)	455.0	0.07
Manganese (Mn)	257.6	0.21
Iron (Fe)	259.9	0.8
Copper (Cu)	324.7	2.36
Zinc (Zn)	213.9	0.6
Mercury (Hg)	184.9	1.10

The action of various metal ions on pepsin action varies according to the metal species and source of pepsin. Pepsins may either be activated or inhibited by the metal ions. Sodium at low concentration activates the *E. suratensis* pepsin. Potassium ions do not affect the enzyme action but magnesium at higher concentration inhibits the enzyme action. Calcium and barium showed an enhancing activity while manganese showed an inhibitory effect according to their increase in concentration (**Fig.2.5.8**). In the case of *O. mossambicus* pepsin, only mercury ions showed a slight inhibitory effect and all other metal ions do not have any significant effect on pepsin like acid proteases (**Fig.2.5.7**).

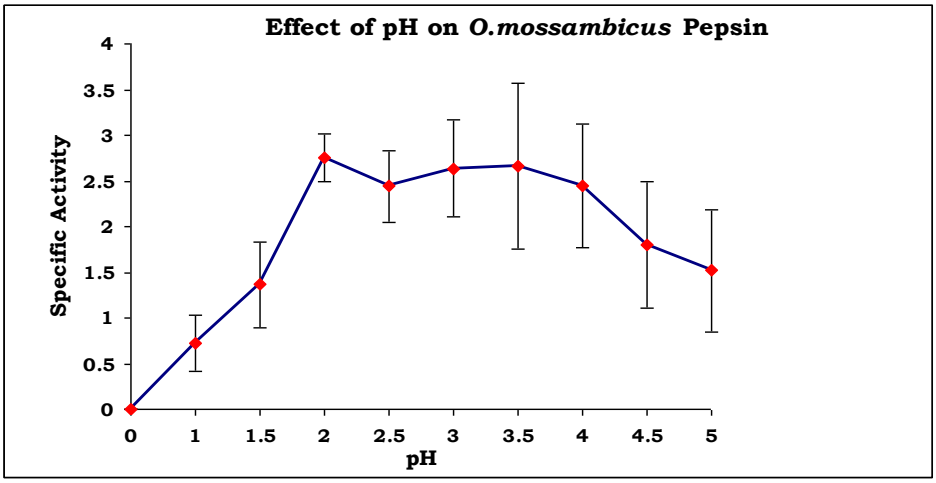


Fig. 2.5.1. Effect of pH on *O. mossambicus* pepsin

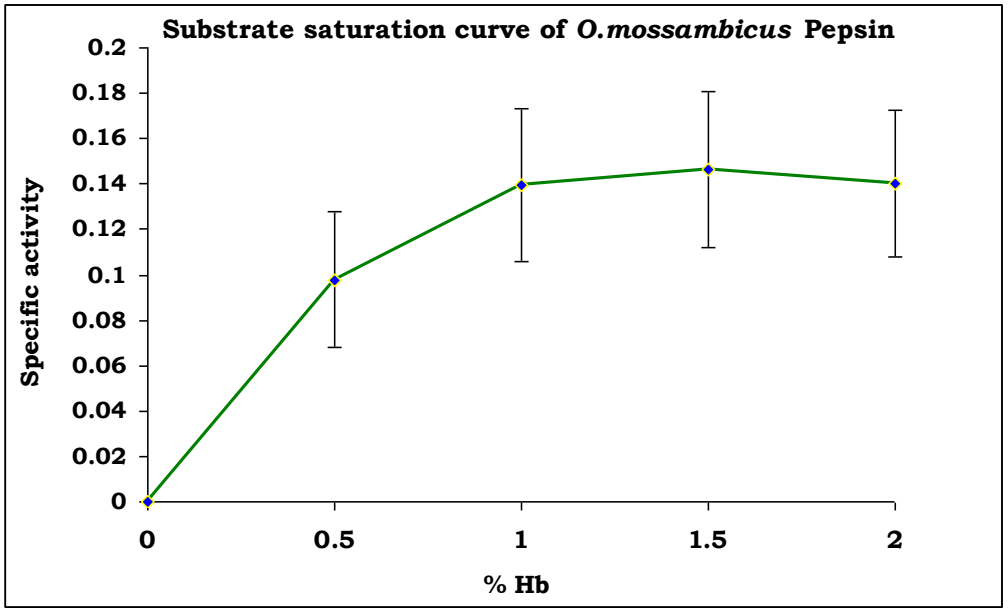


Fig. 2.5.2. Substrate saturation curve of *O. mossambicus* pepsin

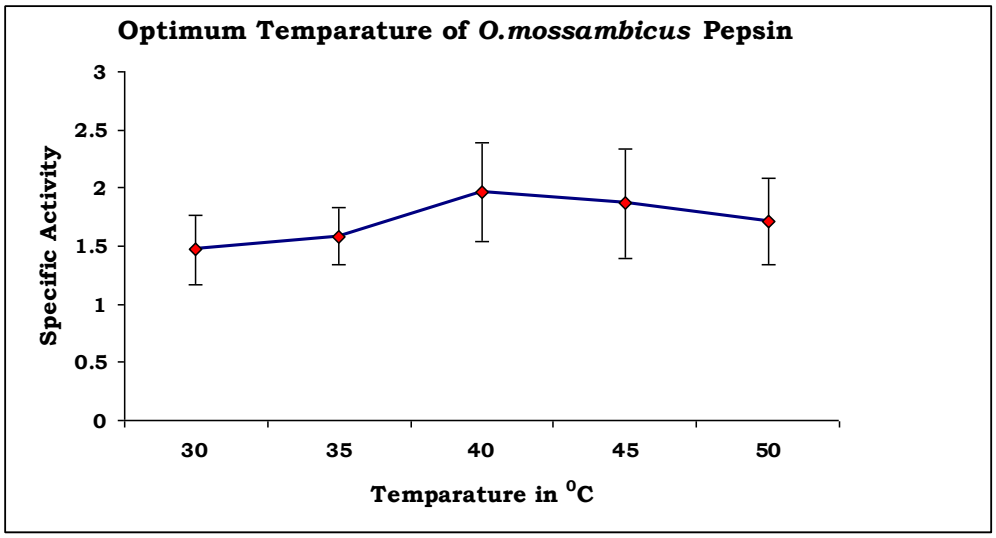


Fig. 2.5.3. Thermal optimization curve of *O. mossambicus* pepsin

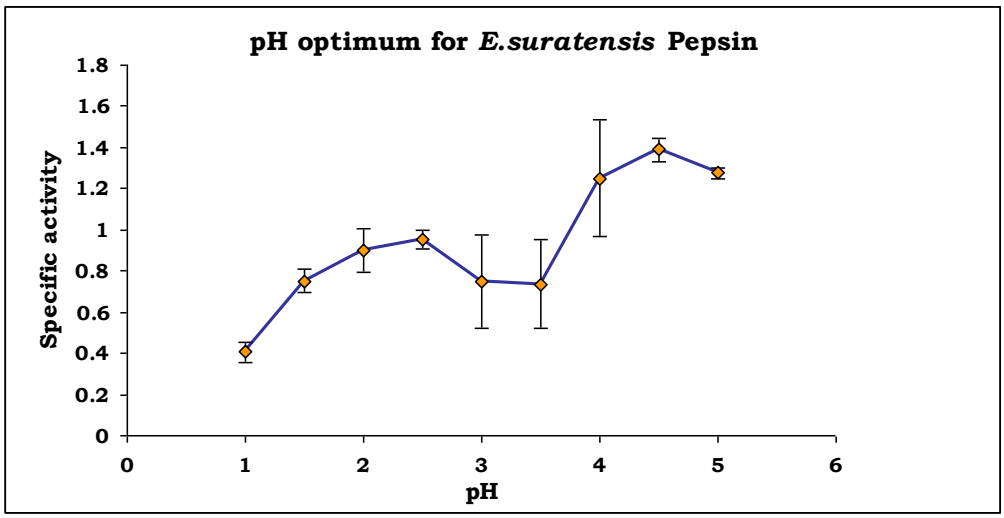


Fig. 2.5.4. Effect of pH on *E. suratensis* pepsin

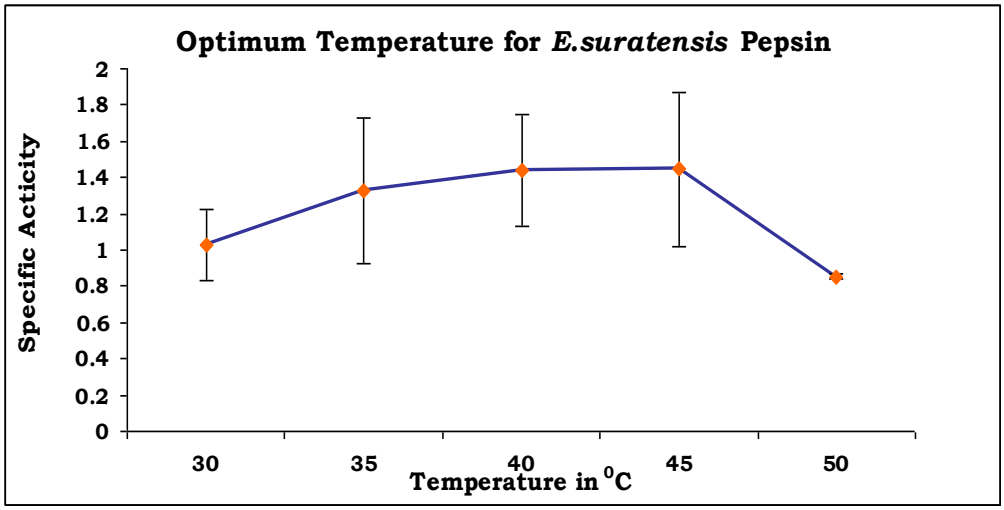


Fig. 2.5.5. Thermal optimization curve of *E. suratensis* pepsin

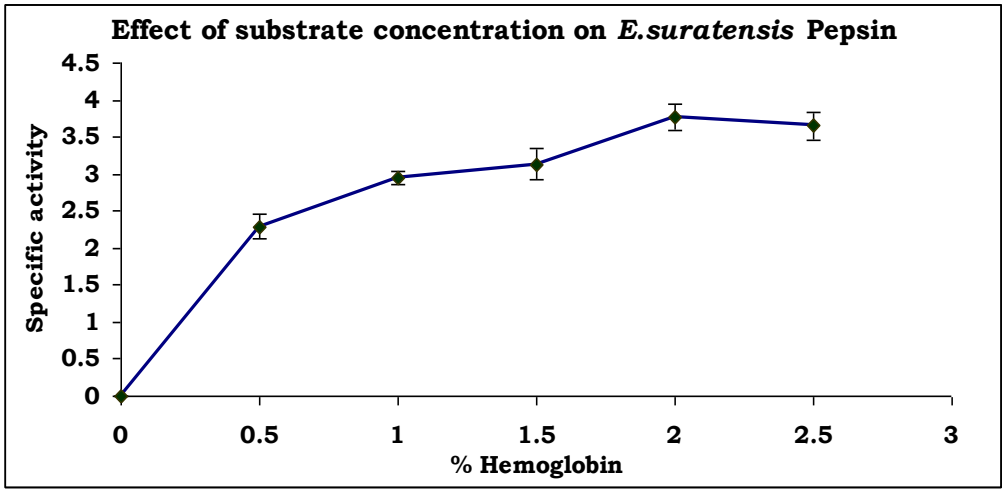


Fig.2.5.6. Substrate saturation curve of *E. suratensis* pepsin

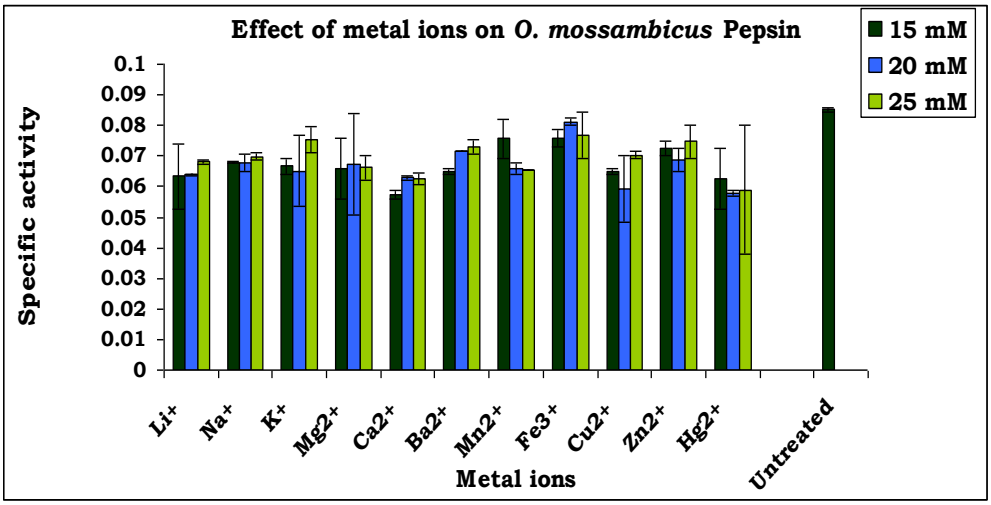


Fig.2.5.7. Effect of metal ions on *O. mossambicus* pepsin

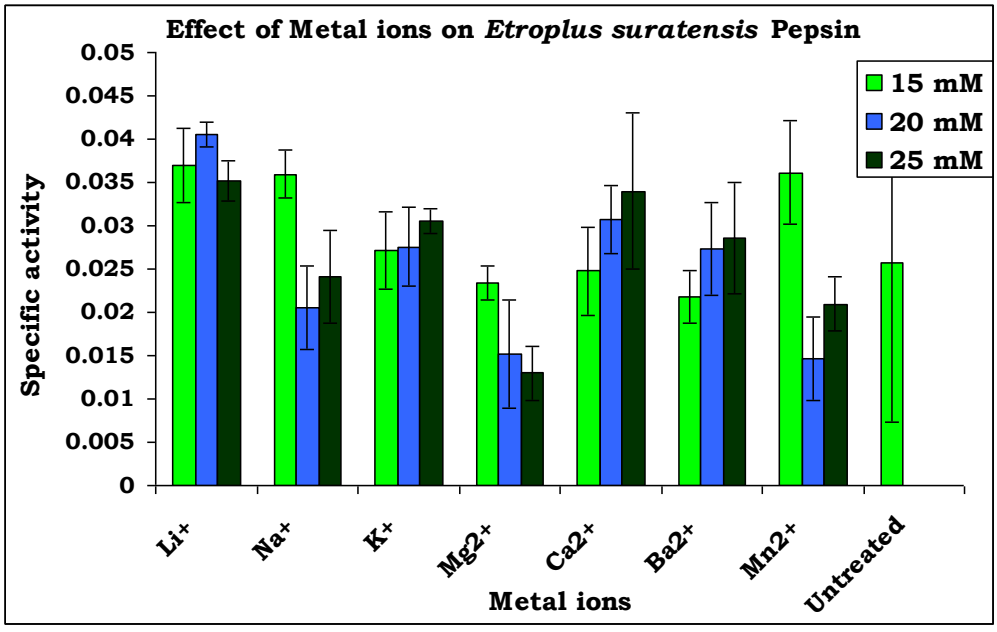


Fig. 2.5.8. Effect of metal ions on *E. suratensis* pepsin

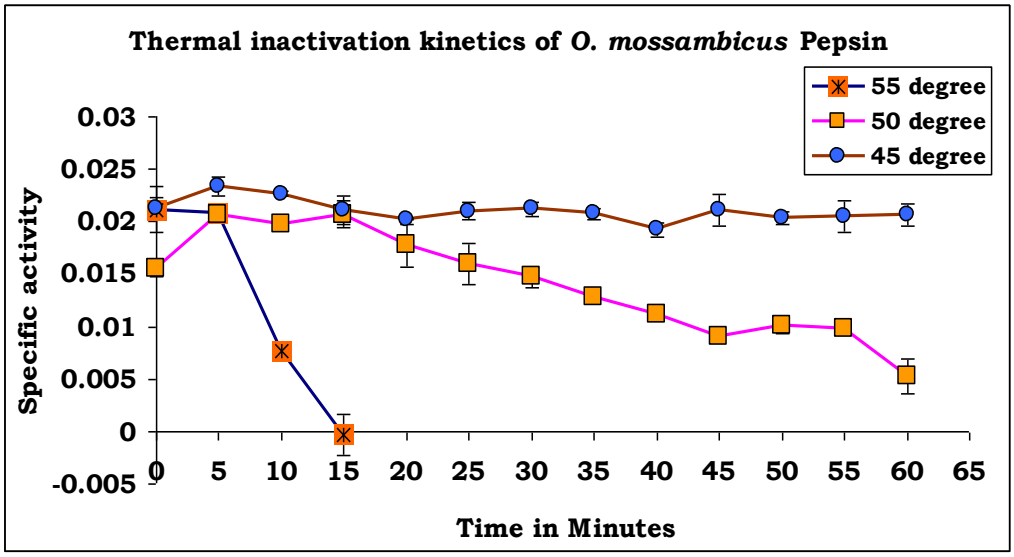


Fig. 2.5.9. Thermal inactivation kinetics of *O. mossambicus* pepsin

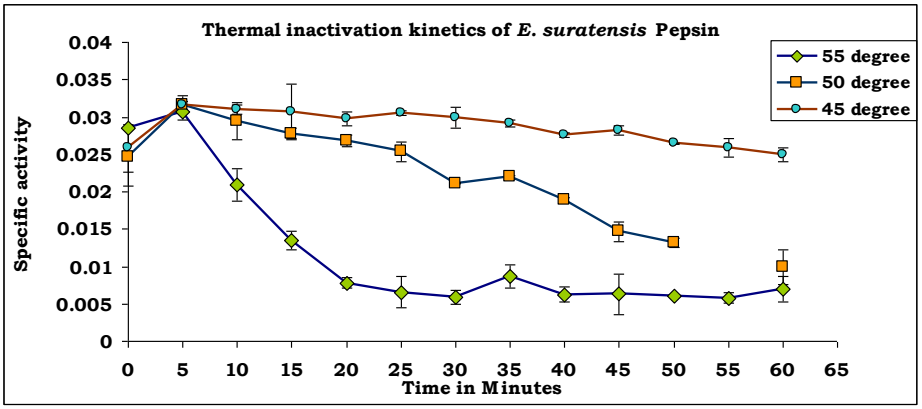


Fig. 2.5.10. Thermal inactivation kinetics of *E. suratensis* pepsin

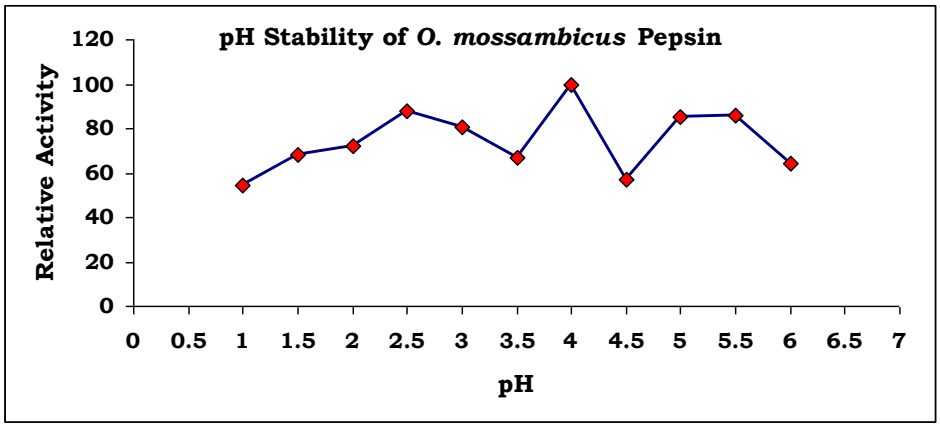


Fig. 2.5.11. pH stability curve of *O. mossambicus* pepsin

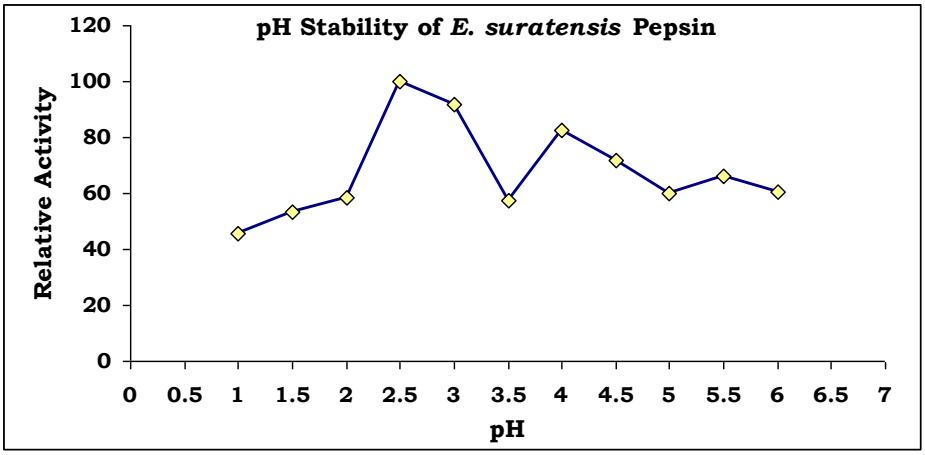


Fig. 2.5.12. pH stability curve of *E. suratensis* pepsin

2.6. Discussion

In aquaculture, investigations on protease activity of various marine as well as fresh water organisms have been carried out to develop cost effective feeds for meticulous farming of such species through thorough knowledge about their digestive capabilities (Clark *et al*., 1985; Alarcon *et al*., 1998). This may help in the selection of feed ingredients especially the proteins containing some indispensable amino acids (Lan and Pan, 1993). Several investigations devised to characterize digestive enzymes and to develop practical applications for

digestive proteases have been performed in recent years (Jiang *et al*., 1991; Garcıa-Carreno, 1992; Haard, 1992). The rate of digestion and absorption of essential amino acids during proteolysis can be obtained with proper knowledge of activity of proteases (Eshel *et al*., 1993). Knowledge of digestive enzymes might clarify the nutritional biochemistry and physiology of fish and it also helps to determine its digestive capabilities, which in turn helps the selection of ingredients to be included in a diet. It may be useful to avoid autolysis and to ascertain the maximum shelf life period of both formulated feed and the organism itself. Pepsin has an extracellular function as the major gastric proteinase (Klomklao *et al*., 2007). Multiple forms of pepsinogens are known to occur in various animals (Foltmann, 1981) and most of them are considered to be products of different genes as in human (Zelle *et al*., 1988) and cow (Lu *et al*., 1988). The presence of multiple forms of pepsin A in some species may be correlated to the type of food or to the feeding habitat (Kageyama, 2000). Fish pepsin may also be present in many isoenzyme forms (Squires *et al*., 1986) and different fishes have different amounts of pepsinogens and pepsins. Bougatef *et al* (2008) isolated only one type of pepsinogen from the stomach of smooth hound. Gildberg (1983) successfully isolated two pepsinogens from the arctic fish capelin. Tanji *et al* (2007) found three types of pepsinogens namely pepsinogen-I, pepsinogen-II and pepsinogen-III, which corresponded to three different types of pepsins in African coelacanth stomach. Wu *et al* (2009) found three kinds of pepsinogens in the stomach of European eel. Sea bream stomachs contained four types of pepsinogen (Zhou *et al*., 2007). The mucosa of mandarin fish contained four pepsinogens (Zhou *et al*., 2008). Pepsins and pepsin-like enzymes have been isolated and characterized in several fish species such as Atlantic cod (Gildberg and Overbo., 1990), monterey sardine (Castillo-Yañez *et al*., 2004) and pectoral rattail (Klomklao *et al*., 2007). Among fishes, pepsins

and pepsinogens from cod and tuna were the most thoroughly investigated. The enzymes from Cod have been studied and sequenced by several researchers (Gildberg *et al*., 1990; Karlsen *et al*., 1998). As a cold water fish, cod has pepsins and pepsinogens which are more active at low temperature than those in warm water fishes, which can be particularly useful in food processing (Gidlberg, 2004).

The pH has a considerable effect on the activity of fish pepsin. Both the optimum pH (the pH at which the maximum enzymatic activity) and pH stability (the pH range in which superior enzyme stability is shown) are very important. Mammalian pepsins are usually most active at pH of 1.5 –2.0 while most fish pepsins show high activity in less acidic conditions (Gildberg, 1988; Gildberg, 2004). Bjelland *et al*. (1988) stated that there are two different types of pepsins Pepsin I and Pepsin II contained in cod stomach. Pepsin-I functions under relatively weak acidic condition (pH=4.0) while Pepsin-II is similar to mammalian pepsin and is more active in strong acidic environment (pH=2.0). *O. mossambicus* pepsin showed maximum hydrolytic activity in 2% acidified haemoglobin at pH 2.0 and 3.5, while *E. suratensis* pepsin showed its maximum activity at pH 2.5 and 4-4.5 **(Fig.2.5.1 and Fig. 2.5.4)**. Thus the presence of two types of pepsins, Pepsin I and Pepsin II, can be confirmed in these two species with alternative dominance. Earlier reports have shown pepsin activity at a slightly higher pH range of 3–5 (Uys and Hecht, 1987; Gildberg, 1988; Martinez and Serra, 1989). The mesophilic porcine enzyme exhibited 100 % relative activity at pH 2.0, but a fast decline in activity at pH 2.5. In contrast, both Antarctic pepsin isoenzymes, pepsin A1 and pepsin A2 from rock cod *Trematomus bernacchii* were less active at their optimum pH (2.5 for fish pepsin A1 and 2.0 for fish pepsin A2), and showed a slow decline of their relative activity at pH values above their optimum (Brier *et al*., 2007).

Albacore tuna have only one type of pepsinogen and pepsin in their body (Nalinanon *et al*., 2009). This pepsin had similar properties to those found in tropical fishes (Nalinanon *et al*., 2009). Tongol tuna contains two isoforms of pepsin while only one type of pepsin was found in skip jack tuna stomach (Nalinanon *et al*., 2008). Pepsin in tuna has a different molecular weight and active temperature than those of bovine pepsin (Nalinanon *et al*., 2009). There are three pepsinogen (pepsinogen-I, pepsinogen-II and pepsinogen-III) detected in pacific blue fin tuna (Tanji, 2009), which contain a greater number of basic residues than mammalian pepsinogens (Tanji *et al*., 1988). These three pepsins in blue fin tuna have been found to be most active at pH 2.5. Pepsins in blue fin tuna have unique properties in enzymatic activity which are quite different from those of cod or porcine pepsin (Tanji *et al*., 1988; Tanji, 2009). **Fig 2.5.11** illustrates the stability of pepsin like acid proteases from *O.* mossambicus. It is highly stable at pH 4 and about 80% stability was exhibited at pH 2.5 and 5-5.5 range. Further they showed decreased stability at higher pH values. The pH stability of *E.* suratensis pepsin is shown in **Fig.2.5.12**. Its stability is higher at pH 2-3 and over pH-4 more than 50% of the relative activity was lost. Acidic protease from the viscera of bolti fish (*Tilapia nilotica*) showed the optimal pH at 2.5 and stability between pH 2-6 (El-Beltagy *et al*., 2004). Pepsin from yellow fin tuna has an optimum pH of 2.5. Its crystal structure, specificity, alkaline stability and other properties are different from other pepsins (Norris and Mathies, 1953; Northrop, 1931). Most fish species contain two or three major pepsins with an optimum haemoglobin digestion at pH between 2 and 4 (Gildberg, 1983). Fish aspartic proteases include cathepsin D which was also assayed using haemoglobin as substrate with only a minor modification in the pH and temperature of the reaction (Anson, 1938). Cathepsin D is an intracellular aspartic proteases found in fish

and plays a major role in digesting yolk proteins in developing eggs (Romano *et al*., 2004). Acidic pepsin-like enzymes were detected in stomach region of arowana fish (*Scleropages formosus*) at optimum pH of 1.5–2.0 (Natalia *et al*., 2004). Two pepsins (A and B) purified from the stomach of pectoral rattail (*Coryphaenoides pectoralis*) showed the maximal activity at pH 3.0 and 3.5, respectively, and had the same optimal temperature at 45°C using haemoglobin as a substrate (Klomklao *et al*., 2007). Compared to those in mammals, pepsinogens and pepsins in fish have some distinct characteristics including, less acidity and higher specific activity (Norris and Mathies, 1953), low optimum temperature (Simpson and Haard, 1987) and higher heat sensitivity (Martinez and Olsen, 1989).

Like pH, the optimum temperature and thermal stability range are very important. Temperature has a great influence on the activity of fish pepsin. The optimum temperature (the temperature of maximum enzymatic activity) of fish pepsin depends greatly on species and its habitat (such as cold or warm water species) (Gilderg, 1988; Pavlisko *et al*., 1997; Shahidi and Kamil, 2001). Fishes from cold water habitats were found to have lower optimum temperature than those from warm aquatic environments (Bjelland *et al*., 1988; Noda and Murakami, 1981; Squires *et al*., 1986; Chiang *et al*., 1987). Pepsin from the viscera of bolti fish (*Tilapia nilotica*) showed the optimal temperature at 35°C and it retained more than 50% of its activity after heating between 50 and 60^0C (El-Beltagy *et al*., 2004). The optimum temperature range of *Scophthalus maximus* is 40–50^0C for stomach protease (Wang *et al*., 2006). Two pepsins (A and B) purified from the stomach of pectoral rattail (*Coryphaenoides pectoralis*) showed the same optimal temperature at 45°C (Klomklao *et al*., 2007). The highest pepsin I activity was found at 38^0C and the highest pepsin II activity at 43°C (Gildberg, 1983). Pepsin from yellow fin tuna has an optimum temperature of 45°C (Norris and Mathies, 1953). The

optimum temperature range of pepsin from Pacific bluefin tuna *Thunnus orientalis* was 45°C with 70% of the remnant activity at 25–35°C (de la Parra *et al*., 2007). *O.* mossambicus showed an optimum temperature of pepsin like acid proteases at 40°C (**Fig. 2.5.3**) and it is highly stable up to 45°C. Further increase in temperature diminishes the enzyme activity and above 55°C it is unstable (**Fig.2.5.9**). The curve of optimum temperature for *E*. suratensis pepsin is shown in **Fig. 2.5.5**. It exhibits a range, between 40-45°C, as its optimum temperature. Its stability extends strongly up to 45^0C and it is moderately stable till the temperature 50°C. The activity of pepsin like acid protease lost on further increase in temperature. Cold water fish enzymes have a low Arrhenius activation energy, explaining their low optimal temperature and high heat lability in contrast with warm water complements (Simpson and Haard, 1987). The sharp decrease of thermal stability was attributed to the obliteration of tertiary structure of enzymes and denaturation of pepsin (Haard, 1988; Nalinanon *et al*., 2009).

In the present study Sodium ions at low concentration activates the *E. suratensis* pepsin. Potassium ions do not affect the enzyme action but magnesium at higher concentration inhibits the enzyme action. Calcium and barium showed an enhancing activity while manganese showed an inhibitory effect according to their increase in concentration **(Fig.2.5.8).** Only mercury ions showed a little inhibitory effect and all other metal ions does not have any effect on pepsin like acid proteases in the case of *O. mossambicus* **(Fig.2.5.7).** The results of the kinetic experiments indicated that Cu2+ particularly, but also Ni^{2+}, increases pepsin activity, while Fe^{3+} and Zn^{2+} do not. The influence of Cu^{2+} and Ni^{2+} depends on their concentrations (Kirchgessner *et al*., 1976). In studies of in vitro digestion of pepsin with soya-bean protein as substrate, Beyer *et al* (1975) found a greater inhibitory effect in the presence of Cu^{2+}, Ni^{2+} and Fe^{3+} than with Zn^{2+}.

Various metal ions influence the enzyme activity differently according to the nature of the ions and the enzymes concerned. All of the digestive enzymes selected for this study are not metallo-enzymes. However, the activity of enzyme lipase which catalyzes the hydrolysis of lipids is enhanced by the presence of calcium ions because calcium ions form salts with the fatty acids liberated by hydrolysis and block the re-esterification reaction. The removal of fatty acids as calcium salts from the reaction center apparently decreases the product concentration and thereby increases the forward reaction rate. A study on such enhancement or inhibition by these metal ions in digestive enzyme activities is very important since the fishes are being exposed to various metal ion concentrations in the aquatic ecosystem.

The present study can be summarized as the pepsin of *E. suratensis* has a broad pH optimum and optimum temperature for its maximum activity when compared to that of *O. mossambicus*. The gastric pepsin from both fishes exhibited similar temperature stability. The *O. mossambicus* pepsin is able to withstand the effect of many of the metal ions studied and this ability may help those fishes to attain a better digestibility in brackish waters where the metal ion concentrations are high in comparison with fresh water habitats.

Chapter 3

ALPHA AMYLASE

Contents

After the gastric digestion, the nutrients are further hydrolysed by intestinal enzymes. The carbohydrates in the diet are digested by intestinal alpha amylase. This chapter deals with the characteristics of alpha amylases in *E. suratensis* and *O. mossambicus*. An effort to identify potent alpha amylase inhibitors from various natural sources has also been made and the results are reported in this chapter. The chapter is divided as similar to the second chapter.

3.1. Introduction

There are many hydrolytic enzymes within the digestive tract of animals, which catalyze the breakdown of polymeric macromolecules. Rates of enzyme action are very reliant on situations such as temperature, pH, ionic concentration and many other physiological and environmental factors. Carbohydrates are one of the major dietary component and show wide stereo chemical variations. A hexasaccharide can exist over 10^{12} possible isomers; this diversity exceeds by far the number of protein folds (Laine, 1994). Organisms exploit this diversity of oligosaccharides and polysaccharides for a multitude of biological functions, from storage and structure to highly specific signalling systems. Careful specific hydrolysis of glycosidic bonds is therefore necessary for metabolic processes like energy uptake, cell wall development

and degradation and turnover of signalling molecules. This saccharide diversity could leads to the structural, functional and evolutionary diversities amongst the enzymes that act on glycosidic bonds. Carbohydrate-active enzymes include glycoside hydrolases (Henrissat, 1991), glycosyltransferases (Coutinho *et al.*, 2003), carbohydrate esterases and polysaccharide lyases (Davies and Henrissat, 1995). Besides cellulose, starch ranks among the most abundant carbohydrate polymers on Earth. The digestion of starch is not a simple, single chemical process. The process can be partially enumerated by several measures that differ from one another depending on the enzyme characteristics and reaction conditions used in catalytic hydrolysis. These measures include the rate of decrease in starch concentration, rate of appearance of reducing sugar moieties and various oligosaccharides (Dona *et al*., 2010). It is an important source of energy for animals, higher plants and especially microorganisms. Starch has rather complex structure (α-l, 4-1inked α-D-glucose units with α-l, 6- branch points) and it is metabolized by a set of enzymes, commonly named as starch hydrolases. The main metabolic role carbohydrate is that of fuel, which when oxidized offers energy for other metabolic functions. Carbohydrates are used for this purpose in the form of mono saccharides, mainly glucose. In this chapter, we concentrate on the action of specific hydrolase, amylase, in *Etroplus suratensis* and *Oreochromis mossambicus* in the digestion of carbohydrates.

3.2. Objectives of the Study

The objective of the present study is to characterize the alpha-amylase from the *E. suratensis* and *O. mossambicus* biochemically by analysing the optimum temperature, pH and substrate concentration. The study also investigates the effect of different metal ions on the activity of the selected

enzymes. The temperature denaturation kinetics of these enzymes is another aim of this research. pH stability is one of the important aspects with respect to enzymes and this chapter is included with the estimation of pH stability of alpha amylases. The screening of alpha amylase inhibitors from various plants and their mechanism of inhibition have also been investigated by a kinetic approach.

3.3. Review of Literature

Glycosidases are the key enzymes for carbohydrate digestion, probably secreted by the pancreas into the intestine. It acts on complex poly, oligo and di-saccharides, such as starch, glycogen, maltose and sucrose, hydrolyzing them up into glucose, fructose, maltose, maltotriose and a combination of branched (1:6) oligosaccharides (limit dextrins) (Jobling, 1995). A variety of glycosidases such as amylase, sucrase, maltase and cellulase were detected in the stomach, duodenum and ileum of gut of fishes like *O. niloticus* (Fagberno *et al*., 2005). In contrary to other higher vertebrates, fishes accomplish their energy requirements from proteins and lipids. Because of the fact that fish do not mobilize liver glycogen during starvation, no nutritional requirements have been described for carbohydrates and it does not seem to be nutritionally important for fish (Cowey and Sargent, 1979). However, enzymatic digestion of carbohydrates has been detected in the gastrointestinal tract of all species so far studied. Accordingly, omnivorous cyprinids and cichlids represent the majority of cultivated species on a worldwide basis. Cultivation of these species has long traditions in Asian and African countries and supplies large populations with essential, high-quality protein (Krogdahl *et al*., 2005). Efficient feed formulations are essential for good culture practices. Carbohydrate-containing feedstuffs are available in great quantities at low prices. Grains or grain products are the main carbohydrate

sources in diets for cultivated fish (Tacon, 1993). Carbohydrates in fish feed range from highly digestible mono-, di- and oligosaccharides to insoluble and indigestible hemicelluloses and cellulose, with sources ranging from seaweed, algae and plankton to refined grain and soybean products. Carbohydrates from plants, algae, plankton and other feeds comprise oligo- and polysaccharides of monomers with various substitutions, whereas starches constitute the major carbohydrate component of grains. Numerous investigations on digestion of starch along with other carbohydrates in fish have been published and the existing information is scarce. The physiological mechanisms behind the species differences are not known (Krogdahl *et al*., 2005)

3.3.1. Classification of Amylases

In 1991, Bernard Henrissat proposed the classification of the glycosyl hydrolases into different families on the basis of their amino acid sequences (Henrissat, 1991). The glycosyl hydrolase enzymes have been grouped into more than 80 families (MacGregor *et al*., 2001). Based on the type of reaction that the enzyme catalyses and on their substrate-specificity (Webb, 1989), these enzymes are named as glycosyl hydrolases (EC 3.2.1.x). The first three digits designate enzymes hydrolyzing O-glycosyl linkages while the last number for the substrate specificity and sometimes indicates the molecular mechanism (Henrissat, 1991). Alpha-amylase (α-l, 4 glucan-4-glucanohydrolase (EC 3.2.1.1) catalyses the hydrolysis of α- 1, 4 glycosidic linkages of starch, glycogen and various other oligosaccharides. Glycosyl hydrolases have broad substrate specificities, thus enzyme commission (EC) classification is not sufficient for these enzymes (Henrissat, 1991). There is a direct relationship between sequences of a protein and its folding pattern. Therefore, considerable sequence similarities are a strong indication of folding

similarities (Chothina and Lesk, 1986). A precise comparison of primary sequences of the glycosyl hydrolases has been conducted (Henrissat *et al*., 1989; Henrissat, 1990; 1991; Svensson, 1988; MacGregor and Svensson, 1989). It was found that it is possible to have same family containing several E.C. entries and enzymes with similar substrate specificities that belong to non-related families (Henrissat, 1991). The glycosyl hydrolases of every family share a common three dimensional structures and common mechanism of action and have several sequence similarities. Lysozymes were the first glycosyl hydrolases to have their three-dimensional structures established (Mathews *et al*., 1974; Blake *et al*., 1965).

Glycosidases catalyse the hydrolysis of various substrates such as aryl glucosides, malto-oligosaccharides (maltose and isomaltose) and p-nitrophenyl α-glucopyranoside from the non-reducing terminals (Iwanami *et al*., 1995; Chiba, 1995). Starch-converting enzymes basically are divided into four groups: endo-amylases, exo-amylases, debranching enzymes, and transferases. According to the hydrolytic types and end products of amylose, it is divided into three types: α-amylase 1, 4-alpha-D-glucan glucanohydrolase (EC. 3.2.1.1), β-amylase EC.3.2.1.2 (1, 4-α-D-glucan maltohydrolase; glycogenase; saccharogen amylase) and glucoamylase (γ-Amylase) EC.3.2.1.3, are the best known enzymes operating on starch. Despite their closely related functions, these three enzymes are structurally different and perhaps evolutionarily distantly related (Janecek, 1994). α-amylase can hydrolyze the glucan 1,4-α-glucoside of amylose and produce glucose, oligomaltose, and dextrin. The main functional difference, except for the place that the enzyme attacks starch and distinguishing the α-amylase from both β-amylase and glucoamylase, is the mechanism of glycosidic bond cleaving: the α-amylase uses the retaining mechanism (the resulting hydroxyl group retains the α-configuration) while

the other two use the inverting mechanism (inversion of the anomeric configuration to β).

Among endo-amylases, α-amylases (α -1, 4-glucan-4-glucanohydrolases, EC 3.2.1.1), belong to glycoside hydrolase family 13 which includes cyclodextrin glucanotransferases and pullulanases (Henrissat, 1991). It acts on internal α-1-4-glycosidic bonds of starch, glycogen and related polysaccharides and oligosaccharides in a random manner; reducing groups are liberated in the alpha-configuration. The term "alpha" relates to the initial anomeric configuration of the free sugar group released and not to the configuration of the linkage hydrolysed (MacGregor *et al*., 2001; Da Lage *et al*., 2004; Darias *et al*., 2006). β-Amylase (α -1,4-glucan maltohydrolase; EC 3.2.1.2) catalyzes the release of β-anomeric maltose from the non-reducing ends of starch and has been isolated from higher plants and some microorganisms (Thoma *et al*., 1971). Type beta is found in plants and type alpha is mainly found in animals (Vonk and Western, 1984). The three major kingdoms (archaebacteria, eubacteria and eukaryotes) possess various glycosyl hydrolases as key enzymes of carbohydrate metabolism. On the basis of its sequence alignment, β-amylase has been classified as belonging to the glycoside hydrolase family 14 (Henrissat and Davies, 1997; Davies and Henrissat, 1995). *β*-amylase that can hydrolyze glucan 1,4-α-glucoside of amylose with a non-reducing end and produce maltose; and *γ*-amylase that can hydrolyze glucan 1,4-α glucoside of amylose with a non-reducing end and produces glucose, maltose, maltotriose, and oligomaltose (Wong, 1995). In addition to their biochemical interest, α-amylases have a number of important biotechnological applications in food and starch processing industries (Vihinen and Mantsala, 1989).

In humans, α-amylase is present in both salivary and pancreatic secretions and they play a key role in catalyzing the hydrolysis of starch in human food intake. These enzymes are encoded by two different loci, Amy 1 and Amy 2, respectively and are found on chromosome 1, regulated such that the various isozymes are expressed specifically in either salivary gland or pancreas (Gumucio *et al*., 1988). These enzymes are highly homologous, with a sequence identity of over 99% and adopt very similar structures (Brayer *et al*., 1995; Ramasubbu *et al*., 1996). Human salivary amylase belongs to family 13 of the glycoside hydrolases that catalyze reactions such as hydrolysis, transglycosylation, cyclization and condensation. Lactose intolerance (Auricchio *et al*., 1963) or mucopolysaccharidosis (Neufeld and Muenzer., 1975) are genetically based syndromes due to heritable deficiencies in glycosyl hydrolases in human beings.

3.3.2. Active site and Catalysis

α-amylases and closely related glycosyl hydrolases of family 13 (Davies and Henrissat, 1995) use a pair of amino acids, aspartate (Asp) and glutamate (Glu), residues at the active site in order to cleave the glycosidic bond with net retention of anomeric configuration via a double displacement mechanism (Koshland, 1953; McCarter and Withers, 1996). These residues are commonly found to be catalytic in glycosyl hydrolases, either as a proton donor in their protonated form or as a nucleophile or oxocarbonium stabilizing agents in their charged form (Sinnot *et al*., 1990). The structure of α-amylase was first determined using the α-amylase from *Aspergillus oryzae* (Matsuura *et al.,* 1980) and has been known to contain a $(\beta/\alpha)_8$-barrel, that is often called TAKA-amylase A. This folding motif is composed of eight parallel β-strands forming the inner β-barrel sheet that is surrounded by eight α-helices in such a

way that there are eight repeated (βα)-units in a regular $(\beta/\alpha)_8$-barrel, with the active site being at the C-terminal end of the barrel β-strands. Crystal structures of α-amylases and related glycosyl hydrolases invariably display three essential amino carboxylic acids (Asp 174, Glu 200, and Asp 264), in the active site which is formed as a long cleft and situated in domain A at the carboxyl end of the $(\beta/\alpha)_8$-barrel. During the reaction, the anomeric centre is subjected to nucleophilic attack along with general acid catalysis and protonation of the glycosidic oxygen, resulting in the formation α-D-glycopyranosyl-enzyme. Hydrolysis of this intermediate by a water molecule requires general base catalytic assistance, presumably from the same residue (Asp 174) acting as acid/base catalyst (McCarter and Withers, 1996). Human salivary amylase occurs as glycosylated (62 kDa) as well as non-glycosylated (55 kDa) proteins in human saliva, and consists of a single polypeptide chain of 496 amino acid residues (Bank *et al*., 1991; Nishide *et al*., 1984). The architecture of human salivary amylase also consists of three structural domains, domain A, domain B and domain C. Domain A adopts a $(\beta/\alpha)_8$ barrel structure and contains the three catalytic residues Asp197, Glu233 and Asp300. Domain B occurs as an extention from domain A and is the structurally least ordered of the three domains. It contains one calcium-binding site. Domain C forms an all-β structure and seems to be an independent domain with unknown function (Ramasubbu *et al*., 1996).

The catalytic machinery of TAKA amylase like enzymes has a general agreement for the assignment of the general acid function to Glu 200 (Svensson and Sogaard, 1993), whereas the third essential acid residue, Asp 264, possibly stabilizes the protonated state of the glutamic side chain (Strokopytov *et al*., 1995). Another characteristic feature of the active site is

the presence of aromatic residues, which are considered as stacking partners for substrates and substrate analogues (Kadziola, 1993; Qian *et al*., 1994). Aromatic residues are expected to play a crucial role in substrate binding. Tyrosine 50, which is found at one extremity of the active site, is conserved in all alpha amylase structures indicating an essential role in the recognition of substrates. At the opposite end of the active site a phenylalanine (Phe 223) has been observed in mammalian (Buisson *et al*., 1987; Qian *et al*., 1993; Larson *et al*., 1994; Brayer *et al*., 1995; Ramasubbu *et al*., 1996), and plant (Kadziola, 1993; Kadziola *et al*., 1994) α-amylases. This phenylalanine has been replaced by a tyrosine in TAKA (Matsuura *et al*., 1984; Swift *et al*., 1991) and acid-alpha amylases (Boel *et al*., 1990; Brady *et al*., 1991). In mammalian α-amylases, two consecutive tryptophans (46 and 47) are found next to Tyr 50, while only one tryptophan is located here in barley α-amylase. The differences in number and nature of aromatic residues lining up the active site region could lead to specific behaviours in the catalysis resulting in different reaction products and transglycosylation patterns. α- amylases are known to contain Ca^{2+} which interacts with the side chains of residues. In all known three-dimensional structures of α-amylases, a well ordered water molecule, Wat 1004, bridges Asp 264 and Glu 200. Kadziola (1994) suggested that it may play a role in the catalytic process. This conserved water molecule is firmly bound to Glu 200 is the most possible proton donor involved in the protonation step of the oxygen atom of the substrate glycosidic bond. Subsequent inter glycosidic bond cleavage would lead to the formation of an oxocarbonium ion intermediate with a partial positive charge on the sugar carbon atom C_1, which then will be covalently bound to the catalytic nucleophile, Asp 174. On one occasion, the leaving group has departed from the active site, Glu 200 acts as a base which may be responsible for proton

abstraction from Wat 1004, inducing a hydroxyl ion prepared for nucleophilic attack at the C_1 atom. A chloride ion situated in the near environment of this water molecule is suggested to participate in its activation, and to promote the movement of the formed hydroxyl ion, via electrostatic repulsion, toward the substrate to be hydrolyzed (Kadziola, 1994; Kadziola *et al*., 1998).

3.3.3. Application

In food industries, amylase is frequently employed in baking buns, breads, and cakes and to produce dextrin, maltose, beer, alcohol, miso (a fermented rice or bean), and cheese in fermentation (Nikolov and Reilly, 1991; Shen *et al*., 1988; Arai *et al*., 1991; Takasaki, 1987). In biological function, it plays an important role in carbohydrate digestion and metabolism in plants and animals (Vonk and Western, 1984).

3.3.4. Alpha-amylase inhibitor from natural source

Glycosidase inhibitors were suggested for pharmaceutical applications like the treatment of conditions like obesity, hyperlipidemia (arteriosclerosis), diabetes, pre-diabetes, gastritis, gastric ulcer, duodenal ulcer and caries in man, or as food additive for various purposes in farm animals (Frommer *et al*., 1975; 1977a; 1977b). World Health Organization (WHO) reported that more than 220 million people worldwide suffer from diabetes mellitus. In 2005, an estimated 1.1 million people died from diabetes. The actual number is likely to be much larger, because although people may live for years with diabetes, their cause of death is often recorded as heart disease or kidney failure. Almost 80% of diabetes deaths occur in low- and middle-income countries and WHO projects that diabetes death will double between 2005 and 2030 (Shaw *et al*., 2010). The severity of the disease points to the need for continuing advanced research on the pharmacology of diabetes mellitus. Hyperglycemia has been a classical risk

factor in the development of diabetes and its complications associated with diabetes. Therefore control of blood glucose levels is critical in the early treatment of diabetes mellitus and reduction of macro- and micro vascular complications. Amylases are considered as target sites for the treatment of disorders in carbohydrate uptake, such as diabetes (Laar *et al*., 2008), and obesity (Yanovski and Yanovski, 2002), as well as, dental caries and periodontal diseases (Touger-Decker and Loveren, 2003), which intimidate an escalating mankind. One therapeutic approach is the prevention of carbohydrate absorption after food intake, which is facilitated by inhibition of the enteric enzymes including α-glucosidase and α-amylase present in the brush borders of intestine (Toeller, 1994; Inzucchi, 2002). The inhibition of such enzymes has been a strong option in the prevention of diabetes and inhibitors like acarbose, voglibose, and miglitol are widely used in type 2 diabetic patients. However, it is well documented that synthetic inhibitors have undesirable side effects such as diarrhea and abdominal cramping (Chakrabarti and Rajagopalan, 2002). Plant based drugs are considered to be less toxic and free from side effects than synthetic ones.

Diabetes mellitus is a metabolic disorder resulting from deficiency in insulin secretion, insulin action, or both, promoting disturbances in carbohydrate, fat and protein metabolism. The word 'Diabetes' is a Greek word that means 'to pass through' was first used by Aretaeous of Capadocia in the 2nd century AD to describe a condition that is characterized by excess of sugar in blood and urine (Mac frlance *et al*., 1997) and the adjective 'mellitus' a Greek word that means 'honey' was introduced by the English Physician John Rollo (Rollo, 1798) so as to distinguish the condition from other polyuric diseases in which glycosuria does not occur. Long term complications of diabetes mellitus include retinopathy, nephropathy, neuropathy, microangiopathy and increased

risk of cardiovascular disease (Laar *et al*., 2008; Cheng and Fantus, 2005; Aguiar *et al*., 2007). Diabetes mellitus is classified in to Type-I and Type-II based on clinical manifestations. Type I, formerly called juvenile onset diabetes, occurs typically before the age of 20. The cause of Type1 diabetes is that the pancreas, the organ that secretes insulin, is destroyed by auto antibodies. Thus patients with Type 1 diabetes always need insulin injected or through insulin pump. Type II diabetes is usually diagnosed after the age of 35. The cause of Type II diabetes is primarily a complicated medical condition called 'insulin resistance'. 'Insulin resistance' refers to the body's inability to respond properly to insulin. Resistance develops because of many factors, including genetics, obesity, increasing age and having high blood sugar for a long time. The therapeutic strategies for the treatment of type II diabetes include the reduction of the demand for insulin, stimulation of endogenous insulin secretion, enhancement of the action of insulin at the target tissues and the inhibition of degradation of oligo and disaccharides (Funke and Melzing, 2006). The drugs commonly used in clinic to handle or control diabetes are insulin, sulfonylureas, biguanide, glucosidase inhibitors, aldose reductase inhibitor, thiazolidinediones, carbamoylmethyl benzoic acid, insulin-like growth factor. The effect of these drugs is aimed to lower the level of blood glucose (Cheng and Fantus, 2005; Inzucchi, 2002; Chakrabarti and Rajagopalan, 2002). One therapeutic approach for treating type II diabetes mellitus is to decrease the post-prandial glucose levels and surge in blood glucose after absorption. This could be done by retarding the absorption of glucose through the inhibition of the carbohydrates-hydrolysing enzymes, α-glucosidase and α-amylase, present in the small intestinal brush border that are responsible for the breakdown of oligosaccharides and disaccharides into monosaccharide suitable for absorption (Laar *et al*., 2008; Inzucchi, 2002; Goke and Herrmann-Rinke, 1998; Lebowitz,

1998). Inhibitors of these enzymes, like acarbose, delay carbohydrate digestion and prolong overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently blunting the postprandial plasma glucose rise (Laar *et al*., 2008; Cheng and Fantus, 2005). Traditional herbal medicine has been used since ancient time in many parts of the world. A wide range of plant-derived principles belonging to compounds, mainly alkaloids, glycosides, galactomannan gum, polysaccharides, hypoglycans, peptidoglycans, guanidine, steroids, glycopeptides and terpenoids, have demonstrated bioactivity against hyperglycaemia (Mentreddy, 2007). Ayurveda, the traditional Indian herbal medicinal system practiced for over thousands of years have reports of antidiabetic plants with no apparent known side effects (Bhat *et al*., 2011; Bhutani and Gohil, 2010). Nowadays several glucosidase inhibitors have been developed from natural sources, especially from plants (Matsui *et al*., 2006; Bhat *et al*., 2008; Kumarappan and Mandal, 2008).

3.4. Materials and Methods

3.4.1. Fish and Preparation of Crude Enzyme Extract

The fish collection, acclimation and preparation of homogenates were done as described in Chapter 2. The only difference is in the organs selected for enzyme extraction. Among bony fishes, the pancreatic tissue is usually diffused in or around the liver (Bond, 1979). The exocrine pancreatic tissue of *O. niloticus* has a diffused distribution in the hepatic parenchyma and is separated from the hepatocyte cords by means of thin septa of connective tissue (Vicentini *et al*., 2005). Thus, in the present study the whole liver consisting of diffused pancreatic tissue (hepatopancreas) and the stomach are taken as digestive organ. The intestine of tilapia and pearlspot were taken without squeezing or rinsing because of the presence of enzymes in intestinal mucus.

3.4.2. Methods

The soluble protein content of enzyme homogenates was measured according to Lowry *et al* (1951) as described in Chapter 2.

3.4.2.1. Estimation of Amylase

The reducing sugars liberated by the action of alpha- amylase on starch was estimated by Somogyi–Nelson method using 3,5-dinitrosalicylic acid (DNSA) (Bernfeld, 1955). 1 ml of starch substrate (1% W/V) in phosphate buffer (pH-6.9) has been mixed with 1ml of phosphate buffer and 50µl of tissue homogenate was added. The reaction system is mixed thoroughly and incubated for 10 minutes. At the end of incubation period, 1 ml of DNSA was added and kept in a boiling water bath after vigorous mixing for 15 minutes. The test tubes were cooled and the resulting coloured solutions were diluted with 20ml of distilled water. The optical density of test solutions were read agains appropriate reagent blanks at 540nm. One unit of activity (U) is defined as the amount of enzyme need to produce 1 mg of maltose/min/ml of homogenate at 37^0 C. The specific activity is expressed as U/mg protein.

3.4.2.2. Screening of Phytochemical Inhibitor for alpha- Amylase

The plants were selected on the basis that each plant represents a specific group. *Cerbera odollam* represents minor element in mangrove classification, *Sonneratia caseolaris* represents true mangrove (major element) which are found exclusively in the mangrove habitat and *Murraya koenigii* (Curry leaves) represents a plant which is commonly being used in the Indian cuisines.

The fruits of *Sonneratia caseolaris* were collected from Thalassery and *Cerbera odolam and Murraya koenigii* were collected from Kochi. The *S. caseolaris* fruits, *M. koenigii* leaves and *C. odollam* fruits were crushed in a

blender and around 800g of each plant tissues were mixed with 800ml of 90% methanol and kept in a shaker (250rpm) over night. The process was repeated three times with fresh methanol. The extracts were filtered and concentrated to around 200ml in a rotary evaporator under vacuum at 40^0C. The crude extracts were preliminarily screened for their alpha amylase inhibitory activity. The extracts with inhibitory activity were further mixed thoroughly with equal volumes of ethyl acetate in a separating funnel. The ethyl acetate fraction was removed and the process repeated three times by adding fresh ethyl acetate. The remaining methanol fraction was then mixed with equal volume of hexane and treated as done before. Finally, the fraction left behind is considered as polar aqueous extracts of the selected plants after evaporation of methanol under vacuum. All the extracts were assayed for their amylase inhibition activity. The active fraction was further estimated for their effect on the Km and Vmax of amylase. The kinetic parameters were determined in the preparation of intestinal amylase of the fish, *Oreochromis mossambicus* intestinal amylase by DNSA method, using starch as substrate. The reaction mixture without enzyme inhibitor was used as positive control and the reaction mixture without enzyme homogenate (homogenate is replaced by phosphate buffer pH-6.9) was used as negative control.

3.5. Results

The optimum temperature, pH and substrate concentration for intestinal alpha-amylase activity of both *Oreochromis mossambicus* and *Etroplus suratensis* were estimated. The *O. mossambicus* possesses its maximum alpha amylase activity at its optimum temperature 35^0C (**Fig.3.5.1**). The pH optimum was found as 7.5 (**Fig.3.5.2**) and it has an optimum substrate concentration of 2% starch concentration (**Fig.3.5.3**). On the other hand, *E.*

suratensis alpha amylase has its optimum temperature at 45^0C (**Fig.3.5.4**). The pH optimum for its activity has been determined to be 7.5, which is similar to that of *O. mossambicus*. The alpha-amylase of *E. suratensis* has its maximum activity at a lower starch concentration, 1.5% (**Fig 3.5.6**) than *O. mossambicus*.

Manganese ions enhanced the *O. mossambicus* alpha-amylase at all the concentrations tested. Copper (Cu^{2+}) and mercury (Hg^{2+}) ions completely inhibit the alpha amylase activity at all the selected concentrations, while the activity is inhibited by Fe^{3+} and the extent of inhibition rises with increase in concentration of the Fe^{3+} ions (**Fig.3.5.7**). However, the *E. suratensis* alpha amylase was completely inhibited by iron ($Fe^{3+),}$ copper (Cu^{2+}) and mercury (Hg^{2+}) ions. Similar to *O. mossambicus* alpha-amylase, manganese ions have an enhancement effect on *E. suratensis* alpha-amylase at low concentrations. In contrast with *O. mossambicus* alpha amylase, zinc (Zn^{2+}) ions showed an inhibitory activity on *E. suratensis* alpha amylase and could completely inhibit the enzyme activity at 25mM (**Fig 3.5.8**).

O. mossambicus intestinal alpha-amylase is stable up to 65^0C and the activity diminishes drastically above this temperature. The alpha-amylase activity was completely destroyed within 30 minutes at 70^0C (**Fig.3.5.9**). The same effect of temperature was shown by alpha-amylase from hepatopancreas of *O. mossambicus* except that the stability was extended to 45minutes at 70^0C (**Fig.3.5.10**). *E. suratensis* intestinal alpha-amylase is less stable than *O. mossambicus*. It is stable only up to a maximum of 55^0C and the enzyme loses its activity with further increase in temperature. The enzyme lost its action within 45 minutes at 65^0C (**Fig.3.5.11**). However, the hepatopancreatic alpha amylase was found to be stable up to 60^0C and further increase in temperature lead to a decrease in specific enzyme activity. The *E. suratensis*

hepatopancreatic alpha amylase completely lost its activity within 45 minutes at 65^0C (**Fig.3.5.12**).

In comparison with *E. suratensis* alpha amylase, *O. mossambicus* enzyme was more stable at acidic pH and around 80% of the relative activity remained at pH 3 and the enzyme restored its 100% relative activity at pH 5.5. The enzyme is inactive at high acidic pH up to 2.5 (**Fig.3.5.13**). The study on pH stability of *E. suratensis* alpha- amylase showed the presence of acid stable alpha-amylase. Intestinal amylase with around 100% relative activity at pH 3.5 has been identified in the study. The hepatopancreatic counter part of alpha-amylase showed 80% relative activity at pH 3.5. The intestinal alpha-amylase restored 80% of its relative activity at high acidic range (4.5-6.5) and 100% activity was restored at pH 7. On the other hand the intestinal alpha-amylase showed stability with 80% relative activity at higher alkaline pH up to 10.5. The hepatopancreas alpha-amylase also restored 100% of its relative activity at around pH 8 (**Fig.3.5.14**). At low acidic pH range (4-6.5) it is able to restore around 50% of its specific activity and at higher alkaline pH the activity tends to decrease and reached 80% of its relative activity at pH 10.5.

Reaction	Equation of Curve	Km	Vmax	Vmax/Km
Uninhibited reaction	Y=3.3575X+10.486	5.9773	7.2464	1.2123
Inhibited reaction	Y=0.8251X-0.138	0.3202	0.0953	0.2976

The equation of inhibition kinetics is 1/Vo= (Km/Vmax) 1/[S] + 1/Vmax

Fig 3.5.15. shows the inhibitory activity of *S. caseolaris* and *C. odollam* extract on α-amylase activities. However, in the present study *M. koenigii* did not show any inhibitory activity of alpha-amylase. Percentage relative activity of different phyto extracts on *O. mossambicus* alpha-amylase is shown in **table 3.5.1.** In vitro studies demonstrated that all the extract of *S. caseolaris* fruits had α-glucosidase inhibitory activity. Activity of free and

inhibitor bound alpha amylase with respect to different substrate concentrations have been demonstrated in **Fig.3.5.16**. Dixon plot (**Fig.3.5.18**) indicated a concentration-dependent increase in α-amylase inhibitory activity. The mode of inhibition of the *S. caseolaris* extract α-amylase was also investigated by Lineweaver–Burk plots using the data derived from enzyme assays containing various concentrations of the starch substrate. The results are presented in **Fig.3.5.17**. Double-reciprocal plots of enzyme kinetics demonstrated a non competitive – uncompetitive mixed type inhibition. The Km of inhibited reaction is much lower than the uninhibited reaction. From the LB plot it is obvious that the mechanism of inhibition is different from the classical inhibition patterns and it exhibits non competitive – uncompetitive mixed type inhibition kinetics. It implies that the inhibitor in *Sonneratia caseolaris* fruit is very effective in reducing the rate of catalysis by *Oreochromis mossambicus* intestinal alpha amylase (**Fig.3.5.17**).

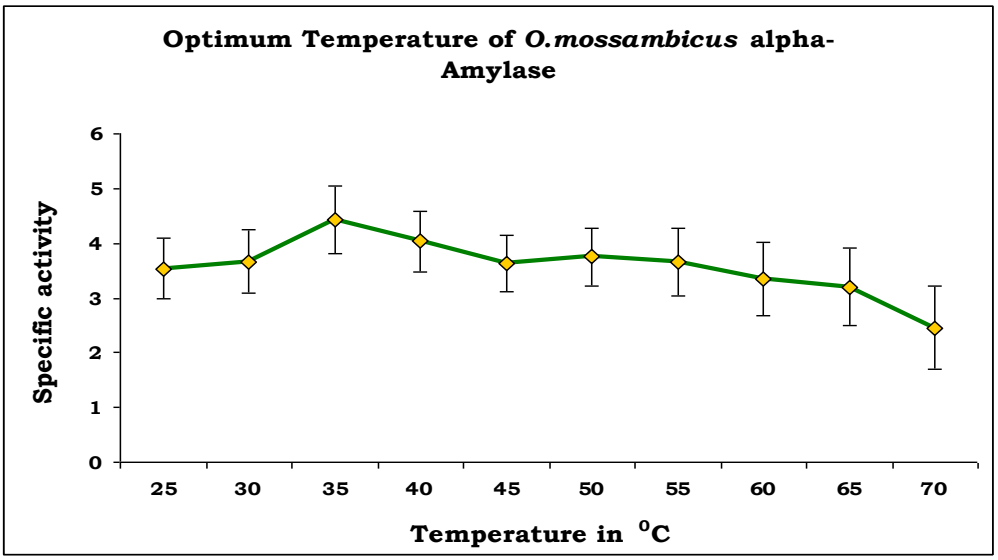


Fig. 3.5.1. Thermal optimization curve of *O. mossambicus* alpha amylase

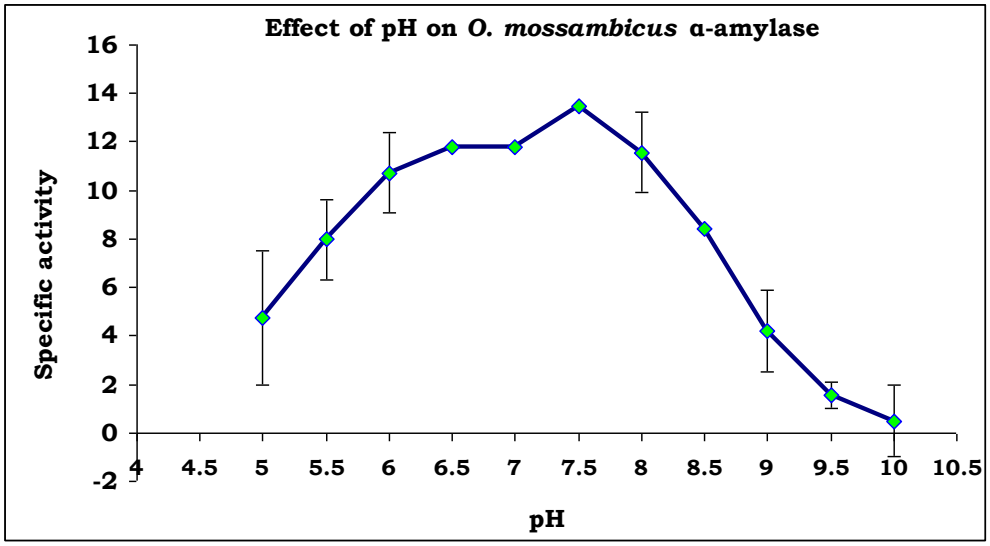


Fig. 3.5.2. Effect of pH on *O. mossambicus* alpha amylase

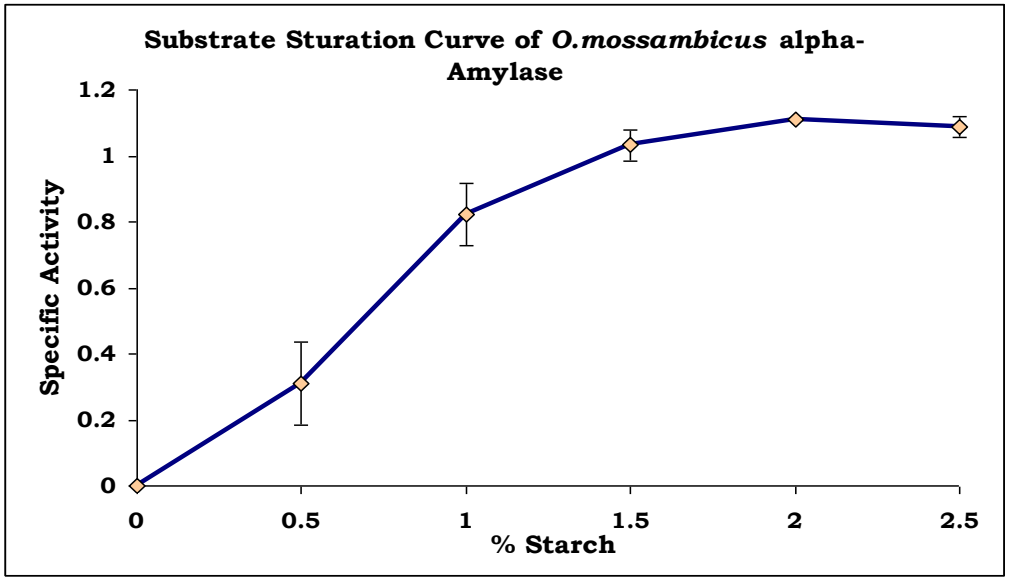


Fig. 3.5.3. Substrate saturation curve of *O. mossambicus* alpha amylase

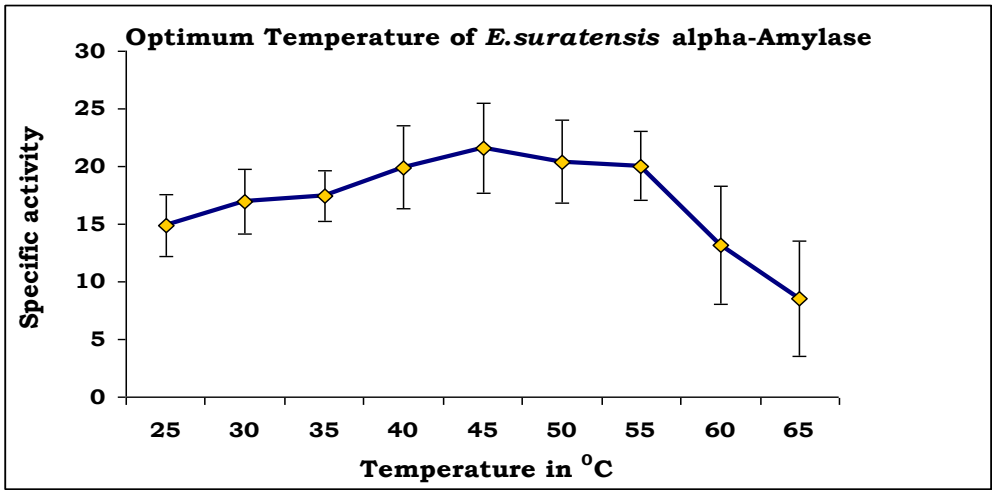


Fig. 3.5.4. Thermal optimization curve of *E. suratensis* alpha-amylase

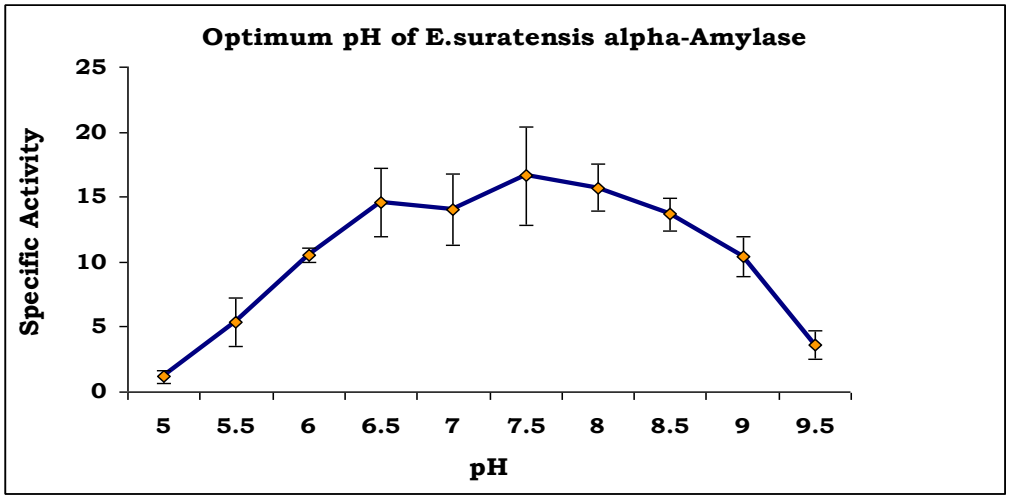


Fig. 3.5.5. Optimum pH of *E. suratensis* alpha amylase

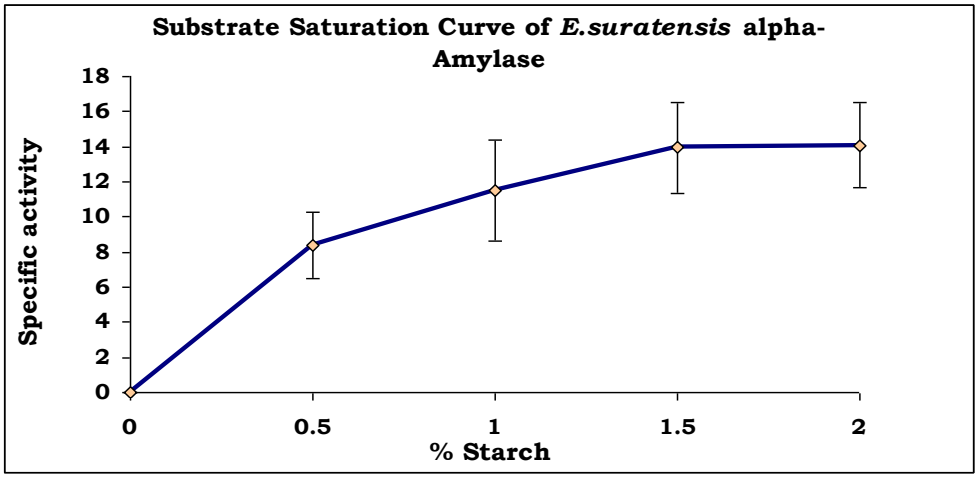


Fig. 3.5.6. Substrate saturation curve of *E. suratensis* alpha amylase

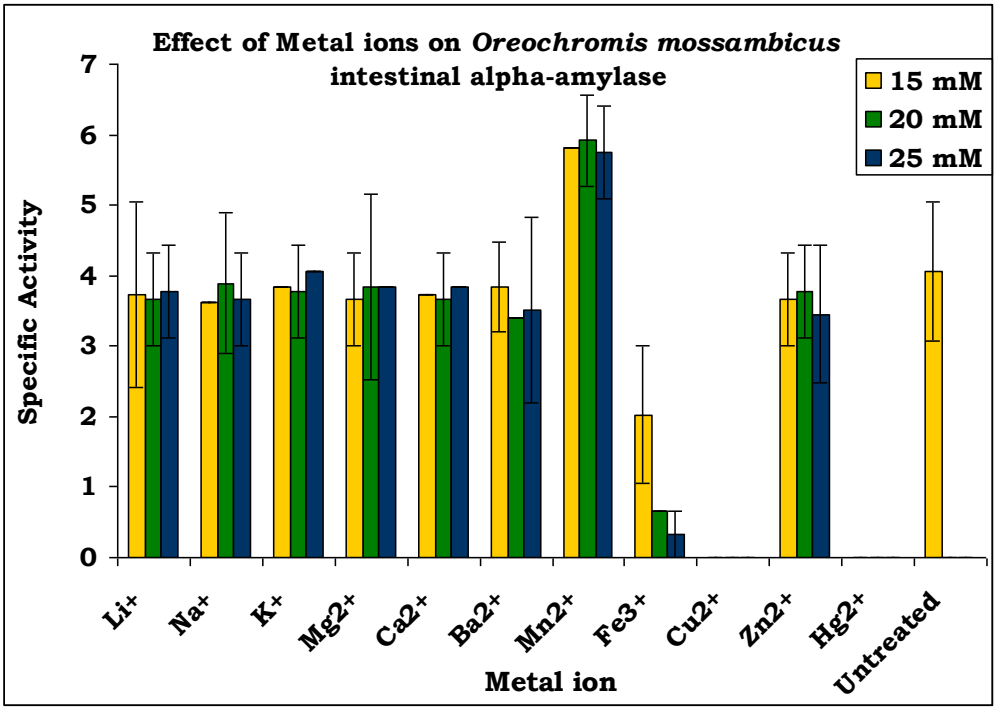


Fig. 3.5.7. Effect of metal ions on *O. mossambicus* intestinal alpha amylase

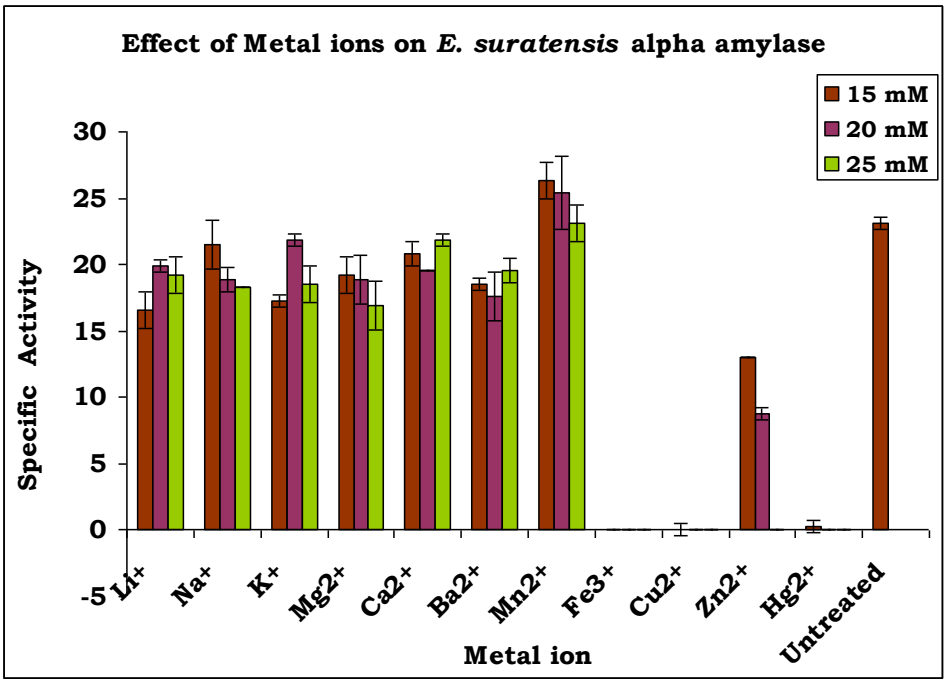


Fig. 3.5.8. Effect of metal ions on *E. suratensis* intestinal alpha amylase

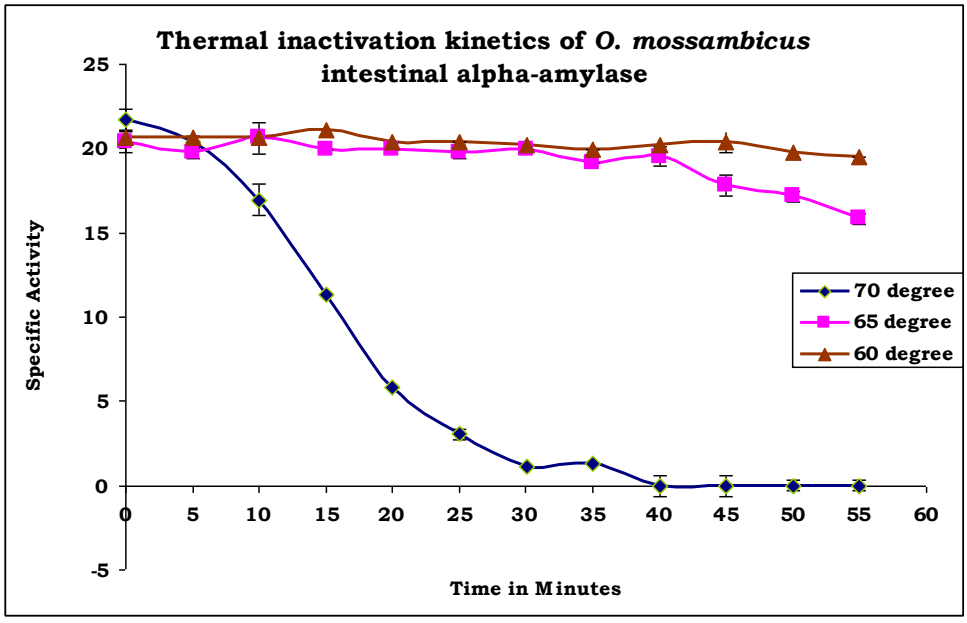


Fig. 3.5.9. Thermal inactivation kinetics of *O. mossambicus* intestinal alpha amylase

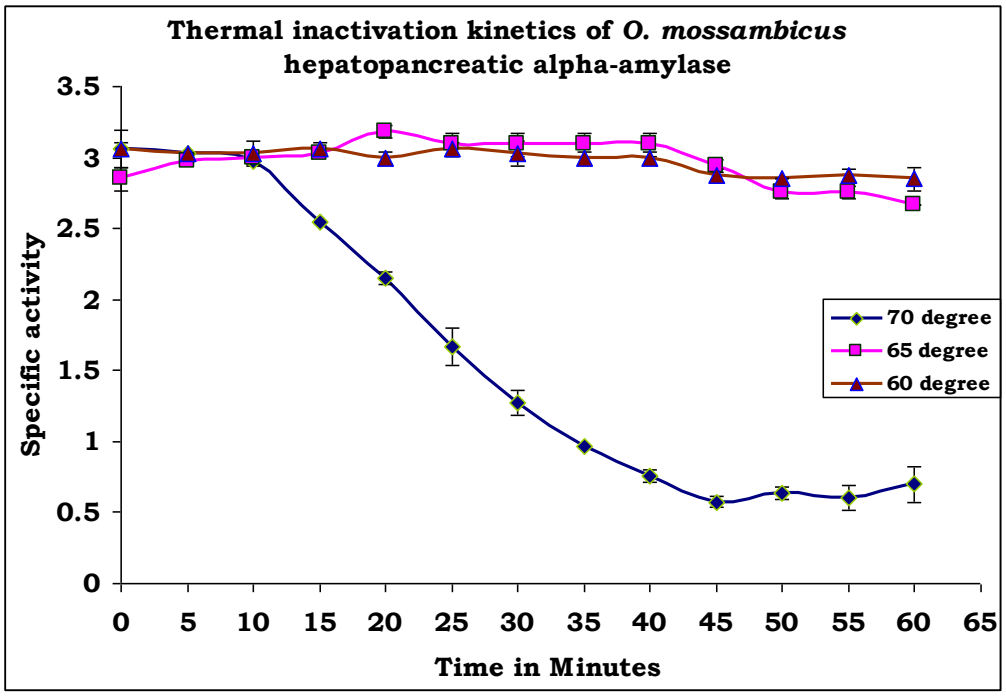


Fig. 3.5.10. Thermal inactivation kinetics of *O. mossambicus* hepatopancreatic alpha amylase

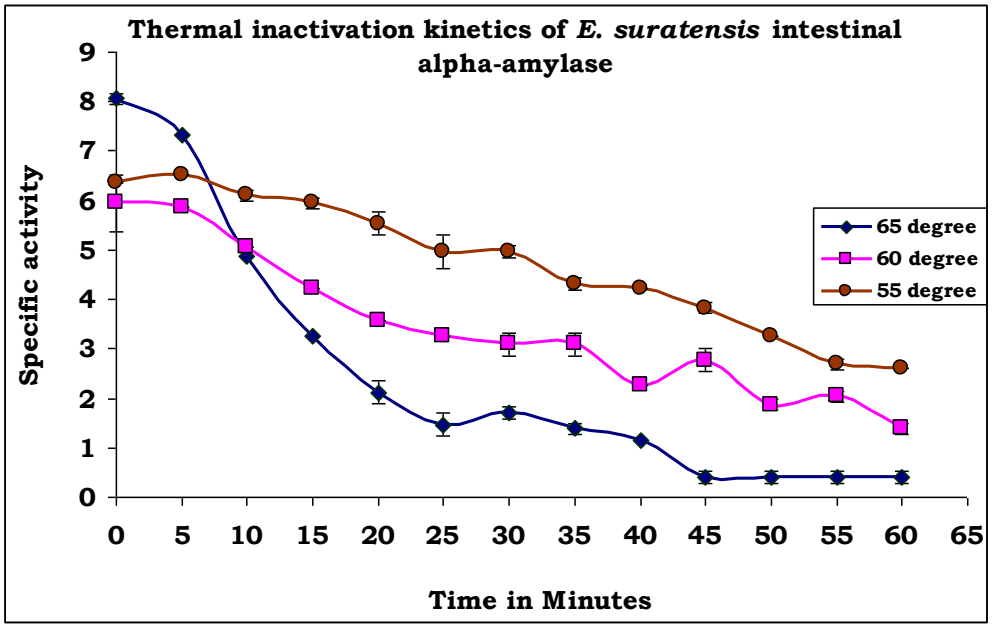


Fig. 3.5.11. Thermal inactivation kinetics of *E. suratensis* intestinal alpha amylase

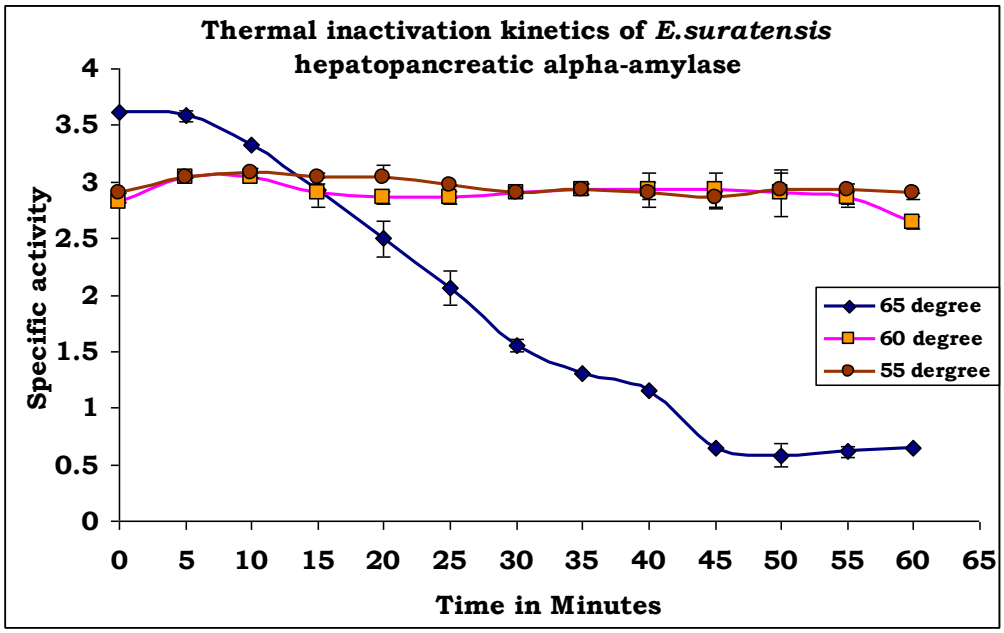


Fig.3.5.12. Thermal inactivation kinetics of *E. suratensis* hepatopancreatic alpha amylase

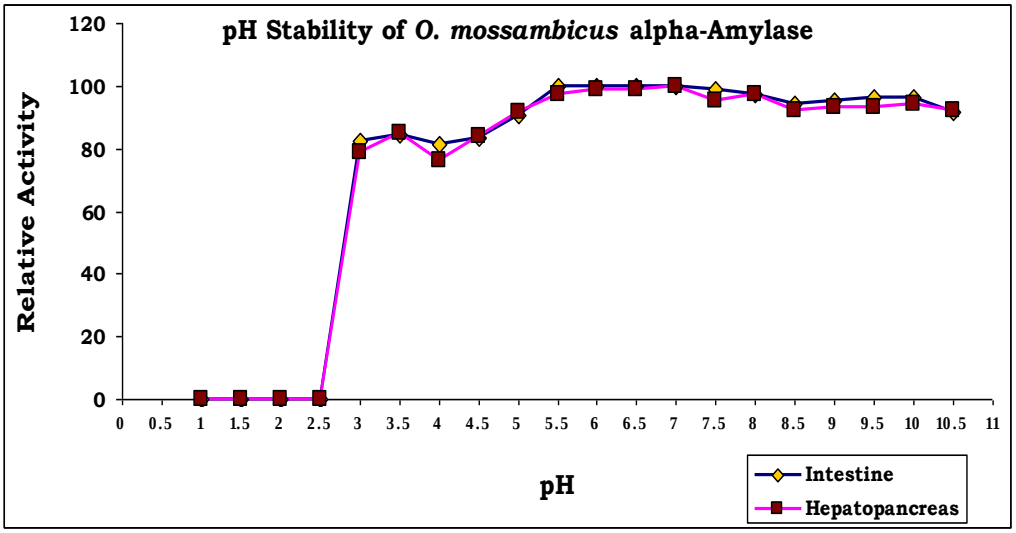


Fig. 3.5.13. pH stability of *O. mossambicus* alpha amylase

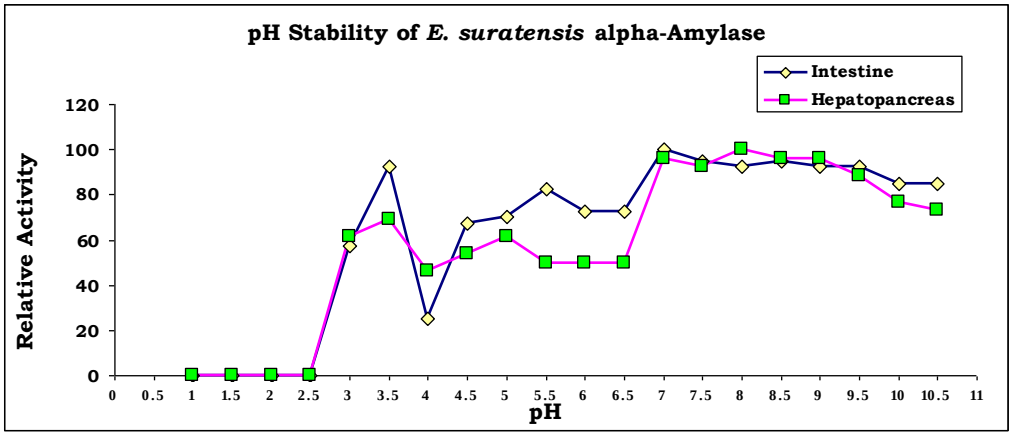


Fig. 3.5.14. pH stability of *E. suratensis* alpha amylase

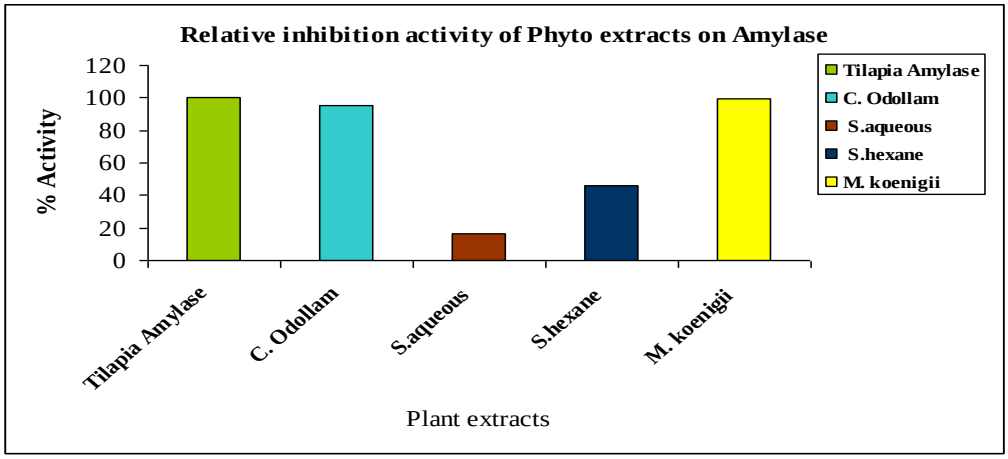


Fig. 3.5.15. Relative inhibition activity of different phyto extracts on *O. mossambicus* alpha-amylase

Table. 3.5.1. Percentage relative activity of different phyto extracts on *O. mossambicus* alpha-amylase

Plant Extract	% Activity
Tilapia Amylase	100
Cerbera odollam	94.96
Sonneratia aqueous extract	16.67
Sonneratia hexane fraction	45.83
Murraya koenigii	99.32

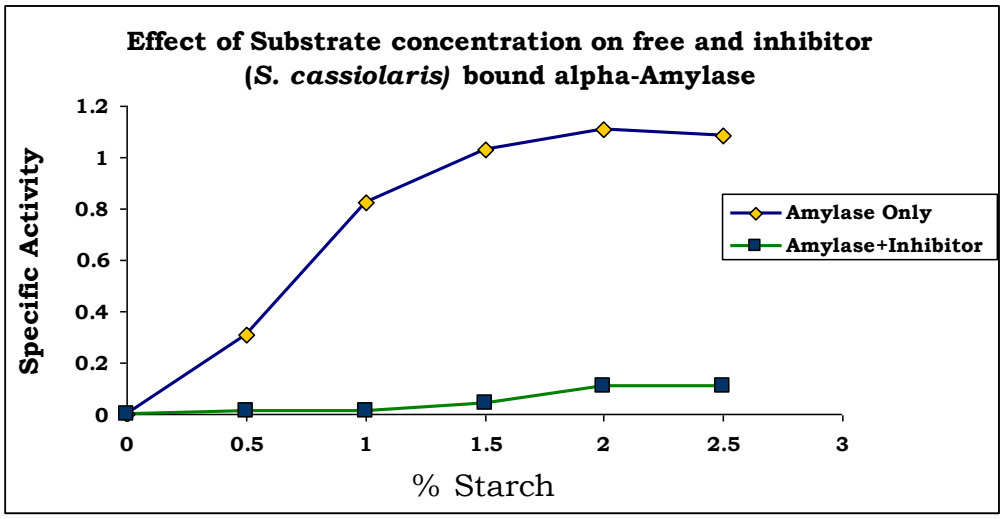


Fig. 3.5.16. Activity of free and inhibitor bound alpha amylase with respect to different substrate concentration

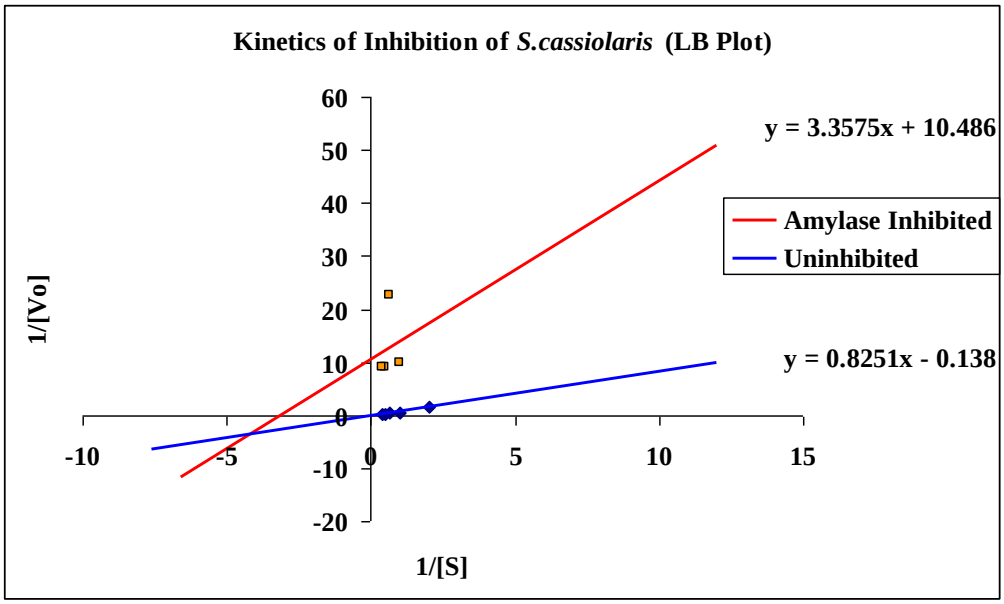
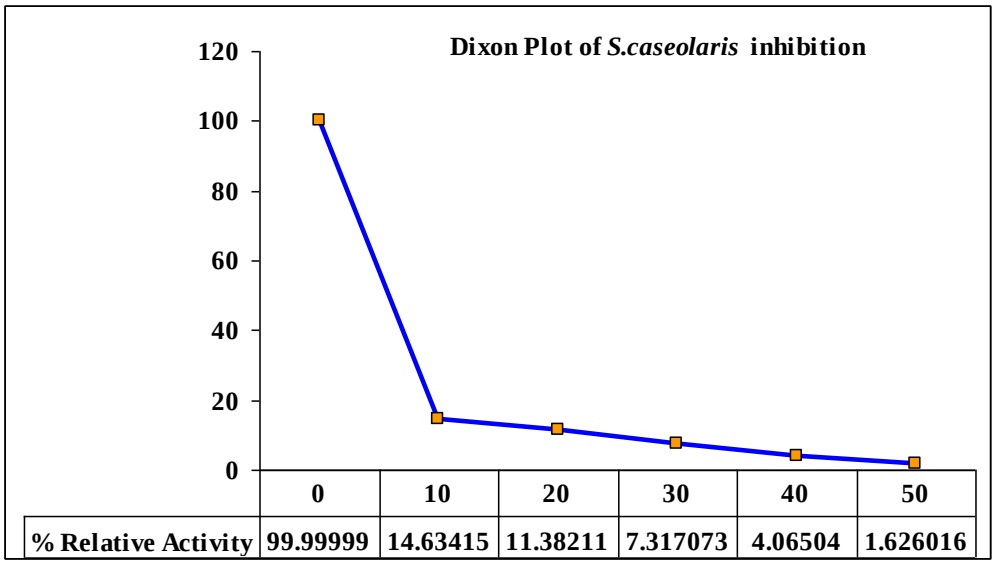


Fig. 3.5.17. Lineweaver–Burk plot of inhibition by *S. caseolaris* fruit aqueous extract



	0	10	20	30	40	50
% Relative Activity	99.99999	14.63415	11.38211	7.317073	4.06504	1.626016

Fig. 3.5.18. Dixon Plot of relative inhibition activity by *S. caseolaris* aqueous extract

3.6. Discussion

Pancreatic α-amylases are common α-amylases of vertebrates; they are secreted from pancreas and can digest starch in the intestine (Machius *et al*, 1996; Froystad *et al*., 2006; Ma *et al*., 2004). In contrast to mammals, where amylase is produced by salivary and pancreatic cells, the only source of α-amylase in fish appears to be the exocrine pancreas. Several teleosts do not possess a distinct pancreas, the organ responsible for the synthesis of digestive enzymes like amylase, proteases and lipases. While the studies showed highest activity in gastro intestinal tissues, the data on amounts of such proteins in the tissues indicated highest amount of enzyme proteins in the hepatopancreas. The gut tissue has relatively low amounts and thus the present studies are consistent with the assumption that the hepatopancreas is the site of synthesis of amylase-like digestive enzyme. This assumption is also supported by the liver immunohistochemical studies showing amylase localized in presumptive

pancreatic tissues which surrounds hepatic portal vessels (Yardley, 1990). Digestive α-amylase has been localised throughout the entire gastrointestinal tract of many fish species (Kawai and Ikeda, 1971; Chiu and Benitez, 1981; Fagbenro, 1990; Ugwumba, 1993; Sabapathy and Teo, 1993; Chakrabarti *et al*., 1995; Kuz'mina, 1996; Peres *et al*., 1998; Hidalgo *et al*., 1999; de Seixas *et al*., 1999; Fagbenro *et al*., 2000; Tengjaroenkul *et al*., 2000; Alarcon *et al*., 2001; Fernandez *et al*., 2001). The enzyme is present in the distal parts of the intestine and in some species it appears in the oesophagus also. As in mammals, amylase is produced in the pancreas and, when required, secreted into the gut (Fish, 1960; Barrington, 1957) where the enzyme is mainly adsorbed onto the mucosa of the intestine and the pyloric caeca (if present) (Munilla-Morgn and Stark, 1990; Kawai and lkeda, 1971; Ugolev *et al.*, 1983). Some authors have not found amylase activity in stomach homogenates (Sabapathy and Teo, 1993; Anderson, 1991; Uys and Hecht, 1971) and they have proposed that gastric amylase activity might be due to exogenous contamination either from the intestinal contents (by regurgitation) or from the ingested food. Not surprisingly, the exocrine pancreas has the highest activities (Overnell, 1973; Yardley and Wild, 1991). Together with other pancreatic enzymes, amylase activity is detected within the lumen of the intestine and in the chyme attached to the mucosal membrane (Ugolev and Kuz'mina, 1994; Hoehne-Reitan *et al*., 2001). Characteristics of amylase differ among species with respect to its pH optima and temperature stability. Studies of six Mediterranean sparid fishes have shown pH optima between 4 and 9 (Fernandez *et al*., 2001; Alarcon *et al*., 2001). Among them, most fish amylase showed more than one pH optimum, and some possessed two isoforms of the enzyme. Amylase from two tilapias, *Oreochromis niloticus* and *Sarotherodon melanotheron*, has shown molecular masses in the same range as the sparid amylases, around 56 kDa, and their pH optima are in the neutral range. Other features were in

common to the α-amylase family of enzymes (Moreau *et al*., 2001) and both enzymes occurred in two isoforms. Similar results have been observed in other fish species also (Munilla-Moran and Saborido-Rey, 1996b). In addition to the common characteristics, fish amylases also reveal distinct differences, for instance regarding dependence on metal ions and ionic concentrations (Munilla-Moran and Saborido-Rey, 1996a). Amylase levels are affected by the filling degree of the gut (Bitterlich, 1985; Takii1 *et al*., 985) and the nutritional condition of the animal. Higher levels are detected when the fish is not starved (Munilla-Morgn and Stark, 1990). Also, herbivorous and omnivorous species have been reported to have more amylase activity than carnivorous species (Fish, 1960; Sabapathy and Teo, 1993). Higher enzymatic levels have also been reported for younger animals than adults in the same species (Munilla-Morgn and Stark, 1990; Kawai and lkeda, 1971).

The importance of gaining knowledge about amylase activity in fish species has been indicated by Buddington and Doroshov (1986). They concluded that the low amylase levels are responsible for the scarce potential of this species to exploit diets with high carbohydrate contents. Despite this, there is a lack of information about the characterization of amylase activity in fishes. No previous attempt has been made to know what type of amylase is present in selected fish species. On the other hand, comparison of information about digestive enzymes in fishes is hampered by the use of heterogeneous substrates and methods of measurement (Bitterlich, 1985; Uys and Hecht, 1971). However, the glycosidase activity both in the digestive system organs and in the whole organism of ichthyophages is much lower than that of invertebrate animals (Kuz'mina, 2008).

The activity of amylase of seabream and turbot showed its maximum at neutral pH (7.0-7.5); meanwhile, the amylase activity of redfish had an

optimum pH at 4.5-5.0. The function ranged between 35 and 45°C for the three species. The Arrhenius plots of the intestinal amylase activities of seabream and turbot showed breakpoints at temperatures close to those of their physiological activities. High saline concentrations inhibited the activity of seabream and turbot amylases and enhanced the activity of redfish amylase. Seabream amylase activity was absolutely dependent on calcium ions (Munilla-Morán and Saborido-Rey, 1996b). On the contrary, redfish amylase activity was only detected in the absence of this metal. Studies carried out by using several effectors suggested that the activities found in these three species are different.

The pH optimum of α-amylase and proteinases in different fish species varies from 6.5 to 8.5 (Vonk, 1927; Ushiyama *et al*., 1965; Ugolev and Kuz'mina, 1993) and from 7.5 to 10 (Kalac, 1978; Murakami and Noda, 1981; Munilla-Moran and Saborido-Rey, 1996b; Garcıa-Carreno *et al*., 2002; Hau and Benjakul, 2006) accordingly. The glycosidase pH optimum of the mucosa and intestinal microbiota is 7.0, whereas that of the chyme varies from 6.0 in roach to 8.0 in bream (Kuz'mina *et al*., 2011). Fish also possesses additional glucosidase activities, including an acid α-glucosidase, these enzymes are usually present only in liver and are involved in the breakdown of endogenous glycogen (Mehrani and Storey, 1993) by a route that bypasses the better known glycogen phosphorylase (Moon *et al*., 1999) or at least complements its activity. In the fishes which differed in their feeding habits, considerable differences of the pH dependence of the enzymes were found, ensuring the hydrolysis of carbohydrate components of food, especially in the case of the cavity hydrolases (Kuz'mina *et al*., 2011).

Nikapitiya *et al* (2009) demonstrated the ability of the α-amylase gene to be regulated at a transcriptional level when the availability of food varies.

Consequently, feed availability constitutes an external regulatory factor for the α-amylase gene. Diet quality is to be expected to have an effect on amylase activity, because different classes of algal species vary in carbohydrate and starch content (Moal *et al*., 1987). Alteration in digestive enzymes, such as amylase, also has been observed in shrimp, depending upon casein and protein sources in the diet (Le Moullac *et al*., 1997). This gives emphasis to the impending effects of food quality on the regulation processes of polysaccharide degrading gene expressions. The fishes like disk abalone may adapt to digestion according to food availability in the environment. Such an adaptive response of digestive enzymes probably affects digestion and absorption efficiencies for the corresponding substrate. Moreover, the diversity in the feeding habit is reflected in the structural adaptations in the gastro intestinal system which can be further clarified by the morphological and physiological adaptations. Munilla-Morán and Saborido-Rey (1996b) noted that digestion of carbohydrates was at low rates in some carnivorous fish species, and α-amylase was not considered fundamental in their digestive processes. It had earlier been reported that carbohydrases and proteolytic activities were higher in the detritivorous fishes compared to the omnivorous and carnivorous fishes (Lopez-Vasquez *et al*., 2009; Chaudhari *et al*., 2012). Usually, detritivorous fishes consume immense quantities of vegetable detritus in the form of minute amorphous material of undetermined origin. Much of the fine organic particulate material taken up by detritivorous fishes is obtained from algae, even in systems in which aquatic macrophytes dominate aquatic primary production (Winemiller and Jepsen, 1998). Higher digestive enzyme activity in detritivorous fishes is an adaptation to extract high nutrient levels from detritus, which is considered as poor nutrient source. This adaptation

may be species specific and may be used extensively by fishes to survive specific environmental conditions.

3.6.1. Alpha-amylase Inhibitor From Natural Sources

Diabetes mellitus is responsible for about 9% of all global death (Bothon *et al*., 2013). Diabetes mellitus is one of the most common chronic diseases in nearly all countries and continues to increase in numbers and significance, as changing lifestyles lead to reduced physical activity and increased obesity. Regardless of the immense treads that have been made in the understanding and management of diabetes, the disease and its complications are increasing unabated. The incidence and prevalence of type II diabetes mellitus continue to rise in world populations (King *et al*., 1998). The global incidence for all age groups was estimated to be 2.8% in 2000 and the estimate is expected to reach 4.4% in 2030 (Sarah *et al*., 2004). The WHO published estimates for the years 2000 and 2030, using data from 40 countries but extrapolated to the 191 WHO member states (Wild *et al*., 2004). Shaw *et al* (2010) suggested that in 2010 there will be 285 million people worldwide with diabetes and the pattern of diabetes will vary considerably according to the country's economic status. In developed countries, the majority with diabetes will be aged over 60 years, whereas for developing countries most people with diabetes will be of working age, between 40 and 60 years. This difference is likely to be present even in 2030, although less marked, as the average age of developing country's populations will increase slightly more than in the developed countries. Population growth, ageing of populations and urbanization with associated lifestyle change is likely to lead to a 54% increase in worldwide numbers with diabetes by 2030. Diabetes mellitus is a complex metabolic disorder resulting from either insulin insufficiency or

insulin dysfunction. Type I diabetes (insulin dependent) is caused by insulin insufficiency due to immunological destruction of pancreatic β cells that leads to insulin deficiency. Type II diabetes is characterized with insulin resistance. It is the more common form of diabetes constituting 90% of the diabetic population. α-Amylase inhibitors are prescribed to manage blood sugar levels in type II diabetes mellitus and these drugs lower blood sugar levels by slowing or decreasing carbohydrate breakdown in the intestine (Scheen, 2003). Pancreatic and intestinal amylases are the key enzymes in dietary carbohydrate digestion and inhibitors of these enzymes are found to be effective in retarding glucose absorption to suppress hyperglycemia. The fundamental mechanism underlying hyperglycemia includes the excessive hepatic glycogenolysis and gluconeogenesis associated with decreased utilization of glucose by tissues. Inhibition of these enzymes reportedly decreased blood glucose levels in diabetic patients (van de Laar *et al*., 2005). Oral hypoglycemic agents/drugs like metformin and α-amylase inhibitors including acarbose, miglitol, and voglibose may be effective for glycemic control, but they have several side effects such as liver disorders, flatulence, abdominal pain, renal tumours, hepatic injury, acute hepatitis, abdominal fullness and diarrhea (Kwon *et al*., 2007; Chakrabarti and Rajagopalan, 2002). Hence, there is an urgent need to identify safer drugs from indigenous natural resources without or with fewer side effects. Plants are reservoirs of natural products with anti-diabetic potential. With respect to effective therapeutic approaches to treatment of diabetes mellitus, much effort has been made to investigate potential inhibitors against α-glucosidase and α-amylase from natural sources. Recently, many traditionally used medicinally important plants have been tested for their antidiabetic potential in various investigations (Sugihara *et al*., 2000; Youn *et al*., 2004; Andrade-Cetto *et al*., 2008). There is increased interest in the

screening of phytochemicals with the ability to delay or prevent glucose absorption (Tiwari and Madhusudana Rao, 2002). Plants have been the major source of drugs in Indian system of medicine and other ancient systems in the world. Earliest description of curative properties of medicinal plants is found in Rigveda (2500 - 1800 BC). Charaka Samhita and Sushruta Samhita give extensive description on various medicinal herbs (Kirtikar and Basu, 1975). Information on medicinal plants in India has been systematically organized (Kirtikar and Basu, 1975; Rastogi and Malhotra, 1989; Satyavati *et al*., 1976; Satyavati *et al*., 1987). The World Health Organization expert committee on diabetes has listed as one of its recommendations that traditional methods of treatment of diabetes should be further investigated (WHO, 1980). Plant extracts have long been used for the ethnomedical treatment of diabetes in various systems of medicine and are currently accepted as an alternative method for diabetic therapy (Gupta *et al*., 2009). Ethnobotanical studies are today recognized as the most viable method of identifying new medicinal plants or refocusing on those earlier reported for bioactive constituents (Fansworth, 1996). The method is reported to show greater percentage yield of bioactive useful medicinal compounds over other methods of random selection and screening (Khafagi and Dewedar, 2000). Grover *et al* (2002) reported that more than 1,100 plant species have been used ethnopharmacologically or experimentally to treat diabetes mellitus. The mechanism of inhibition by the widely occurring natural inhibitors is mostly unclear. A recent structural analysis of alpha-amylase complexed with acarbose sheds light on inhibition by carbohydrate-based compounds (Qian *et al.*, 1994). Acarbose represents a pharmacological approach to achieving the metabolic benefits of a slower carbohydrate absorption in diabetes, by acting as a potent competitive inhibitor of intestinal α-glucosidases. Acarbose molecules attach to the carbohydrate

binding sites of α-glucosidases, with a much higher affinity constant than the normal substrate (Salvatore and Giugliano, 1996). However, the conversion of oligosaccharides to monosaccharides is only delayed rather than completely blocked due to the reversible nature of the inhibitor-enzyme interactions. Recent interest in plant polyphenols has focused on their potential benefits to human health. The polyphenols are capable not only reducing oxidative stress but also inhibiting carbohydrate hydrolyzing enzymes and thus preventing hyperglycemia (de Sousa *et al*., 2004; Hanamura *et al*., 2005). During the work on identification of antidiabetic principles from Indian mangrove flora, we noticed moderate intestinal α-glucosidase inhibitory activity in the methanolic extract of fruits of *S. caseolaris* and *C. odollam* (**Fig. 3.5.15 and Table.3.5.1**).

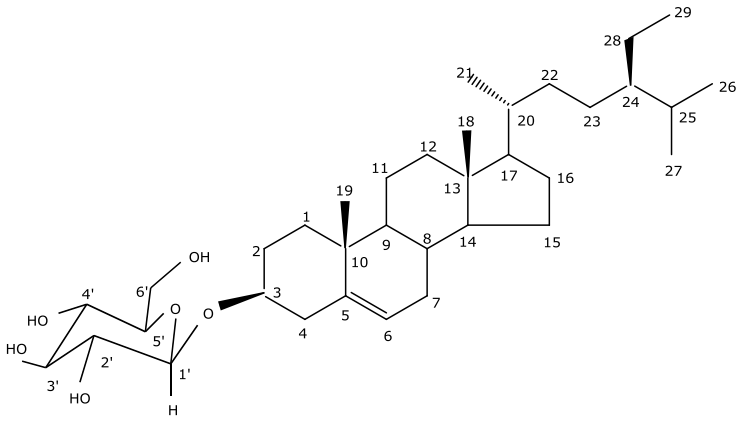
Murraya koenigii (Curry veppu) is widely used as relish and condiment in India and other tropical countries. It belongs to the family *Rutaceae* (citrus family). It has been cited as a treatment for diabetics in Ayurveda (Satyavati *et al*., 1999). Several studies have mentioned the antidiabetic effect of the *M. koenigii* leaves on diabetic animal models (Vinuthan *et al*., 2004; Kesari *et al*., 2005; Tembhurne and Sakarkar, 2010; El Amin *et al*., 2013). Phytochemical profile of *M. koenigii* leaves shows the presence of some vitamins, carbazole alkaloids, triterpenoids, mineral contents such as iron, calcium, zinc and vanadium, and phenolic compounds (Chakrabarty *et al*., 1997; Narendhirakannan *et al*., 2005). The antioxidant activity in *M. koenigii* is mainly attributed to carbazole alkaloids (Tachibana *et al*., 2003). The antidiabetic effect of *Murraya koenigii* leaf extract is attributed to its insulin secretagogue effect which could be due to the stimulation of the beta cells or regeneration of beta cell functioning by alleviating the oxidative stress (Vinuthan *et al*., 2004, Kesari *et al*., 2005; El Amin *et al*., 2013). The present

study reveals that the *M. koenigii* does not possesses alpha-amylase inhibition activity **(Fig.3.5.15)**. However, Bhat *et al* (2011) showed that chloroform extracts of *M. koenigii* showed porcine pancreatic α-amylase inhibitory activity which is higher than that of acarbose standard (1.9mM) and glucosidase activity. Dineshkumar *et al* (2010) reported that petroleum ether extract of *M. koenigii* leaves showed a higher inhibition of alpha-amylase activity than it hexane, chloroform, ethanol and aqueous extract.

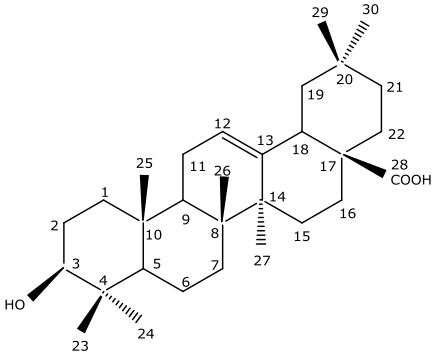
Cerbera odollam is a mangrove plant belonging to the *Apocynaceae* and distributed widely in the coastal areas of South East Asia and Indian Ocean. Two *Cerbera* species, *Cerbera odollam* and *Cerbera manghas*, are distributed widely throughout Bangladesh, India, Malaysia, Thailand, China, Australia and Philippine mostly on the sea coast (Cheenpracha *et al*., 2004; Rehman *et al*., 2011). *Cerbera venenifera*, a related species is found in Madagascar (Gillard *et al*., 2004). The seeds are extremely toxic, containing cerberin as the main active cardenolide and the *C. odollam* tree is responsible for about 50% of the plant poisoning cases and 10% of the total poisoning cases in Kerala, India (Gillard *et al*., 2004). Its root and fruits are purgative and are used for the treatment of rheumatism (Rollet, 1981). A number of research works have been performed to evaluate its biological activities as cytotoxic activity (Laphookhieo *et al*., 2004), effect on central nervous system (Hien *et al*., 1991), purgative and antirheumatic activity (Yamauchi and Abe, 1987), cardiac stimulant activity (Chen and Zheng, 1987), neurological manifestations (Iyer and Narendranath, 1975), cardiotoxic activity (Kini and Pai, 1965), etc. However, information of the specific enzyme inhibition of *Cerbera odollam* is scares. In the present study, methanol extract of *C. odollam* seed showed mild inhibition (95% relatively) of alpha-amylase activity **(Table 3.5.1).**

Sonneratia caseolaris is a true mangrove (Keng, 1969) species belonging to family *Sonneratiaceae* (WanJusoh and Hashim, 2009) generally found near the banks of tidal rivers in brackish water. This species is widespread and can be found in Bangladesh, Brunei Darussalam, Cambodia, China (Hainan Island), India, Indonesia, Malaysia, Myanmar, Philippines, Singapore, Sri Lanka, Thailand, Viet Nam, Northeast Australia, Papua New Guinea, Solomon Islands, Vanuatu, New Caledonia, and the Maldives.

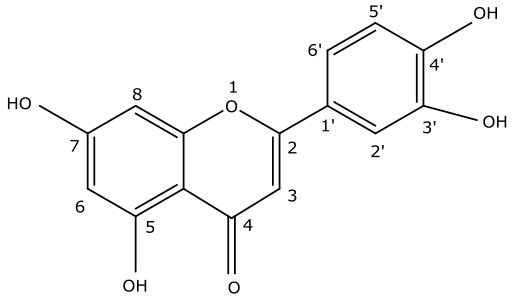
The inhibitory compounds present in the *S. caseolaris* are found to be β -Sitosterol-3-O-D-glucopyranoside, oleanolic acid and luteolin (Tiwari *et al*., 2010)



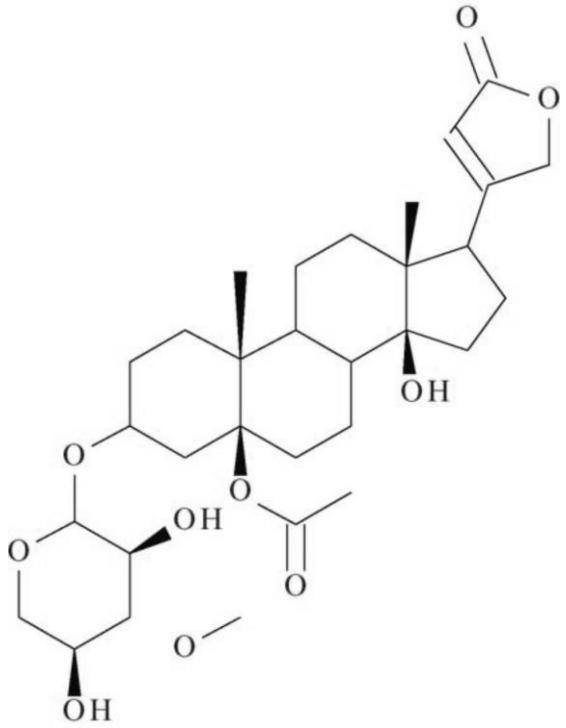
β -Sitosterol-3-O--D-glucopyranoside



Oleanolic acid



Luteolin



Cerberin- Toxin from *Cerbera odollam* (Gaillard *et al,* 2004)

Our study shows that the extract of *S. caseolaris* by its inhibition on alpha-amylase can act as an agent that can delay the absorption of glucose and cause a reduction of postprandial hyperglycemia. The presence of phytochemicals such as flavanoids, saponins and tannins may be responsible for the inhibitory effect of such aqueous plant extracts. Three compounds namely oleanolic acid, β-sistosterol-β-D-glucopyranoside and luteolin were

isolated and identified from the bioactive methanolic extract of *S. caseolaris*. In vitro pre-incubation of crude rat intestinal α-glucosidase with oleanolic acid showed potent α-glucosidase inhibitory activity. Similarly triterpenoids from *R. mangle, A. ilicifolius, A. corniculatum, B. rumphii, C. candolleana, K. rheedii*, L. racemosa, N. fruiticans, S. sericea (plumieri) and *A. ebracteatus* have shown pottential use in the control of diabetes mellitus (Bandaranayake, 2002). The characteristic non-competitive inhibition displayed by the *S. caseolaris* towards alpha-amylase indicates that the phytochemical moieties present in the aqueous extracts bind to a site other than the active site of the enzyme and combine with either free enzyme or the enzyme-substrate complex, possibly interfering with the action of both (Mayur *et al*., 2010). Also the Lineweaver–Burk plot showed that the same extract inhibited the enzyme competitively and thereby it can be inferred that saccharide based inhibitors also may be present and they compete with the substrate for binding to the active site. The action mechanism proposed for inhibitory capacity of flavonoids correlated the potency of inhibition of these compounds with the number of hydroxyl groups on the B ring of the flavonoid skeleton with the formation of hydrogen bounds between the hydroxyl groups of the polyphenol ligands and the catalytic residues of the binding site of the enzyme. High inhibitory capacity is observed in flavonols and flavones. The main inhibitory effect of the tannins is related to its ability to strongly bind to carbohydrates and proteins. However, Kandra *et al* (2004) suggested that the interaction between tannins, such galloylated quinic acid and human α-amylase is also correlated with free -OH groups in the tannin that are able to participate in hydrogen bonding. However, tannins are not effective inhibitors of α-amylase (de Sales *et al*, 2012).

Cerberin is the toxic compound present in *C. odollam* (Gaillard *et al*, 2004) however the exact α-amylase inhibitor from this plant is not yet identified.

The observations in this chapter indicated that at ambient conditions *O. mossambicus* alpha-amylase is more active than *E. suratensis* alpha-amylase since the optimum temperature and pH for maximum enzyme activity of the former are more near to the average ambient conditions of our natural habitat. The high stability at low pH values and the high thermal stability of *O. mossambicus* alpha-amylase suggest the need to develop new industrial applications by further studies of these enzymes at a larger scale.

The higher inhibitory property of *S. caseolaris* fruit extracts on alpha amylase activity in comparison with other plant extracts is proved using kinetic studies in the present chapter. The concentration of specific inhibitory molecules in the *C. odollam* fruit extracts could be the cause of decreased inhibitory activity. Its inhibitory property can be improved by further purification and the molecules can be identified and characterised for better applications. The mechanism of inhibition by aqueous extracts of *S. caseolaris* fruits is a non competitive-un competitive mixed type and is different from the classical reversible inhibition kinetics.

Chapter 4

TOTAL ALKALINE PROTEASE

Contents

The complete hydrolysis of ingested proteins, polypeptides and partially denatured proteins after the gastric digestion is carried out by the alkaline protease present in the intestine of the fishes. The present chapter describes the total alkaline protease present in the intestine of *Etroplus suratensis* and *Oreochromis mossambicus*. The chapter is divided into sections as in the case of previous chapter.

4.1 Introduction

Proteases constitute one of the most important groups of industrial enzymes, and account for at least 60% of all global enzyme sales (Gupta *et al*., 2002; Johnvesly and Naik, 2001; Rao *et al*., 1998). Alkaline proteases are widely distributed in nature. Historically their characteristic properties were first recognized among the digestive enzymes originating in the pancreas of mammals. Alkaline proteases from bacteria, fungi or insects can be exploited commercially (Anwar and Saleemuddin, 1998). The total alkaline protease is a mixture of several serine, cysteine or metallo proteases. The serine-type peptidases have an active serine residue involved in the catalytic process, the cysteine-type peptidases have a cysteine residue in the active centre, and the metallo-peptidases use a metal ion (commonly zinc) in the catalytic mechanism. Furthermore, they are not

restricted to digestive functions in the intestinal tract. Thrombin and plasmin, enzymes involved in the formation and dissolution of blood clots, respectively, show these characteristic properties. Interestingly, serine proteases have also been detected in mammalian mast cells.

4.2. Objective of the Study

The alkaline proteases have immense medicinal, industrial and research applications and the objective of the present study is to characterize the total alkaline protease from the *E. suratensis* and *O. mossambicus* biochemically by analysing their optimum temperature, pH and substrate concentration. The study furthermore investigates the effect of different metal ions on the activity of selected enzymes. The temperature denaturation kinetics of these enzymes is another aim of this research. pH stability is one of the important aspects with respect to enzymes and the estimation of pH stability of total alkaline proteases from gastro-intestinal tract of the selected fishes is also studied.

4.3. Review of Literature

There are many studies on new sources of proteolytic enzymes and especially proteases from fish have been frequently brought to focus. There is a large diversity of aquatic species in the tropical zones. Fishes are ectothermic animals and have many morphological and physiological adaptations, different food habits and characteristics of the digestive tracts. This diversity is reflected in their digestive enzyme activities and properties (Alencar *et al*., 2003; Bezerra *et al*., 2001; Bezerra *et al*., 2005; Cohen *et al*., 1981b; El-Shemy and Levin, 1997; Guizani *et al*., 1991). The proteins present in fish viscera, an important food processing waste that is usually discarded, could be employed as an alternative source of bioactive molecules for biotechnological applications (De Vecchi and Coppes, 1996; Haard, 1992). The search for

proteases from different sources has increased in the last years with estimation that nearly 50% of total industrial enzyme sales consisting of proteases. In order to offer a variety of proteases, particularly those with unique properties, new sources of proteolytic enzymes have been studied including proteases from fish viscera (Souza *et al*., 2007). Although fish proteases are basically similar to their mammalian counterparts, differences in structural and catalytic properties have been reported (Fong *et al*., 1998). Fish proteases have shown higher catalytic activities over a wide range of pH and temperature conditions (Shahidi and Kamil, 2001) at relatively low concentrations (Haard, 1998). As an example, industrial applications of serine proteinases for detergent, food, pharmaceutical, leather and silk industries have been studied (Klomklao, *et al*., 2005). Alkaline proteases have applications in peptide synthesis and to resolve racemic mixtures of amino acids (Sutar *et al.*, 1991; Chen *et al.*, 1991; Chen *et al.*, 1995). Therefore, studies describing enzymes isolated from these animals represent the first step in evaluating their potential for technological application. In fact, experiments at laboratory level are essential for future production at industrial scale (Silva *et al*., 2011).

Enzyme	Peptide bond cleaved
Trypsin	C-Terminus of Lys or Arg
Chymotrypsin, subtilisin	C-Terminus of Tyr, Phe or Trp
Staphylococcus V8 protease	C-Terminus of Asp or Glu
Thermolysin	N- Terminus of Leu or Phe
Pepsin	N- Terminus of Phe, Tyr or Trp
Asp-*N*-protease	N- Terminus of Asp or Glu
Submaxillarus protease	C-Terminus of Arg

Fish viscera, accounting for about 5% of the total body mass, contains the most important digestive protease like pepsin, trypsin, chymotrypsin,

phosphatases etc. (Gildberg and Overbo, 1991; Simpson, 2000; Simpson and Haard, 1987). As a rule, alkaline proteases from tropical fish have thermal stability, a long shelf life and high activity over a wide range of pH levels (Alencar *et al*., 2003; Bezerra *et al*., 2000; Bezerra *et al*., 2001; Bezerra *et al*., 2005; Souza *et al*., 2007). In vertebrates with a distinct pancreas, the proteases are present in the gland and in the exocrine juice as inactive precursors, or zymogens, which must be activated before their application. All of the known zymogens of the alkaline proteases are activated by trypsin. Trypsin itself arises from the activation of pancreatic trypsinogen by an autolytic mechanism; in vivo this process is initiated by an intestinal enzyme, enterokinase (entero peptidases). In teleost fishes, which do not have a distinct pancreas, serine proteases have been found in the pyloric caeca. The knowledge about alkaline proteases of fishes is important because they participate in feed utilization and have a role in larval development. In early larval stage, many fishes like *Acipenser fulvescens* (Buddington, 1985), *Sparus aurata* (Sarasquete *et al*., 1993) and A*cipenser baeri* (Zottowska *et al*., 2007) possess proteases that act only in the alkaline pH range. Fish viscera are potential sources of enzymes such as proteinases (Haard, 1992) that may have some unique properties for industrial applications (Ooshiro, 1971; Kawai and Ikeda, 1972; Kristjansson, 1991). Digestive proteinases have been extensively studied in several species of fish (An and Visessanguan, 2000). Fish food habit would affect their proteolytic activity and thereby growth (Chakrabarti *et al*., 1995; Hidalgo *et al*., 1999). Hofer and Schiemer (1981) reported that proteolytic activity is related to feeding habits of different fishes. Even though carnivorous species have smaller guts compared to herbivore species, they have higher proteolytic activity. Chakrabarti *et al* (1995) and Hidalgo *et al* (1999) reported similar findings. Although it is a common practice to refer to

piscine serine endopeptidases as trypsin- or chymotrypsin-like on the basis of their substrate specificities, the enzymes from fish differ in a number of ways, most notably in their stability and activity under highly alkaline conditions. Understanding the optimum conditions for digestive enzymes activities will also enable better comprehension of nutrient digestibility in fish (Glass *et al*., 1989; Kolkovski, 2001). Characterization of proteases activities in several fish species enables formulation of feed, prediction of inhibition by antinutritional factors in plant-based ingredients and development of suitable feeding regimes based on internal rhythm of protease secretion (Alarcon *et al*., 1999; El-Sayed *et al*., 2000; Chong *et al*., 2002a; Eusebio and Coloso, 2002). The development of an economical and palatable artificial feed with optimized nutrient content is often practised because the use of nutrient utilization in fish depends on the activities of digestive enzymes present in various digestive organs. Protease activity in the digestive tract is a key determinant of the digestibility and assimilation efficiency of ingested proteins. Protease activity studies also helped in the development of more rapid and accurate in vitro digestibility assays (Dimes *et al*., 1994; Chong *et al*., 2002b). For example, the digestive protease activity is correlated to enhanced food conversion efficiency and growth rate in Atlantic cod (Lemieux *et al*., 1999). However, most proteinases from marine organisms are extracellular digestive enzymes with characteristics differing from homologous proteases from warmblooded animals (De Vecchi and Coppes, 1996). Among alkaline proteases, serine proteinases have been described as a group of endoproteinases with a serine residue in their catalytic site. This family of proteinases is characterized by the presence of a serine residue, together with an imidazole group and aspartyl carboxyl group in their catalytic sites (Simpson, 2000). The proteinases in serine subclass all have the same first three digits: EC 3.1.21. Trypsin and

chymotrypsin are the major serine proteinases purified and well characterized from the digestive glands of marine animals.

4.3.1 Fish Trypsin

Trypsin was first described and named in 1876 by Kuhne as the proteolytic activity in pancreatic secretions (Kuhne, 1876; Neurath and Zwilling, 1986). Kuhne differentiated this activity from that of pepsin by the higher optimal pH of trypsin. The proteolytic enzyme trypsin and its inactive precursor, trypsinogen, were first obtained in crystalline form from bovine pancreatic tissue by Northrop and Kunitz (1931). Trypsinogen is transformed into trypsin as the result of the cleavage of a single peptide bond (Lys6-Ile7) near the N-terminus of the zymogen, and the appearance of activity is accompanied by conformational changes. The predominant single chain product is β-trypsin, resulting from the release of a hexapeptide (at the Lys6-Ile7 sequence) from bovine trypsinogen while an octopeptide is released from the porcine zymogen (Charles *et al.*, 1963). Subsequent cleavage of the Lys131-Ser132 bond at the N-terminal end of the molecule of bovine trypsinogen leads to α-trypsin (Schroeder and Shaw, 1968) which is a two-chain structure held together by disulphide bonds. The activation process is catalyzed by different enzymes including enterokinase and trypsin itself. The latter autocatalytic process is accelerated by calcium ions which bind to the N-terminal region of the zymogen and promote the specific bond cleavage.

In the digestive tract of fish, one of the main peptidases is trypsin (EC 3.4.21.4) known as the serine endoprotease that hydrolyze peptide bonds at the carboxylic end of the amino acid residues arginine (Arg) and lysine (Lys). Biologically, trypsin serves as the activator of all the other zymogens of pancreatic tissue (Klomklao *et al*., 2009). A striking feature of the enzyme is

the narrow specificity of its action, which is almost exclusively directed toward L-lysyl and L-argininyl bonds of polypeptides. Thus, the control of the activation of trypsinogen has broad consequences in terms of formation of the endopeptidase and exopeptidase components of pancreatic juice. As separation and characterization of the individual pancreatic proteases was achieved, the name trypsin became associated with the proteolytic activity which cleaved peptide bonds C-terminal to Arg or Lys. The ready availability of trypsin from the pancreas of cattle allowed the enzyme to be purified by crystallization in 1931 (Northrop and Kunitz, 1931). Trypsin can be seen as a prototype of the serine endopeptidases of family S1, and much of the fundamental knowledge about the family has been derived from the study of this enzyme (Perona and Craik, 1995). Trypsin strongly prefers to cleave amide substrates following P1 Arg or Lys residues. The preference for these basic side chains is reflected by relative values for catalytic efficiency (kcat / Km) at least 10^5 greater than for other natural amino acids. The preference for Arg over Lys is 2- to 10-fold (Craik *et al*., 1985). The pH optimum of trypsin is approximately 8, although this varies slightly with species. The reaction buffer is required to contain moderate amounts (20 mM) of $CaCl_2$ for maximal activity and stability of the protease. Trypsins from marine animals resemble mammalian trypsins with respect to their molecular size (22-30 kDa), amino acid composition and sensitivity to inhibitors. Their pH optima for the hydrolysis of various substrates were from 7.5 to 10.0, while their temperature optima for hydrolysis of those substrates ranged from 35 to 65°C (De Vecchi and Coppes, 1996).

Trypsins from marine animals tend to be more stable at alkaline pH, but are unstable at acidic pH. On the other hand, mammalian trypsins are most stable at acidic pH (Simpson, 2000; Klomklao *et al.*, 2006a). Several studies suggest that pH 7.0– 9.0 is the optimal range for trypsin in carnivorous species

such as *Solea solea* (Clark *et al*., 1985), *Euthynnus pelamis* (Joakimsson and Nagayama, 1990) and *S. formosus* (Natalia *et al*., 2004) or herbivorous species like *Ctenopharyngodon idella* (Das and Tripathi, 1991), by comparison with chymotrypsin activity which, although the pH of activation is similar, seems to be present at pH 7.0–8.0 in species with different nutritional habits (Clark *et al*., 1985; Hidalgo *et al*., 1999; Chong *et al*., 2002a). Martinez *et al* (1988) purified two trypsin-like enzymes (trypsin A and trypsin B) from the pyloric caeca and intestine of anchovy (*Engraulis encrasicholus*). Cao *et al* (2000) isolated two anionic trypsins (trypsin A and trypsin B) from the hepatopancreases of carp. Trypsin from the pyloric caeca of Monterey sardine (*Sardinops sagax caerulea*) with molecular weight of 25 kDa, optimum pH 8.0 and temperature at 50°C was purified and characterized by Castillo-Yanez *et al* (2005). Trypsin was reported to be the major form of proteinase in the spleen of tongol tuna (*Thunnus tongol*) (Klomklao *et al*., 2006a) and two anionic trypsins (A and B) were purified from yellowfin tuna (*Thunnus albacores*) spleen (Klomklao *et al*., 2006b). Three trypsin isoforms, trypsins A, B and C were isolated and characterized from skipjack tuna (*Katsuwonus pelamis*) spleen by a series of chromatographies by Klomklao *et al* (2009). They have been isolated from different species of fish including mandarin fish (*Siniperca chuatsi*) (Lu *et al*., 2008), walleye pollock (*Theragra chalcogramma*) (Kishimura *et al*., 2008), spotted goatfish (*Pseudopeneus maculates*) (Souza *et al*., 2007), jacopever (*Sebastes schlegelli*) and elkhorn sculpin (*Alcichthys alcicornis*) (Kishimura *et al*., 2007), spotted mackerel (*Scomber australasicus*) (Kishimura *et al*., 2006a), yellow tail (*Seriola quinqueradiata*) and brown hakeling (*Physiculus japonicus*) (Kishimura *et al*., 2006b), Japanese anchovy (*Engraulis japonica*) (Kishimura *et al*., 2005), tambaqui (*Colossoma macropomum*) (Bezerra *et al*., 2001), carp (*Ciprinus*

carpio) (Cohen *et al*., 1981a), brownstripe red snapper (*Lutjanus vitta*) (Khantaphant and Benjakul, 2010), hybrid catfish (*Clarias macrocephalus* X *Clarias gariepinus*) (Klomklao *et* al., 2011), sardinelle (*Sardinella aurita*) (Khaled *et al*., 2011), silver mojarra (*Diapterus rhombeus*) (Silva *et al*., 2011) and zebra blenny (*Salaria basilisca*) (Ktari *et al*., 2012).

Trypsin and trypsin-like enzyme have been isolated and identified in a wide array of cold water as well as warm water fish species (Shahidi and Kamil, 2001). Trypsin and trypsin-like proteolytic enzymes have been extracted, purified and characterized in several fish species including pancreatic tissue of carp (Cohen *et al.,* 1981a), pyloric caeca of rainbow trout (Kristjansson, 1991), pyloric caeca of tambaqui (Bezerra *et al*., 2001), pyloric caeca of starfish (Kishimura and Hayashi, 2002), pyloric caeca of silk snapper (Rivera, 2003), intestine and pyloric caeca of white grunt (Munõz, 2004), intestine of Nile tilapia (Bezerra *et al*., 2005), viscera of Japanese anchovy (Kishimura *et al*., 2005), pyloric caeca of big eye snapper (Hau and Benjakul, 2006), spleen of tongol tuna (Klomklao *et al*., 2006a), pyloric caeca of chinook salmon (Kurtovic *et al*., 2006), viscera of sardine (Bougatef *et al*., 2007), pyloric caeca of jacopever, elkhorn sculpin (Kishimura *et al*., 2007), pyloric caeca of spotted goatfish (Souza *et al*., 2007), pyloric caeca of walleye Pollock (Kishimura *et al*., 2008), pyloric caeca of mandarin fish (Lu *et al*., 2008) and hepatopancreas of cuttle fish (Balti *et al*., 2009). The characteristics of enzymes from marine invertebrates resemble those of mammalian and fish trypsins in molecular weight, cleavage specificities, pH stability and reaction with inhibitors (Balti *et al*., 2009). However, marine invertebrate trypsins were unstable at acidic pH and were not activated or stabilized by adding calcium ions, unlike mammalian pancreatic trypsin (Kishimura and Hayashi, 2002). Generally, fish trypsin has been reported to have the molecular weight in the

range of 23–28 kDa (Hau and Benjakul, 2006). Castillo-Yanez *et al* (2004) found that the molecular weight of isolated trypsin from the pyloric caeca of Monterey sardine was 25 kDa. El-Beltagy *et al* (2005) reported that purified alkaline protease from the viscera of Bolti fish had a molecular weight of 23 kDa. The molecular weights of trypsin purified from the pyloric caeca of arabesque greenling, skipjack tuna and walleye pollock were estimated to be 24 kDa (Kishimura *et al*., 2006a; Klomklao *et al*., 2007; Kishimura *et al*., 2008). Their pH optima for the hydrolysis of various substrates have been reported to range from 8 to 11, while their optimal temperature for hydrolysis of those substrates ranged from 35 to 70ºC. However the stability of trypsins at a particular pH might be related to the net charge of the enzyme at that pH (Castillo-Yanez *et al.*, 2005). Trypsin might undergo the denaturation under acidic conditions, where the conformational change takes place and the enzyme cannot bind to the substrate properly (Klomklao *et al.*, 2006a).

4.3.2. Fish Chymotrypsin

Chymotrypsin, an endopeptidase, is a digestive enzyme existing in pancreatic tissues of vertebrates and invertebrates which is secreted into the duodenum (Geiger, 1985). Chymotrypsin preferentially cleaves peptide amide bonds where the carboxyl side of the amide bond (the P1 position, the first amino acid residue in the N-terminal direction from the cleaved bond) is a tyrosine, tryptophan, or phenylalanine. These amino acids contain an aromatic ring in their side chain that fits into a 'hydrophobic pocket' (the S1 position) of the enzyme. Chymotrypsin hydrolyzes peptide bonds with various α-amino acid carbonyl groups and attacks larger nonpolar aromatic groups such as tyrosine, phenylalanine and tryotophan (Folk *et al*., 1970; Appel, 1986; Galvao *et al*., 2001). It also attacks nonpolar groups like leucine but the

reaction is slower. It is also reported that leucyl and glutamyl bonds could be cleaved by chymotrypsin (Geiger, 1985; Appel, 1986). Appel (1986) indicated that the hydrolyzing power increases according to the type of substrate in the following sequence:

Proteins < Peptides < Amides < Esters < N-tyrosine esters.

Chymotrypsin has been widely reported in the gut of a variety of fish including: discus (*Symphysodon* sp.), carp (*Cyprinus carpto*), rainbow trout (*Oncorhyncus mykiss*), coho salmon (*O. kistuch*), chinook salmon (*O. tshawytscha*), gilthead seabream (*Sparus awrata*) and dentex (*Dentes dentex*) (Dimes *et al*., 1994; Alarcón *et al*., 1998; Chong *et al*., 2002a). Chymotrypsin concentration is 105 times higher in the gut than in non-gut tissue but the activity and concentration vary according to fish species and the environment in which the fish live (Elert *et al*., 2004; de la Parra *et al*., 2007). Chymotrypsin exists in three zymogen forms (chymotrypsinogens A, B and C) in the zymogen granules of the pancreas (Smith *et al*., 1951; Geiger, 1985; Raae, 1995; Leth-Larsen *et al*., 1996). The three types of chymotrypsin (A, B and C) have been found in mammals but only two types of chymotrypsin (A and B) have been found in fish (Yang *et al*., 2009). Specifically two different forms are cationic (chymotrypsin B) and anionic (chymotrypsin A) (Yang *et al*., 2009). The isoelectric points of fish chymotrypsinogens A and B are at the pH of 9.1 and 5.2, respectively. The two isoforms of chymotrypsin have also been extracted from rainbow trout (Kristjansson and Nielson, 1992), grass carp (Fong *et al*., 1998), Altantic cod (Ásgeirsson *et al*., 1993), anchovy (Heu *et al*., 1995), Monterey sardine (Castillo-Yañez *et al*., 2006) and crucian carp (Yang *et al*., 2009). Chymotrypsin has 245 amino acid residues and 5 pairs of disulfide linkages with a molecular mass around 24000. Generally, the single polypeptide molecular weight is 25-28 kDa (Haard *et al*., 2000). The amino

acid sequences of chymotrypsins A and B are very similar with only minor differences and the activated enzymes contain three polypeptide chains. Raae *et al* (1995) found specificity differences between two types of chymotrypsins (ChT1 and ChT2) isolated from cod. The inactive form of chymotrypsin (chymotrypsinogen) can be activated by trypsin which partially cleaves it into two parts while still maintaining an S-S bond. In the zymogen activation process, chymotrypsinogen goes through different forms of intermediate products (α-chymotrypsin, γ- chymotrypsin, δ-chymotrypsin, π-chymotrypsin) before forming chymotrypsin A. However, the type of intermediate products generated depends on trypsin concentration and reaction velocities as shown in **Fig. 4.2.1.**

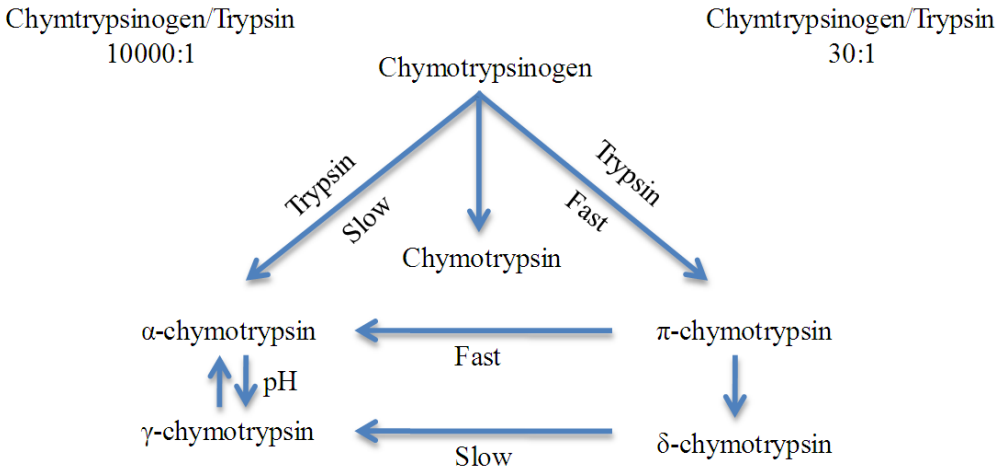


Fig. 4.2.1 Activation of chymotrypsinogen (Appel, 1986)

Hudáky *et al* (1999) reported that chymotrypsins A and B have similar specificities in hydrolyzing peptides with Leu and aromatic amino acid residues like Tyr, Trp and Phe. The pH, temperature, source and storage are the most significant factors influencing the activity and stability of chymotrypsin. The pH range of chymotrypsin activity is 7.5-9.0 (Kristjansson *et al*., 1992). Castillo-Yanez *et al* (2009) reported that fish chymotrypsin was

stable only under alkaline pH condition but animal chymotrypsin was stable at both alkaline and acidic pH. Studies on the effect of pH on the activity of sardine and bovine chymotrypsins revealed that the activity of chymotrypsin was significantly inhibited when the pH was less than 6 and the enzyme was denatured at a pH of 4 but was stable at a pH of 8 (Castillo-Yanez *et al*., 2009). Sabapathy and Teo (1995) found the optimal activity of rabbitfish chymotrypsin to be within the pH range of 7.5-8.5 and for 2 types of chymotrypsin (CTR1 and CTR2) extracted from *Locusta migratoria*, the optimal activities were in the pH ranges of 8-10 and 8-11, respectively (Lam *et al*., 1999) Comparable results were reported for *European sea bass* (Alliot *et al*., 1973), *Dover sole* (Clark *et al*., 1985), *skipjack* (Joakimsson and Nagayama, 1990), *Siganus canaliculatus* (Sabapathy and Teo, 1995), anchovy (Heu *et al*., 1995) and Bluefin tuna (de la Parra *et al*., 2007).

4.4. Materials and Methods

The collection and acclimation of *E. suratensis* and *O. mossambicus* were conducted as explained in Chapter 2. As the hepatopancreatic tissue is responsible for the production and storing of zymogens in the granules of acinar cell, the hepatopancreas of the selected fishes is also investigated for their total alkaline protease activity.

4.4.1 Methods

The soluble protein content of enzyme homogenates was measured according to Lowry *et al* (1951) as described in Chapter 2.

4.4.2. Estimation of Total Alkaline Protease

The total alkaline protease was estimated by using casein as substrate (Lundstedt *et al*., 2004) with a slight modification. The pH of tris buffer was 8.

Briefly, 50µl of homogenate was mixed with 0.1M Tris buffer pH-8 having 20mM $CaCl_2$ and preincubated for 5 minutes at 37^0C and mixed thoroughly with 1% substrate solution. The reaction was stopped by adding 12% ice cold TCA and tyrosine in the supernatant was measured at 280nm after centrifugation at 8000 rpm for 15 min. The homogenate was added to the blank tubes at the end of the incubation after adding the TCA. L-tyrosine was used as a standard, and one unit of enzyme activity (U) is defined as the amount of enzyme needed to catalyse the formation of 1 µM of tyrosine/min/ ml of homogenate at 37^0 C. The specific activity is expressed as U/mg protein.

4.5. Results

A casein concentration of 1.75% is sufficient to attain maximum saturation of total alkaline proteases in the crude homogenate of *O. mossambicus* intestine (**Fig.4.5.1**). The *O. mossambicus* hepatopancreatic total alkaline protease maximum saturation has been attained at 1.25% casein concentration (**Fig.4.5.2**). The slight decrease in reaction velocity at 2% casein substrate saturation is negligible. The optimum temperature of *O. mossambicus* intestinal Total Alkaline Protease has been obtained as 65^0C (**Fig.4.5.3**). The optimum temperature of *O. mossambicus* hepatopancreatic Total Alkaline Protease is found to be 50^0C (**Fig.4.5.4**).

At all alkaline pH values, enzyme showed higher activity than neutral and the optimum pH of intestinal total alkaline protease of *O. mossambicus* is around 9 (**Fig.4.5.5**). In contrast, the hepatopancreatic total alkaline protease of *O. mossambicus* possessed its maximum activity at pH 8.5 and also showed comparatively higher activity beyond pH 9.5, which extended up to 10.5 (**Fig.4.5.6**). In the case of intestinal total alkaline protease of *E. suratensis*, the maximum velocity was obtained at 1.25% casein (**Fig.4.5.7**). The

hepatopancreas total alkaline protease similarly possessed substrate saturation concentration at 1.25% Casein (**Fig.4.5.8**). The optimum temperature of *E. suratensis* intestinal total alkaline protease is found to be around 45^0C (**Fig.4.5.9**). The **Fig.4.5.10** illustrates the effect of temperature on the activity of *E. suratensis* hepatopancreatic total alkaline protease. The optimum temperature is determined to be 60^0C. It is assumed that the enzymes in the hepatopancreatic tissue are stabilized by some other protein factors. Thus enzymes from the hepatopancreatic tissue may be protected from extreme temperatures, pH and other stressors. The intestinal and hepatopancreatic total alkaline proteases of *E. suratensis* exhibit a pH optimum of 7.5 (**Fig.4.5.11** and **Fig.4.5.12**). The intestinal total alkaline protease of both *O. mossambicus* and *E. suratensis* showed comparatively similar extent of activity with respect to 1% casein concentration. However, the hepatopancreatic total alkaline protease of *O. mossambicus* showed lesser activity than that of *E. suratensis* at the same substrate concentration (**Fig.4.5.13**).

The effect of metal ions on alkaline protease was studied. Iron inhibits the *O. mossambicus* total alkaline protease and beyond 20mM concentration the enzyme loses its activity completely. Copper ions showed an increasing inhibition trend with respect to increase in concentration. In the case of *O. mossambicus* total alkaline protease the zinc and mercury ions increased the activity at all the concentrations studied. Even though the increase in concentration of calcium ions showed an inhibitory effect, it showed an enhancement in enzyme activity at low concentration (15mM). Sodium and barium ions exhibited an enhancing capacity all at the concentrations studied. Lithium ions do not significantly affect the enzyme activity. Potassium and magnesium ions showed an identical trend in its action, both of the exhibited an enhancing property in the enzyme action (**Fig.4.5.14**).

Fig.4.5.15 shows the result of metal ions on intestinal total alkaline protease of *E. suratensis*. Lithium ions displayed an enhancing effect. Sodium, copper, zinc and mercury ions do not exert significant effect on the enzyme specific activity. Iron ions inhibit the enzyme activity. Potassium, magnesium, calcium, barium and manganese ions increase the enzyme activity at all concentrations. **Fig.4.5.16** illustrates that both intestinal and hepatopancreatic total alkaline protease of *O. mossambicus* showed pH stability at pH 2 and at pH 2.5 it is less active. At pH 2 the intestinal enzyme retained its 80% relative activity, while the hepatopancreatic counterpart exhibit 100% relative activity. At pH 2.5, the intestinal as well as hepatopancreatic enzymes lose their activity to 30% and 70% respectively. The intestinal total alkaline protease attained its maximum (100%) relative activity at pH 3.5. Both the enzymes were stable up to pH 10.5. The pH stability of *E. suratensis* total alkaline protease has been depicted in **Fig.4.5.17**. The intestinal and hepatopancreatic enzymes retained their maximum activity at 4.5 and beyond that the increase in alkalinity leads to instability of the enzyme, especially in the case of hepatopancreatic total alkaline protease. Both the enzymes retained 60 to 80% relative activity at pH 2. The hepatopancreatic total alkaline protease of *E. suratensis* showed a drop in enzyme stability at pH 3.5, whereas it is at pH 2.5 in the case of *O. mossambicus* (**Fig.4.5.16**). The intestinal and hepatopancreatic enzymes retained their stability up to pH 10.5 with relative activity 80% and 40% respectively.

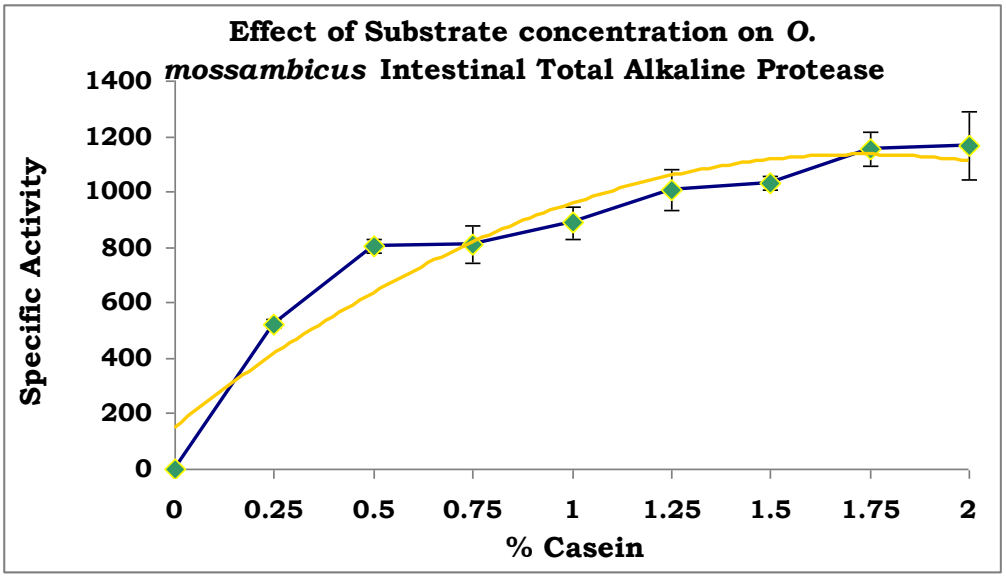


Fig.4.5.1. Substrate saturation curve of *O. mossambicus* intestinal Total Alkaline Protease

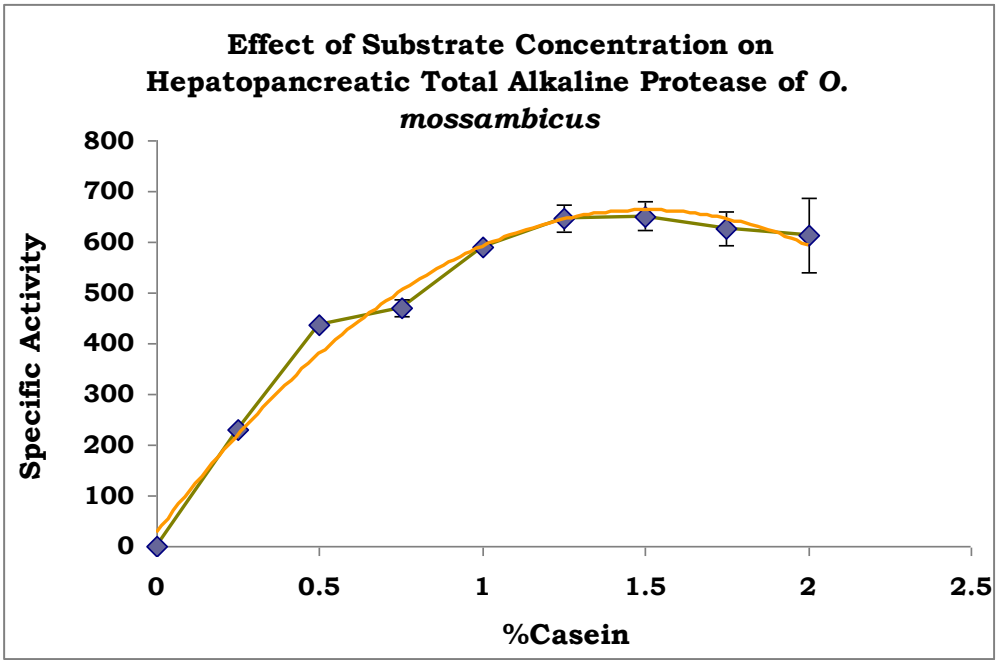


Fig.4.5.2. Substrate saturation curve of *O. mossambicus* hepatopancreatic Total Alkaline Protease

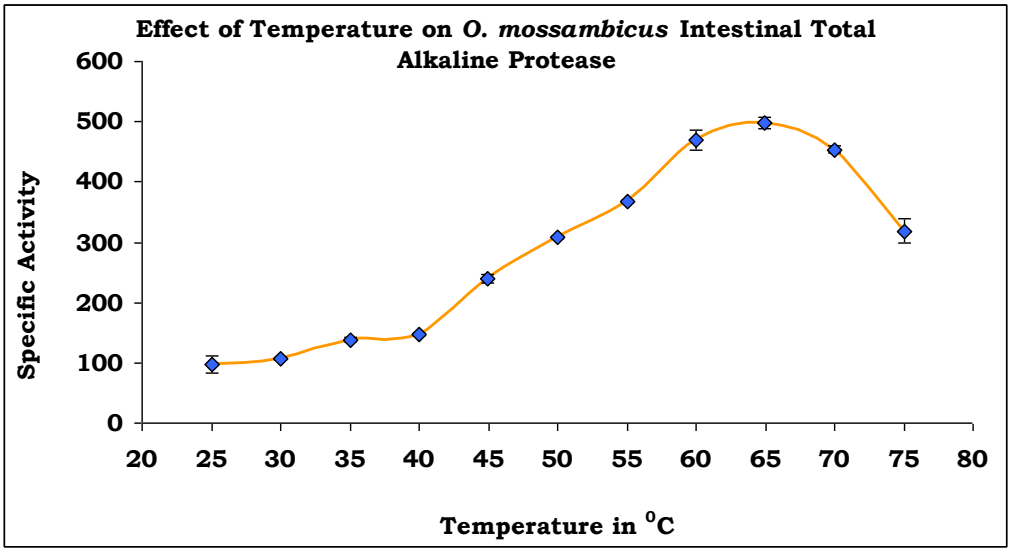


Fig.4.5.3. Optimum Temperature curve of *O. mossambicus* intestinal Total Alkaline Protease

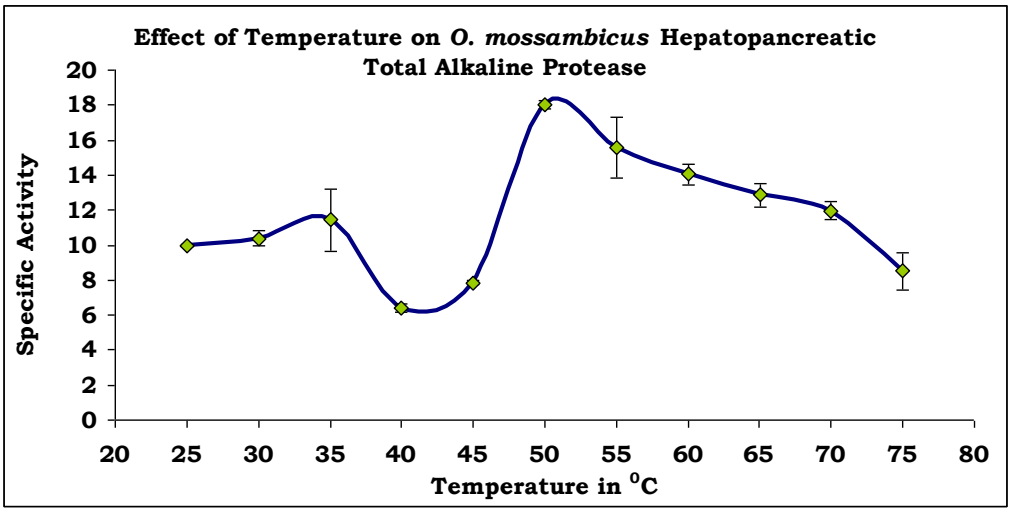


Fig.4.5.4. Optimum Temperature curve of *O. mossambicus* hepatopancreatic Total Alkaline Protease

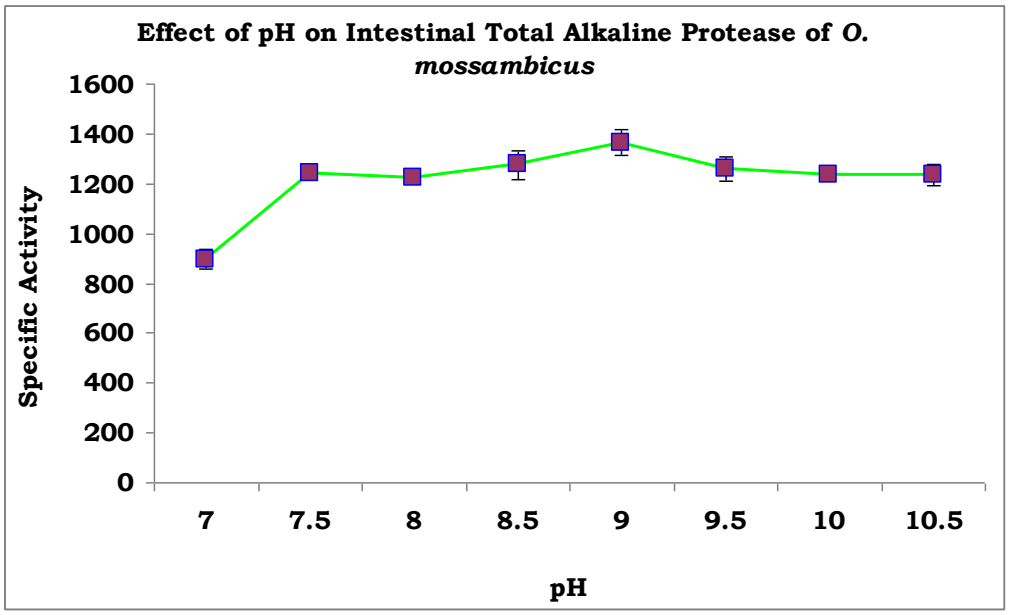


Fig.4.5.5. Optimum pH curve on *O. mossambicus* intestinal Total Alkaline Protease

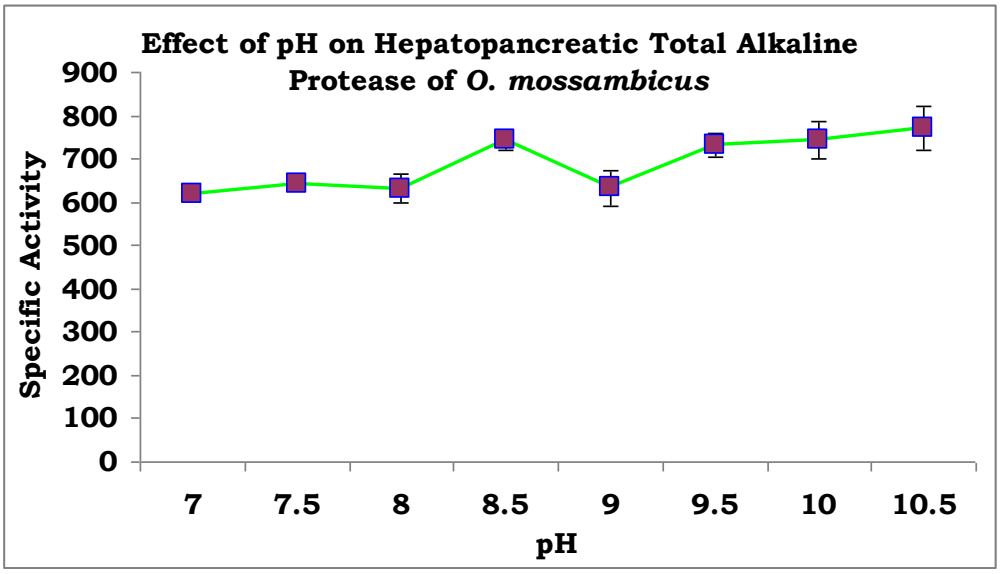


Fig.4.5.6. Optimum pH curve of *O. mossambicus* hepatopancreatic Total Alkaline Protease

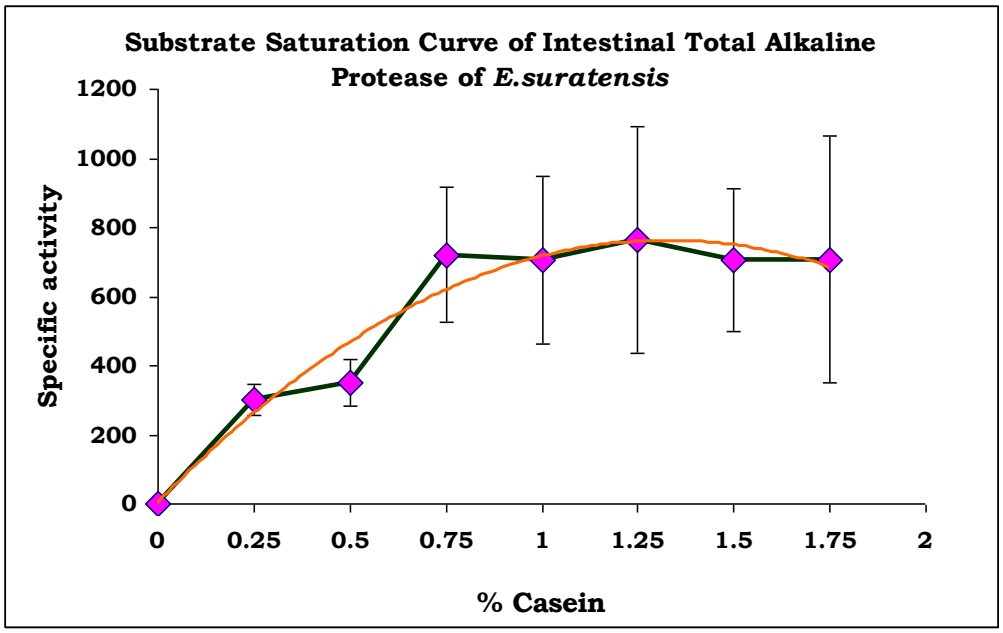


Fig.4.5.7. Substrate saturation curve of *E. suratensis* intestinal Total Alkaline Protease

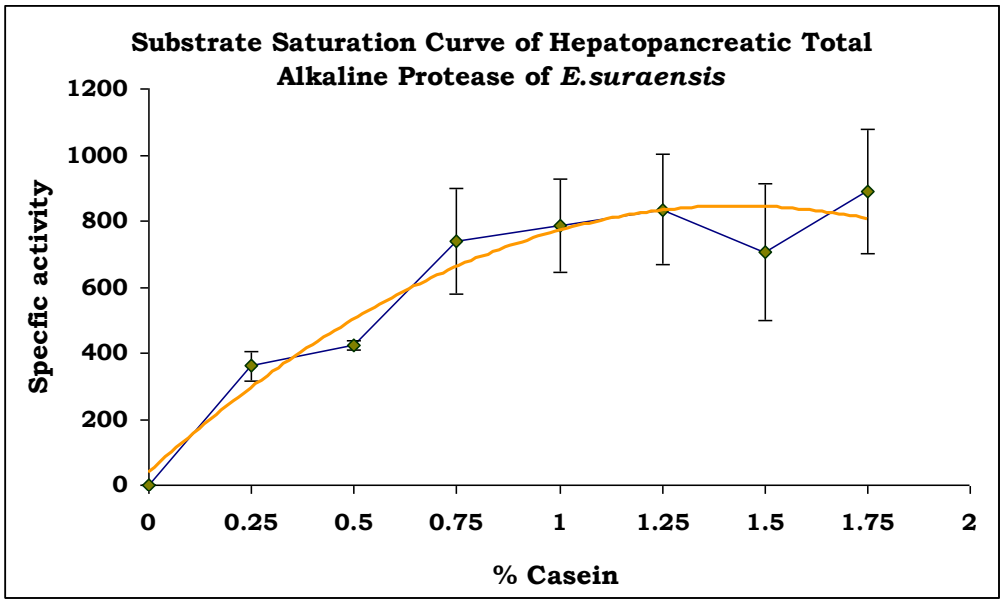


Fig.4.5.8. Substrate saturation curve of *E. suratensis* hepatopancreatic Total Alkaline Protease

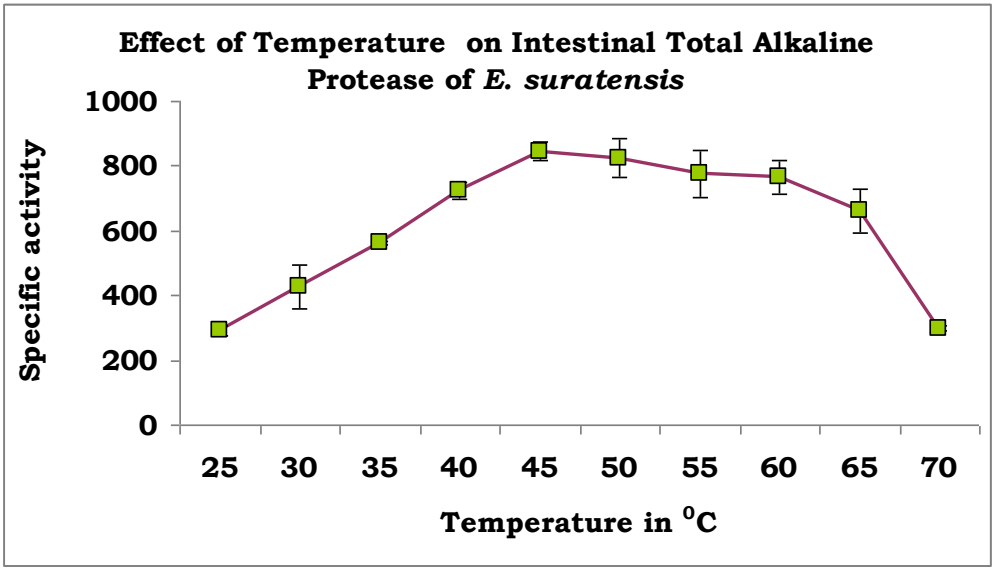


Fig.4.5.9. Optimum temperature curve of *E. suratensis* intestinal Total Alkaline Protease

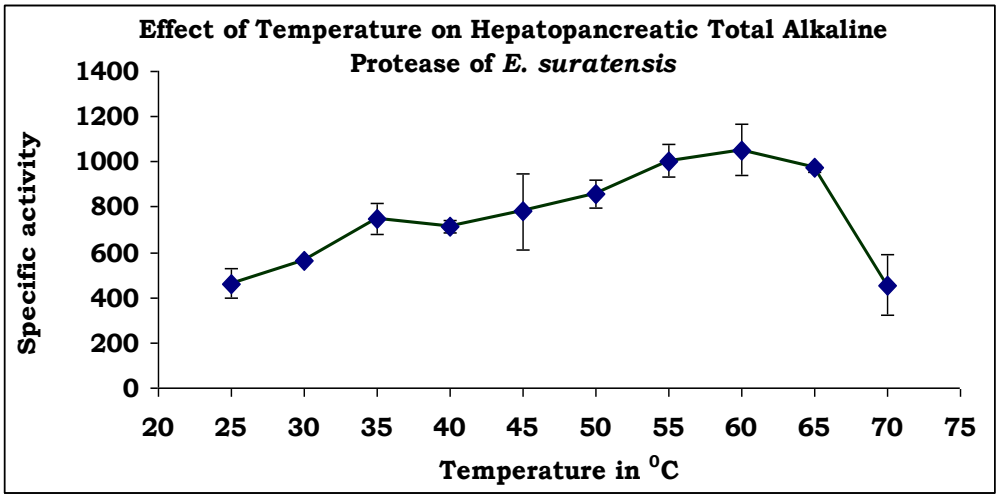


Fig.4.5.10. Optimum temperature curve of *E. suratensis* hepatopancreatic Total Alkaline Protease

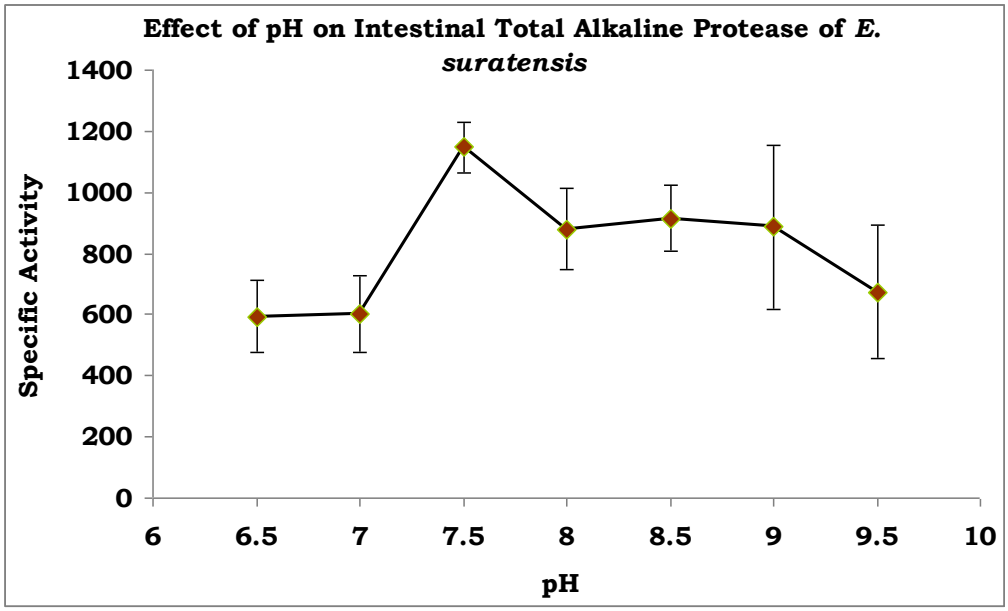


Fig.4.5.11. Optimum pH curve of *E. suratensis* intestinal Total Alkaline Protease

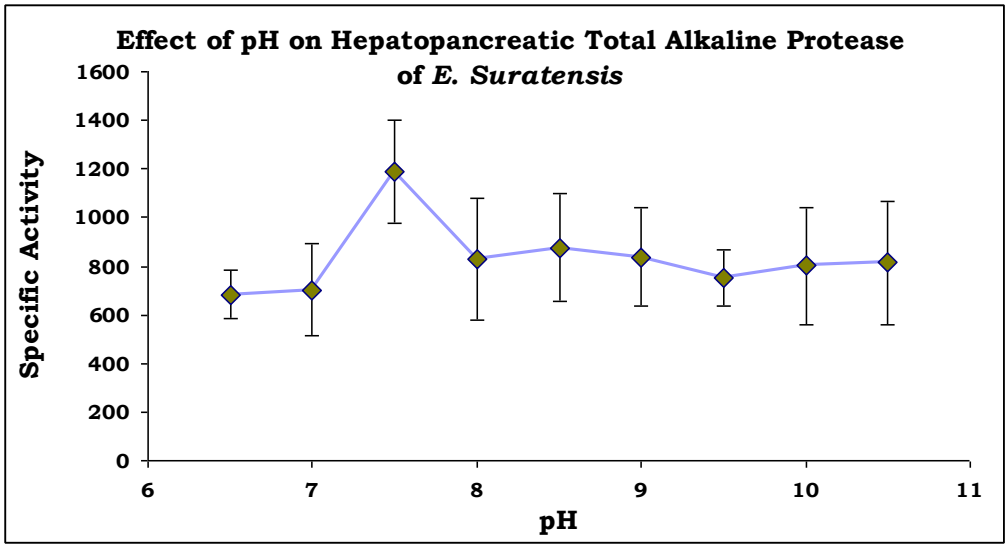


Fig.4.5.12. Optimum pH curve of *E. suratensis* hepatopancreatic Total Alkaline Protease

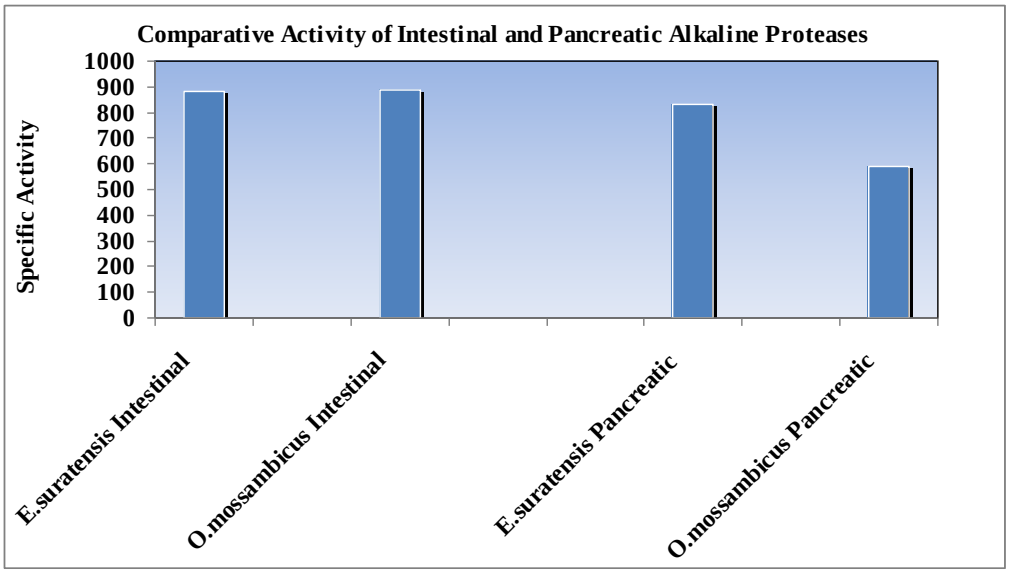


Fig.4.5.13. Comparative activities of intestinal and hepatopancreatic Total Alkaline Protease activity of *O. mossambicus* and *E, suratensis*

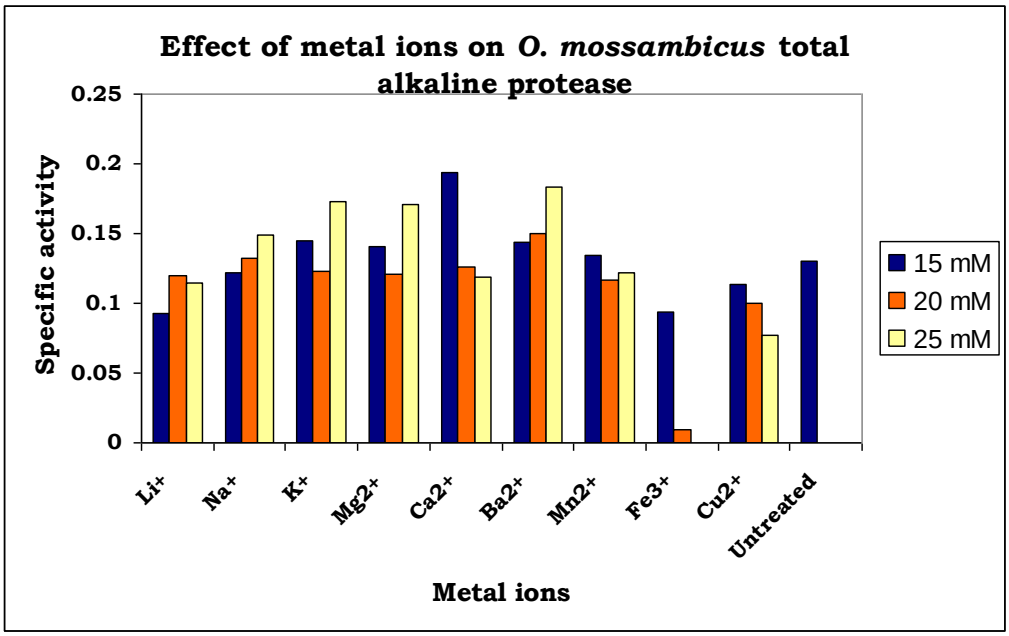


Fig.4.5.14. Effect of metal ions on *O. mossambicus* intestinal Total Alkaline Protease

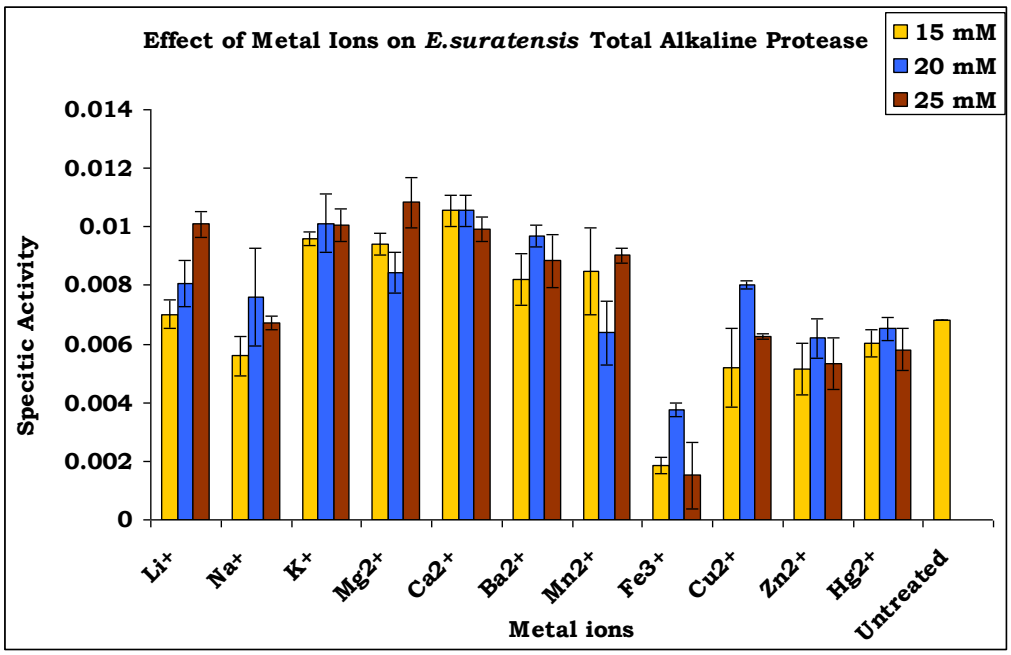


Fig.4.5.15. Effect of metal ions on *E. suratensis* intestinal Total Alkaline Protease

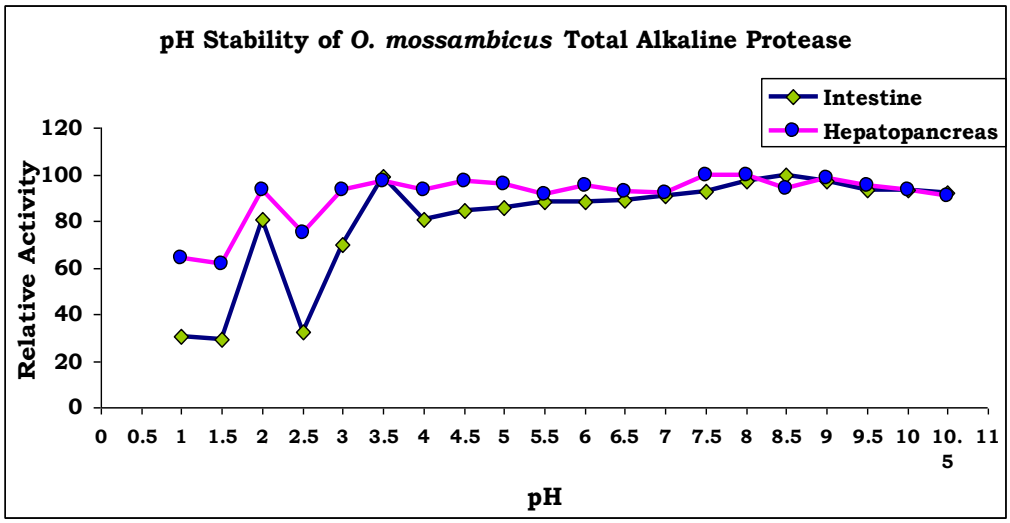


Fig.4.5.16. pH stability curve of *O. mossambicus* Total Alkaline Protease

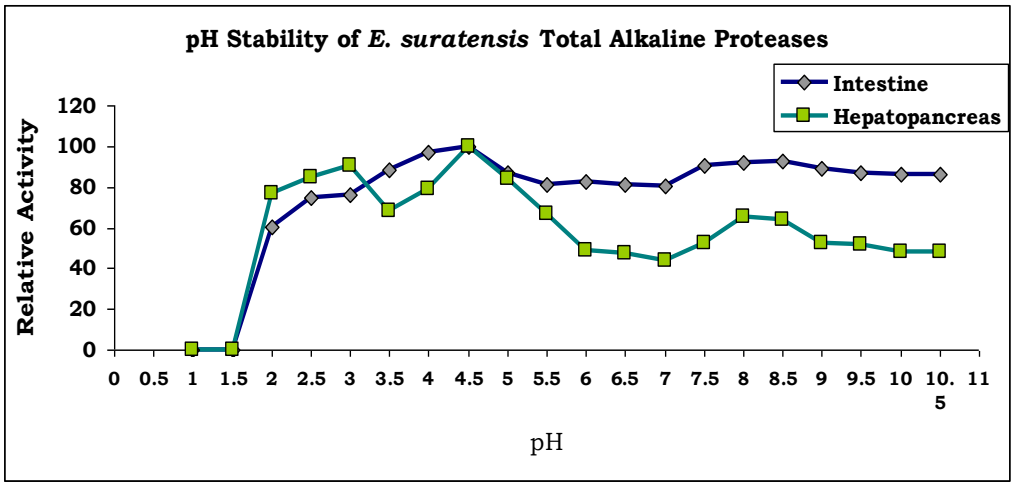


Fig.4.5.17. pH stability curve of *E. suratensis* Total Alkaline Protease

4.6. Discussion

Proteolytic enzymes or proteases catalyse the total hydrolysis of proteins by the cleavage of peptide bonds. They are enzymes of class 3, the hydrolases, and subclass 4, the peptide hydrolases or peptidases and divided as endopeptidases or proteinases (EC 3.4 21-99) and exopeptidases (EC 3.4.11-19) according to the point at which they break the peptide chain. Alkaline proteases (EC.3.4.21-24, 99) are hydrolytic enzymes that catalyze the cleavage of peptide bonds. Alkaline proteases are produced by mammalian tissues, higher animals, plants, fungi, actinomycetes, bacteria etc. Alkaline proteases (EC.3.4.21–24, 99) are defined as those proteases which are active in a neutral to alkaline pH range. They either have a serine center (serine protease) or a metal ion (metalloprotease); and the alkaline serine proteases are the most important group of enzymes exploited commercially. These enzymes have been reported to operate under harsh physiological conditions of temperature of (20^0C to 70^0C), pH (up to 11) and in presence of organic solvents, detergents etc. Fish viscera, accounting for 5% of total mass, have wide

biotechnological potential as a source of digestive enzymes, especially digestive proteases that may have some unique properties of interest for both basic research and industrial applications (Simpson and Haard, 1999). Fishes are poikilothermic, so their survival in cold waters required adaptation of their enzyme activities to low temperatures of their habitats. Enzymes from cold adapted fish species thus often have higher enzymatic activities at low temperatures than their counterparts from warm-blooded animals (Asgeirsson *et al*., 1989; Kristjansson, 1991). High activity of fish enzymes at low temperatures may be interesting for several industrial applications of enzymes, such as in certain food processing operations that require low processing temperatures. The intestinal secretions from fishes with stomach are not adequate in themselves to digest proteins because the aid of acid denaturation and acid protease hydrolysis which takes place in stomach is essential for the complete hydrolysis of dietary proteins by alkaline proteases of intestine. Intestinal secretions of stomachless fishes were sufficient to facilitate complete protein digestion. Such differences in the efficiency of alkaline proteases can be used as an estimate of protein digestibility by fish digestive enzymes. Since the relative activities of the total alkaline proteases, trypsin and chymotrypsin have been proposed as indicators of the nutritional status in fish (Uscanga *et al*., 2010), it is important to know about the functional characteristics of these enzymes. Information about proteases from tropical fishes and their applications is very scarce (De Vecchi and Coppes, 1996). In the present study, at all alkaline pH values, the enzyme showed higher activity than neutral. The optimum pH of intestinal total alkaline protease of *O. mossambicus* is around 9 (**Fig.4.5.5**). Total alkaline proteases of Blue fin tuna *Thunnus orientalis* showed maximal activity at pH 9.0(de la Parra *et al*., 2007). However, the hepatopancreatic total alkaline protease of *O.*

mossambicus possessed its maximum activity at pH 8.5 and also showed comparatively higher activity beyond pH 9.5, which extended up to 10.5 (**Fig.4.5.6**). Probably, the present optimum pH pattern showed in **Fig.4.5.5** and **Fig.4.5.6** is due to the mixture of proteases like trypsin, chymotrypsin and other peptidases in the crude extracts. The intestinal and hepatopancreatic total alkaline protease (**Fig.4.5.11** and **Fig.4.5.12** respectively) of *E. suratensis* exhibit a pH optimum at 7.5. The total alkaline protease from the viscera of *Tilapia nilotica* showed an optimum pH around 8.0 (El-Beltagy *et al*., 2005; Bezerra *et al*., 2005). The optimum pH for total alkaline protease activities of *Z. ophiocephalus*, *R. clavata*, and *S. scrofa* were 8.0-9.0, 8.0, and 10.0 respectively (Nasri *et al*., 2011). The crude enzymes from viscera of yellowfin tuna (*Thunnus albacares*) and skipjack tuna (*Katsuwonus pelamis*) exhibited the highest activities at pH 10.0 whereas it was at pH 9.0 for those from viscera of tonggol tuna (*Thunnus albacares*) (Prasertsan and Prachumratana, 2008). Many fishes possess multiple optimum pH for their digestive proteolytic activity. For example, alkaline protease from four tropical fishes, Crevalle Jack (*Caranx hippos*), Spotted Goatfish (*Pseudupeneus maculatus*), Parrotfish (*Sparisoma* sp.) and Traira (*Hoplias malabaricus*) had more than one optimum pH in a range of 7.0 to 9.0 (Alencar *et al*., 2003). Both intestinal and hepatopancreatic total alkaline protease of *O. mossambicus* showed pH stability at pH 2 and at pH 2.5 it is less active. At pH 2 the intestinal enzyme retained its 80% relative activity, while the hepatopancreatic counter part exhibit 100% relative activity. At pH 2.5, the intestinal as well as hepatopancreatic enzymes lose their activity to 30% and 70% respectively. The intestinal total alkaline protease attained its maximum (100%) relative activity at pH 3.5. Both the enzymes were stable up to pH 10.5 (**Fig.4.5.16)**. The pH stability of *E. suratensis* total alkaline protease has been depicted in

Fig.4.5.17. The intestinal and hepatopancreatic enzymes retained their maximum activity at 4.5 and beyond that the increase in alkalinity leads to instability of the enzyme, especially in the case of hepatopancreatic total alkaline protease. Both the enzymes retained 60 to 80% relative activity at pH 2. The hepatopancreatic total alkaline protease of *E. suratensis* showed a drop in enzyme stability at pH 3.5, whereas it is at pH 2.5 in the case of *O. mossambicus*. The intestinal and hepatopancreatic enzymes of *E. suratensis* retained their stability up to pH 10.5 with relative activity 80 and 40% respectively **(Fig.4.5.17)**. The crude alkaline protease may contain several zymogens that are converted to active forms at an alkaline pH. The relative abundance of zymogens and their active forms may explain the stability of the explored proteases in the alkali as well as in the acidic regions. More than 90% of the enzyme activity has been maintained by Bolti fish (*Tilapia nilotica*) total alkaline protease at pH values between 6.0 and 10.0. In the acidic region (pH 2.0–6.0), the enzyme retained more than 50% of its activity (El-Beltagy *et al*., 2005). The result obtained clearly suggests that there will be at least two different alkaline proteases are present in the crude extract. Generally, trypsin and chymotrypsin are two different proteases present in animals aiding the complete digestion of dietary proteins to aminoacids. They differ in their optimum temperatures. Further separation and purifications are essential for detailed studies and it will be one of the future perspectives. Fascinatingly, all the enzyme preparations from viscera of *Z. ophiocephalus*, *R. clavata*, and *S. scrofa* were highly stable over a wide range of pH from 6.0 to 11.0 (Nasri *et al*., 2011). The examination of pH optimum of the chyme enzymes of zander *Zander lucioperca* (L.), perch *Perca fluviatilis* L., bream *Abramis brama* (L.) and roach *Rutilus rutilus* (L.), revealed that the alkaline protease has pH optimum of in roach 7.0, in bream 8.0 and in perch and zander 10.0. However,

the pH optimum of the mucosa proteinases in all fish species is 10.0 (Kuzmina *et al*., 2011). It should be noted that the four studied fish species differ significantly in their feeding habits. In this study, the selected species, *Etroplus suratensis* and *Oreochromis mossambicus*, differ in their feeding habits and the difference in the enzyme activity and characteristics can be easily understood. The character of pH dependence of proteinase studied can somewhat change in the condition of real digestion. Actually, it is known that some nutrients influence the proteolytic activity (Ugolev and Kuz'mina, 1993; Kuz'mina, 2008). Besides, the temperature of the medium can play some role. An increase in temperature from 20 to 30^0C causes the change of pH optimum from 11.0 to 10.0 and further increase to 50^0C leads to the change of pH optimum to 8.0 (Munilla-Moran and Saborido-Rey, 1996a).

The optimum temperature of *O. mossambicus* intestinal Total Alkaline Protease has been obtained as 65^0C (**Fig.4.5.3**) and that of hepatopancreas is found to be 50^0C (**Fig.4.5.4**). The optimum temperature of *E. suratensis* intestinal total alkaline protease is found to be around 45^0C (**Fig.4.5.9**). The **Fig.4.5.10** illustrates the effect of temperature on the total alkaline protease activity of *E. suratensis* hepatopancreatic total alkaline protease and it is determined to be 60^0C. Digestive proteases are synthesized as inactive zymogens in the pancreatic cells and they are activated by specific proteolysis upon release into the gut lumen (Jobling, 1995). It is assumed that the enzymes at the hepatopancreatic tissue are stabilized by some other protein factors. Thus enzymes from the hepatopancreatic tissue may be protected from extreme temperatures, pH and other stressors. Visceral total alkaline protease of *Tilapia nilotica* exhibits an optimum temperature at 45^0C with casein as a substrate (El-Beltagy *et al*., 2005). Bezerra *et al* (2005) determined the optimum temperature of intestinal trypsin like alkaline protease of Nile tilapia,

Oreochromis niloticus as 50^0C. Blue fin tuna *Thunnus orientalis* total alkaline protease and trypsin showed temperature optimum, at 60^0C (de la Parra *et al*., 2007). The optimum temperature for *S. scrofa* proteases was 55^0C, however, alkaline proteases from goby and thornback ray displayed maximum activity at 50^0C (Nasri *et al*., 2011). The crude total alkaline protease activities of *Z. ophiocephalus*, *R. clavata*, and *S. scrofa* were active at temperatures from 30 to 70^0C (Nasri *et al*., 2011).

The optimum pH and temperature of total alkaline protease vary with respect to the source organ. The study of the proteolytic activity of the mucosa shows that, in species like zander *Zander lucioperca* (L.), perch *Perca fluviatilis* L., bream *Abramis brama* (L.) and roach *Rutilus rutilus* (L.), its level is significantly lower in the mucosa than in the chyme (Kuzmina *et al*., 2011).

The maximum saturation of *O. mossambicus* intestinal total alkaline protease has been attained at 1.75% casein concentration (**Fig.4.5.1**). The maximum saturation of *O. mossambicus* hepatopancreatic total alkaline protease has been attained at 1.25% casein concentration (**Fig.4.5.2**). The slight decrease in reaction velocity at 2% casein substrate saturation is negligible. In the case of intestinal total alkaline protease of *E. suratensis*, the maximum velocity has been obtained 1.25% casein (**Fig.4.5.7**). The hepatopancretic total alkaline protease similarly possessed substrate saturation concentration at 1.25% casein (**Fig.4.5.8**). The intestinal total alkaline protease of both *O. mossambicus* and *E. suratensis* showed comparatively similar extent of activity with respect to 1% Casein concentration. However, the hepatopancreatic total alkaline protease of *O. mossambicus* showed lesser activity than that of *E. suratensis* in the same substrate concentration (**Fig.4.5.13**).

The effect of metal ions on the total alkaline proteases of both the fishes was studied in detail. Iron inhibits the *O. mossambicus* total alkaline protease and at 20mM concentration, the enzyme loses its activity completely. Copper ions showed an increasing inhibition with increase in concentration of Cu^{2+}. Even though the increase in concentration of calcium ions showed an inhibitory effect, Ca^{2+} showed an enhancement in enzyme activity at low concentration (15mM). The Ca^{2+} inhibition effect is unusual since this is a classical trypsin activator. Thermo stable dipeptidase from common carp (*Cyprinus carpio*) intestine (Aranishi *et al*., 1998) and the trypsin from an aquatic invertebrate starfish (*Asterina pectinifera*) have been also reported to be not activated by Ca^{2+} addition (Kishimura *et al*., 2002). Nasri *et al* (2011) identified that the activity of *R. clavata* crude alkaline protease enzyme was not affected by $CaCl_2$.These findings suggest that a difference in the structure of the primary calcium-binding site may exist between mammalian pancreatic trypsin and the enzyme in fish. Sodium and barium ions exhibited an enhancing capacity at all the concentrations studied. Lithium ions do not significantly affect the enzyme activity. Potassium and magnesium showed an identical trend in its action, both exhibited an enhancing action on the enzyme action (**Fig.4.5.14**). **Fig.4.5.15** shows the result of metal ions on intestinal total alkaline protease of *E. suratensis*. Lithium ions displayed an enhancing effect proportional to their concentration. Sodium, copper, zinc and mercury ions do not exert significant effect on the enzyme specific activity. Iron ions inhibit the enzyme activity. Potassium, magnesium, barium and manganese ions increase the enzyme activity at all their concentrations. The presence of NaCl and $CaCl_2$ at 10 mM concentration increased the enzyme activity of Nile tilapia (El-Beltagy *et al*., 2005). However, Nasri *et al* (2011) observed that presence of 5mM NaCl and KCl did not affect alkaline protease activity. Bezerra *et al*

(2005) reported that the trypsin like alkaline protease of *Oreochromis niloticus* was strongly inhibited by Al^{3+} and Cd^{2+}, followed by Cu^{2+}, Hg^{2+}, Zn^{2+} and Co^{2+}. In most of the experiments with marine or euryhaline fish, they were acclimatized to laboratory conditions by using sea water with desired salinity. Since the metal ions cause significant alterations in enzyme activity, it is advisable to maintain the metal ion concentrations constant to avoid ambiguity. However, the salinity itself affects the activation of digestive enzymes (Moutou *et al*., 2004). The activation of a series of zymogens is achieved by trypsin as the common activator (Berg *et al*., 2002). Changes in water salinity might influence zymogen activation since it takes place outside the cell boundaries, in the intestinal lumen, a possible effect of salinity on trypsin production and activity could act as a common regulatory mechanism of the activation of all zymogens. More probably, salinity influences either the activation of each zymogen separately or the activity of each protease itself (Moutou *et al*., 2004).

Studies of proteolytic activity of the intestinal mucosa, the chyme and the intestinal flora in the fishes, zander *Zander lucioperca* (L.), perch *Perca fluviatilis* L., bream *Abramis brama* (L.) and roach *Rutilus rutilus* (L.), revealed that the enzyme activity varies according to the feeding habit (Kuzmina *et al*., 2011). It has been shown that the enzymes present in feeds make a significant contribution to the hydrolysis of nutrients during digestion in fish alimentary tract (Kuz'mina and Golovanova, 2004). Furthermore, the role of microbial enzymes from the intestinal microflora in the hydrolysis of various food components including specific exoenzymes is important (Gatesoupe *et al*., 1997). The separation of total alkaline protease into its specific components, trypsin-like, subtilisin-like or chymotrypsin-like, and further characterization requires special separation techniques along with the use of specific substrate

and protease inhibitors. Proteases acting on BApNA (Benzoyl-L-Arginyl-p-nitro anilide) (1.59 mU mL^{-1}) were strongly inhibited by Benzamidine (100%) and TLCK (98%), classical trypsin-like inhibitors, whereas those hydrolyzing Suc-Phe-p-Nan (0.04 mU mL^{-1}) were strongly inhibited by TPCK (100%), a typical chymotrypsin inhibitor (Esposito *et al*., 2009).

In summary, the observations revealed that the intestinal total alkaline protease of both *E. suratensis* and *O. mossambicus* have similar extent of activities apart from slight deviations in their pH and temperature optima. However, the low specific activity of hepatopancreatic total alkaline protease in *E. suratensis* indicates that the fish has less ability to hydrolyse proteins in comparison with *O. mossambicus*. The high pH and temperature optima and low susceptibility of crude total alkaline protease of *O. mossambicus* to metal ions indicate potential of its industrial and research applications. Further purification will be helpful for a thorough understanding about these enzymes.

Chapter 5

LIPASE

Contents

In the dietary composition of many fishes, lipids play an important role as an essential nutrient and energy source. The present chapter deals with lipid digesting enzyme 'Lipase'. The chapter is designed and divided similar to that of second chapter.

5.1. Introduction

Lipases can be generally defined as enzymes that catalyze the hydrolysis of ester bonds in substrates such as triglycerides (TGs), phospholipids, cholesteryl esters, and vitamin esters (Wong and Schotz, 2002). Although lipases are water soluble proteins, their substrates are insoluble in a purely aqueous system. Within the above classification, the so called "true lipase" (EC 3.1.1.3, triacylglycerol lipase) is distinguished from other lipolytic enzymes such as phospholipases, sterol esterase, and retinyl-palmitate esterase. Lipid esterases have traditionally been separated from lipases on the basis that their substrates have higher solubility in water (Anthonsen *et al*., 1995). Lipases are fat-digesting enzymes that include triacylglycerol- and phospholipases. Triacylglycerol lipase catalyzes the hydrolysis of triacylglycerol to free fatty acid, mono acylglycerol and diacylglycerol. Phospholipase catalyzes the hydrolysis of phospholipids. Phospholipases are

further classified in to phospholipase A, B and C. The class A is again differentiated in to A1, A2 etc. Phospholipase A catalyzes the hydrolysis of phospholipids to free fatty acid and lysophospholipid. Phospholipases A1 and A2 attack ester bonds in positions 1 and 2 of the phospholipid respectively. Phospholipase B catalyzes the generation of free fatty acid and glycerylphospholipid, and phospholipase C catalyzes the generation of diacylglycerol and a phosphoryl base (Mayes *et al*., 1983).

The first published lipase sequence was for porcine pancreatic triacylglycerol lipase (De Caro *et al*., 1981). Mammalian digestive, systemic, and tissue specific lipases continue to be researched very extensively, especially for medical purposes; for instance, to elucidate their roles in the process of atherosclerosis and obesity (Goldberg, 1996; Hui and Howles, 2002; Jansen *et al*., 2002). Microbial lipases have received most of the attention in recent lipase research. They have many commercial applications as a consequence of the convenience of their production, ease of genetic manipulation, and a huge diversity in characteristics and specificities. A comprehensive overview of microbial lipase production, purification, characterization, and applications was provided by Sharma *et al* (2001). Lipases have also been researched and sourced from plants, in which they are widely distributed. Compared with mammalian and microbial lipases, less information is available on plant lipases. Oilseeds and cereal grains are the most common sources for the study and extraction of plant lipases. Understanding the properties of plant lipases is important for maintaining the freshness of oilseeds, cereals, and oily fruits, and for the production of high-quality edible oils (Mukherjee and Hills, 1994; Mukherjee, 2002). However the demand for lipases with novel properties is ongoing. Considering the evolutionary pathways, unusual diets and habitats of fish, piscine lipases may present certain qualities that complement those of the lipases from conventional plant,

mammalian and microbial sources. Lipases are important because of the role they play in the postmortem quality, deterioration of seafood (and other foodstuffs) during handling, chilled and frozen storage. An increase in the use of lipase as food and other industrial processing aids has been noticed, thus there is growing interest in discovering new sources of these enzymes with appropriate characteristics to suit particular applications (Aryee *et al*., 2007). The prospect of extracting enzymes from fish wastes is attractive from an economic viewpoint. Currently, most fish by-products are turned into low-value fish meal and crude oil fractions. Economic returns could be improved if specialty biochemicals were extracted and marketed on a commercial scale. In the present chapter the lipase from *Etroplus suratensis* and *Oreochromis mossambicus* is characterized and the properties are discussed.

5.1.1 Objectives of the study

The objectives of the present study is to characterize lipase from *E. suratensis* and *O. mossambicus* biochemically. The study aims to investigate the optimum pH temperature and optimum substrate concentration of lipase in the selected fishes. The pH stability, the thermal inactivation kinetics and the effect of metal ions on the lipases are also to be examined.

5.2. Review of Literature

Lipases occur widely in animals, plants, and microorganisms as a digestive enzyme and are of considerable physiological significance and industrial potential (Aravindan *et al*., 2007). Lipases (triacylglycerol hydrolases, E.C. 3.1.1.3) are a versatile group of enzymes that catalyze the hydrolysis or synthesis of a broad range of water insoluble esters. Two major classes of hydrolases are of supreme importance: 'true' esterases (EC 3.1.1.1, carboxyl ester hydrolases) and lipases (EC 3.1.1.3, triacylglycerol hydrolases) (Bornscheuer, 2002). Lipases are mainly

active against water-insoluble substrates, such as triglycerides composed by long-chain fatty acids, whereas esterases preferentially hydrolyze 'simple' esters and usually only triglycerides composed by fatty acids shorter than six carbon atoms (Helisto and Korpela, 1998; Kulkarni and Gadre, 2002). They belong to the class of α/β-hydrolases which also contains esterases, acetylcholinesterases, cutinases, carboxylesterases and epoxide hydrolases. Compared with other hydrolytic enzymes (e.g., proteases and carbohydrases), lipases from fish are relatively poorly studied.

Lipases have gained considerable attention as versatile biocatalysts for the hydrolysis/synthesis of a wide range of esters and amides. Most importantly, lipases do not require cofactors for activity. Lipases might be pragmatically redefined as carboxyl-esterases that catalyze the hydrolysis/synthesis of long-chain acylglycerols (Verger and De Haas, 1976). The use of lipases in esterification reactions to produce industrially important products such as emulsifiers, surfactants, wax esters, chiral molecules, biopolymers, modified fats and oils, structured lipids, and flavour esters is very attractive. Although these reactions can also be carried out using inorganic metal-derived catalysts, the interest in using enzymes as biotechnological tools for performing various reactions in both macro- and micro aqueous systems has picked-up tremendously during the last decade. Lipases (EC 3.1.1.3, triacylglycerol hydrolases) have diverse physiological, industrial and research applications mainly in the degradation of food and fat. Lipases are receiving increasing interest due to their effects on the quality of food products, for example, the quality of post-harvest sea foods and their actual and potential applications in modified foods and industrial processes.

Lipases are the hydrolase group of enzymes, which catalyze the hydrolysis of glyceride ester bonds. The fatty acid released is transformed to

water or molecules having a free hydroxyl group or related moiety (nucleophile). Lipases are also termed as acylglycerolases, acyl hydrolases, or triacylglycerol hydrolases. Lipases that catalyze specific reactions and that are active at particular conditions of pH and temperature to suit the requirements of industrial processes are of particular interest. They are shown to have the capacity for synthesising aliphatic, (Gillies *et al*., 1987; Hari Krishna *et al*., 2000a; Hari Krishna *et al*., 2000b), aromatic (Iwai *et al*., 1980) and other (Klibanov *et al*., 1977; Tsujisaka *et al*., 1977; Seino *et al*., 1984) esters in nonaqueous and biphasic systems. Lipases, when employed to catalyze esterification and transesterification reactions in organic solvents, have shown pH memory (Zaks and Klibanov, 1985; 1988), increased enzyme activity and stability at elevated temperatures (Zaks and Klibanov, 1984; Ahern and Klibanov, 1985), regiospecificity and stereoselectivity, and may be affected by water activity (Goderis *et al*., 1987).

The digestive process takes place in the stomach and the lumen of the small intestine and requires several enzymatic processes to convert the insoluble dietary triglyceride emulsion into a soluble and absorbable form. Fat digestion and absorption can be divided into several phases; lipolysis, micellar solubilization of lipolysis products and permeation of lipolysis products across the mucosal cell membrane, intracellular re-esterification into triglycerides, chylomicron formation, and release of chylomicrons from the cell into the lymphatics. The chyme from the stomach enters the duodenum where it mixes with biliary and pancreatic secretions. The biliary secretions contain the bile salts that are required for micellar solubilization of lipolysis products, whereas the pancreatic secretions contain the lipolytic enzymes. The exact complement of pancreatic enzymes involved in glyceride hydrolysis is species specific, but they include pancreatic triglyceride lipase, cholesterol esterase, and a bile-salt-

stimulated type of lipase, termed carboxyl ester hydrolase or nonspecific lipase. Pancreatic lipase acts at the oil-water interface of the emulsified dietary triglyceride to produce fatty acids and 2-monoglycerides (Desnuelle, 1961). Many literature citations report the use of synthetic substrates for the measurement of lipase activity. In particular, *p*-nitrophenyl derivatives of fatty acids (generally lauric or palmitic acids) have been popular (Becker *et al*., 1997; Labuschagne *et al*., 1997; Ushio *et al*., 1996; Kojima *et al*., 1994). Lipases can be distinguished from carboxyl esterases by their substrate spectra, using *p*-nitrophenyl palmitate (cleaved by lipases) versus *p*-nitrophenyl butyrate (cleaved by esterases). Lipases hydrolyze p-nitrophenyl palmitate, but esterases do not, while both can hydrolyze p-nitrophenyl acetate. Lipases can also be distinguished from esterases by the phenomenon of interfacial activation, which is observed only for lipases. Adhered to the lipid-water or micelle-water interface, the enzyme displays a much higher activity than in the aqueous phase and accordingly the activity against water-soluble substrates is negligible. This phenomenon is known as interfacial activation. Both lipases and esterases remain stable in organic solvents, but this property is more noticed for lipases (Bornscheuer, 2002). However, there is no universal assay, and the use of the same method, in the same organism, can result in different values, due to differences in storage time, purification process and source organism of the enzyme.

5.2.1. Structure, Mechanism and Specificity

The comparative analysis of three-dimensional structures of esterases and lipases reveals a feature known as α/β hydrolase fold, which consists of a central β-sheet surrounded by a variable number of α-helices, and accommodates a catalytic triad composed of serine, histidine, and a carboxylic acid (Ollis *et al*.,

1992; Cygler *et al*., 1993; Holmquist, 2000). The carboxylic acid usually is an aspartate or glutamate that is hydrogen bonded to a histidine. Four substrate binding pockets were identified for triglycerides: an oxyanion hole and three pockets accommodating the fatty acids bound at positions sn-1, sn-2, and sn-3. Oxyanion hole is formed by two backbone amides of a residue in the N-terminal region of the lipase and the C-terminal neighbour of the catalytic serine. The differences in size and the hydrophilicity/hydrophobicity of these pockets determine the specificity of a given lipase. Many lipases and esterases have their active site buried under secondary structural elements that must change conformation to allow substrate to access the active site. These secondary structure elements have been called caps, lids or flaps, and have important roles in regulating accessibility of substrates into the catalytic device. The lids constitute the interface recognition and activation sites. The presence of lid covering the active site along with interfacial activation has been generally used to distinguish between lipases and esterases, and to classify an enzyme as a true lipase. The lid is created by a long surface loop and this fragment defies a classical definition of a Ω-loop (Leszczynski and Rose, 1986) in that it exhibits well-defined secondary structure in its central helical fragment.

Lipase can be defined as a carboxyl esterase of rather broad specificity that hydrolyzes only esters of primary alcohols. The activity of lipase is a complex function of the nature of the glyceride substrate, the type and concentration of bile salts, and the presence or absence of colipase. Lipase is oriented so that its active site is near the oil-water boundary. This orientation is achieved by oil-enzyme bonding at the "hydrophobic head" of the enzyme, a region free of electric charges and relatively resistant to unfolding. The interfacial orientation of lipase is further aided by hydrophilic negative charges on the "back" of the enzyme and by a hydrophilic carbohydrate "tail". However many of the lipid molecules also

possess hydrophilic tails. It is suggested that similar hydrophobic heads and hydrophilic tails and asymmetric charge distributions establish the orientation of many enzymes, which act at interfaces. The high turnover rate of lipolysis, 5×10^5 per minute, exceptional even for an enzyme, results from the extremely high substrate concentration near the active site, and from an almost complete extrusion of water because of the hydrophobicity of both the active site and the substrate. In addition, both substrate and enzyme, because of their polarity, are already so favourably positioned at the interface that the formation of the "active complex" requires only a proper two-dimensional alignment, perhaps with partial extraction of the substrate molecule from the lipid phase (Brockerhoff, 1973). The substrate molecule is fixated on the enzyme in a two-dimensional orientation, because its leaving alkoxy group must be received by the serine hydroxyl hydrogen that is directed towards the imidazole ring of the reactive histidine through a hydrogen bond.

Lipases generally catalyze three types of reactions. Hydrolysis of lipids, esterification of glycerol like alcohols with acids like fatty acids and trans esterification reactions. The catalytic action of lipases is reversible. They catalyze hydrolysis in an aqueous system (**Fig.5.2.1**).

(1) Hydrolysis

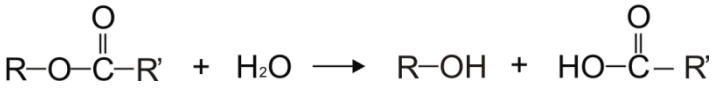


Fig.5.2.1. Hydrolysis

The esterification (reverse reaction of hydrolysis) usually performs in a microaqueous system, where water content is very low (**Fig.5.2.2**).

(2) Ester synthesis

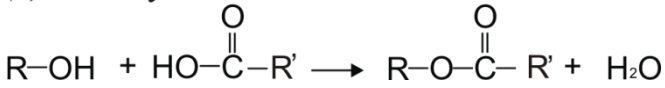
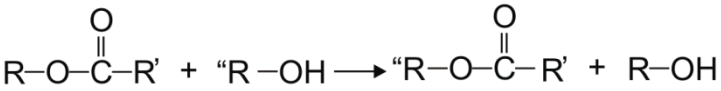


Fig.5.2.2. Esterification

Trans-esterification is categorized into four subclasses according to the chemical species which react with the ester. Alcoholysis is the reaction with an ester and an alcohol, while acidolysis is the one with an ester and an acid (**Fig.5.2.3**).

(3) Trans-esterification

(3.1) Alcoholysis



(3.2) Acidolysis

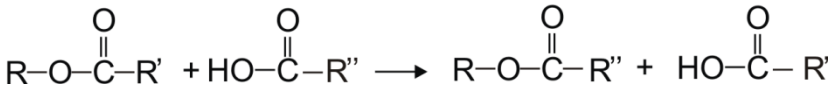


Fig.5.2.3. Trans esterification reactions: Alcoholysis and Acidolysis

Inter-esterification is a reaction between two different esters, where alcohol and acid moieties are exchanged (**Fig.5.2.4**).

(3.3) Interesterification

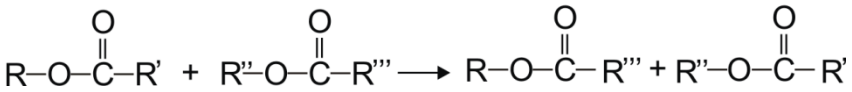


Fig.5.2.4. Trans-esterification reaction by inter-esterification

In aminolysis, an ester is reacted with an amine, generating an amide and an alcohol (**Fig.5.2.5**).

(3.4) Aminolysis

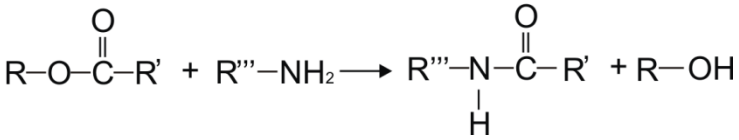


Fig.5.2.5. Trans- esterification by aminolysis

However, the advantage over chemical catalyzed reactions are, the lipase-catalyzed reactions can proceed under modest conditions than the chemical

reactions and undesired side reactions such as thermal degradation of the substrates can be avoided. The extreme specificity of enzyme catalyzed reactions allows specific reactions and thereby specific products can be obtained.

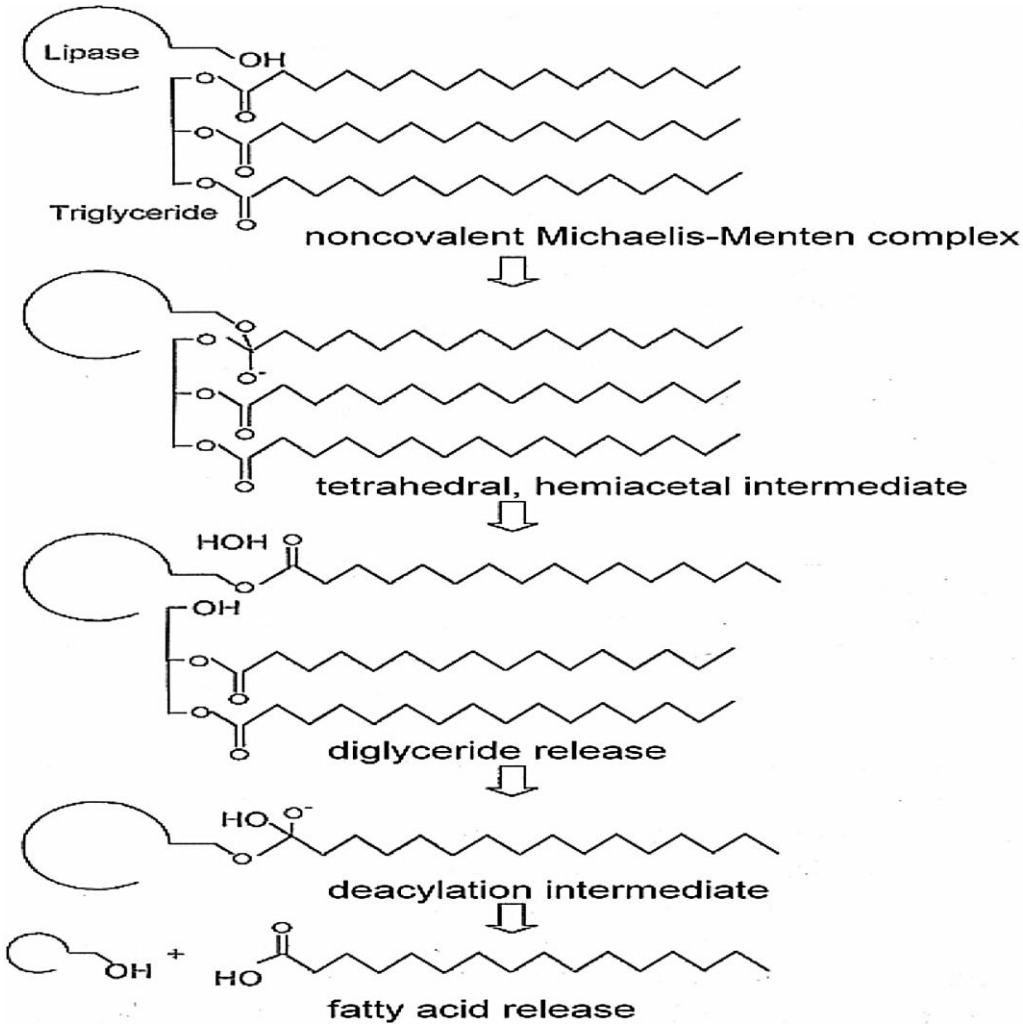


Fig. 5.2.6. Mechanism of triglyceride cleavage by lipase (adopted from Winkler and Gubernator, 1994).

The **Figure 5.2.6** is the proposed catalytic mechanism of lipases by Winkler and Gubernator (1994). The lipase action is chemically analogous to that of the serine proteases, based on the presence of a common catalytic triad.

The difference between the two catalytic systems lies in the handedness of the tetrahedral, hemiacetal, intermediate. The release of a fatty acid from the active site cleft in the last step got special importance since a too tightly bound fatty acid will inhibit the enzyme. According to "the electrostatic catapult model" proposed by Petersen *et al* (2001) the fatty acid formed due to the ester bond cleavage is rapidly ejected from the active site cleft, which exerts a negative electrostatic potential around neutral and basic pH values.

Enzymes are extremely selective catalysts. Unlike most catalysts used in synthetic chemistry, enzymes are specific both for the reaction catalyzed and for a single substrate or a set of closely related substrates. Lipases are the rare class of enzymes which possess various types of specificity like absolute and broad substrate specificity, stereo specificity and positional specificity. Substrate specific lipases differentiate between esters such as TGs, diglycerides, monoglycerides, and phospholipids. Fatty acid-specific lipases show preference for particular fatty acids or a class of fatty acids (short-chain, polyunsaturated, etc.). Lipases can also be regioselective, that is positional specificity can be exhibited by distinguishing between the external, primary (*sn*-1 and *sn*-3 positions) and internal, secondary (*sn*-2 position) ester bonds. Many microbial lipases, as well as gastric and pancreatic lipase are specific for the external ester bonds. The enzymes purified from trout, cod and turbot have 1, 3-specificity, whereas in crude extracts or in vivo studies these fish as well as species like Atlantic salmon, Arctic char, striped bass (*Morone saxatilis*), anchovy (*Engraulis mordax*), pink salmon (*Oncorhynchus gorbuscha*) and speckled char (*Salvelinus fontinalis*) appear to effect complete hydrolysis of triacylglycerols to free fatty acids (Olsen and Ringo, 1997). The lipase purified from sardine hepatopancreas was 1, 3- specific and had relatively high activity towards tributyrin as well as triolein (Mukundan *et al*., 1985). True *sn*-2

selective lipases are very rare. The premier specificity that lipases express is enantioselectivity. It is the ability to differentiate between enantiomers of chiral molecules. This ability in certain microbial lipases has been used recently for producing pure chiral isomers during chemical synthesis. The partial stereo specificity of the lipases shows a preference for either the *sn*-1 or *sn*-3 position of triglycerides (Matori *et al*., 1991; Anthonsen *et al*., 1995; Hou and Yuji, 2002).

5.2.2. Role of Bile Salts, Colipase and Surfactants in Lipase Action

The stability of any enzyme is an essential property when it is considered for industrial, medicinal or research applications. Many factors like pH, temperature, ionic concentration and other chemicals affect enzyme stability. Some molecules are proven as enzyme stabilizers. In lipase action, because of the amphipathic nature of reaction environment, the biological system has been evolved to provide stabilizers like bile salts, albumin and colipases. Bile salts prevent the denaturation of the lipase. Bovine albumin also protects the enzyme. Denaturation of lipase occurs also at the interphase of the substrates (tributyrin or olive oil) and water. Bile salts keep the oil-water interphase free from blockage by unfolded proteins. Kinetic studies show that taurocholate and albumin prevent but cannot reverse the unfolding of the enzyme. The accelerating effect that these agents have on lipolysis can be explained on this basis (Brockerhoff, 1971). It was subsequently realized that pure pancreatic lipase is inhibited by bile salts in concentrations over their critical micellar concentration (CMC). Colipase was first described as a heat-stable protein that is necessary for the activity of lipase in an assay system that contained high concentrations of bile salts (Baskys *et al*, 1963). However, the first indication for the existence of a cofactor for pancreatic lipase was

reported by Rosenheim (1910) and the cofactor had been partially purified and named colipase by Maylie *et al* (1971). Canioni *et al* (1977) and Julien *et al* (1978) demonstrated that the application of Triton X-100 seems to have distinct advantages that result in a homogeneous preparation and the surfactant obviously protects colipase from proteolytic degradation during purification. Colipase, however, has been found in the vertebrate series down to the shark, and a general cross reaction of colipase and lipase from different species has been indicated (Patton, 1978; Rathelot *et al*., 1976). Interestingly, neither colipase, nor bile salts were detected in the crab hepatopancreas. This suggests that colipase evolved in invertebrates simultaneously with the appearance of an exocrine pancreas and a true liver which produce bile salts (Cherif *et al*., 2007). Sea bream (*Sparus aurata*) pyloric caeca-duodenal lipase has a bile salt sodium taurocholate requirement for increased activity (Nolasco *et al*., 2011).

5.3. Materials and Methods

The collection and acclimation of *E. suratensis* and *O. mossambicus* were conducted as explained in Chapter 2.

5.3.1 Methods

The soluble protein content of enzyme homogenates was measured according to Lowry *et al* (1951) as described in Chapter 2.

5.3.2. Estimation of Lipase

Lipase was estimated spectrophotometrically by hydrolysis of p-nitrophenyl palmitate using a modified method based on that of Winkler and Stuckmann (1979). 50µl of 20mM p-nitrophenyl palmitate was mixed with 20mM Tris buffer (pH-8) containing 20mM $CaCl_2$, 5mM sodium cholate and 0.01% gum Arabica. 50µl of tissue homogenate was added to this reaction

mixture and incubated for 10 minutes. The p-nitro phenol liberated was read at 410nm against a reagent blank.

5.4. Results

The pH optima for *Oreochromis mossambicus* intestinal and hepatopancreatic lipase have been estimated as 9.00 and 7.5 respectively (**Fig.5.4.1** and **Fig.5.4.2**). The temperature optimum for *O. mossambicus* intestinal lipase is found to be 45^0C, whereas it is 40^0C for its hepatopancreatic counterpart (**Fig.5.4.3** and **Fig.5.4.4**). At around 25mM, the intestinal lipase is completely saturated with p-nitrophenyl palmitate substrate. But the hepatopancreatic lipase has been saturated by 15mM p-nitrophenyl palmitate (**Fig.5.4.5** and **Fig.5.4.6**). The intestinal lipase of *E. suratensis* possesses optimum activity in a range of pH from 8.5 to 9.5. However, the hepatopancreatic lipase showed its optimum activity in a short range (8.5 to 9) (**Fig.5.4.7** and **Fig.5.4.8**). The intestinal lipase of *E. suratensis* exhibited a specific optimum temperature of 30^0C but the hepatopancreatic lipase acts effectively over a wide temperature range from 40^0C to 60^0C with an optimum around 55-60^0C (**Fig.5.4.9** and **Fig.5.4.10**). Both intestinal and hepatopancreatic lipase of *E. suratensis* showed a substrate saturation at 15mM of p-nitrophenyl palmitate (**Fig.5.4.11** and **Fig.5.4.12**). Alkali metals like calcium, barium and transition metal manganese exerted a tremendous increase in intestinal lipase activity with increase in the respective ion concentrations. Conversely, lithium, sodium, potassium, copper and zinc ions do not affect the enzyme activity significantly. Iron and mercury completely inhibit the lipase activity (**Fig.5.4.13**). In the case of *Etroplus suratensis* intestinal lipase most of the metal ions showed an enhancing activity significantly (**Fig.5.4.14**). The thermal inactivation kinetics of *O. mossambicus*

intestinal lipase showed that the enzyme is more stable below 50^0C. The enzyme exhibited a slow thermal denaturation within 30 minutes at 50^0C and a drastic denaturation at 55^0C. The intestinal lipase of *O. mossambicus* lost its activity within 50 minutes at 60^0C (**Fig.5.4.15**). The hepatopancreatic lipase of *O. mossambicus* exhibited a fast decline in enzyme activity above the temperature 50^0C. The enzyme has been completely thermally inactivated within 15 minutes at 55 and 60^0C (**Fig.5.4.16**). The *E. suratensis* intestinal lipase is stable up to 35^0C and beyond this temperature thermal denaturation of the enzyme starts and inactivation occurs. The enzyme is stable for 50 minutes and for 40 minutes at 40^0C and 45^0C respectively (**Fig.5.4.17**). The lipase from *E. suratensis* hepatopancreas has exhibited thermal stability up to 35^0C and the thermal denaturation gradually starts from 30 minute of the experiment at 40^0C. At 45^0C, the enzyme loses its activity drastically and it is completely denatured within 35 minutes (**Fig.5.4.18**). The hepatopancreatic lipase from *O. mossambicus* is more stable in acidic pH range than the intestinal enzyme. It exhibit 80% stability at pH 3.5 and restored its 100% relative activity at pH 5.5. The intestinal lipase exhibited its maximum stability at pH-7.5 (100 % relative activity). However it is more stable in the alkaline range with about 80% relative activity. The hepatopancreatic lipase exhibited its maximum stability at acidic pH than in alkaline range (**Fig.5.4.19**). The intestinal and hepatopancreatic lipase from *E. suratensis* showed maximum stability in the acidic to neutral pH. Specifically, the intestinal lipase is significantly stable from pH 3.5 to 7.0 and the hepatopancreatic lipase is stable from pH 4.0 to 7.0. Both enzymes have retained around fifty percent of their stability over the alkaline pH range (7.5 to 10.5) (**Fig.5.4.20**).

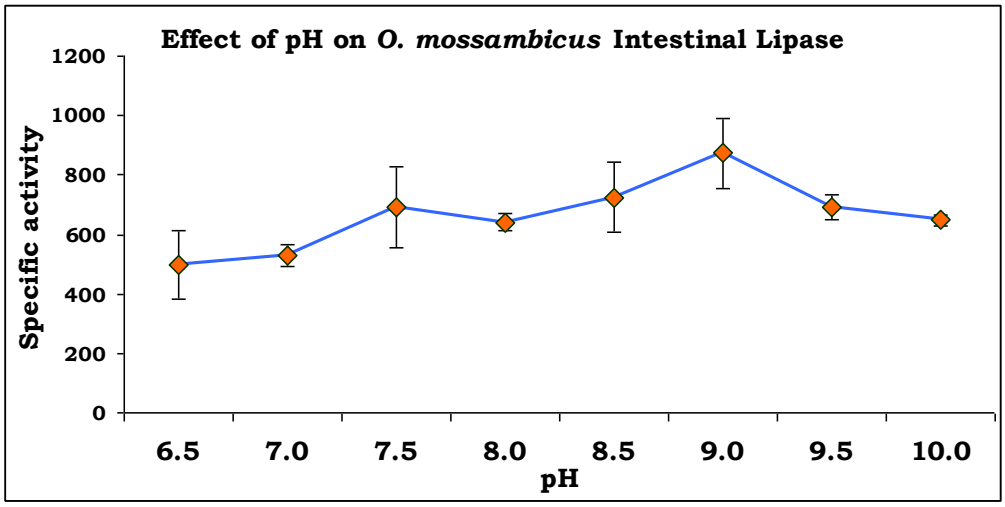


Fig.5.4.1. pH optimum for *O. mossambicus* intestinal Lipase

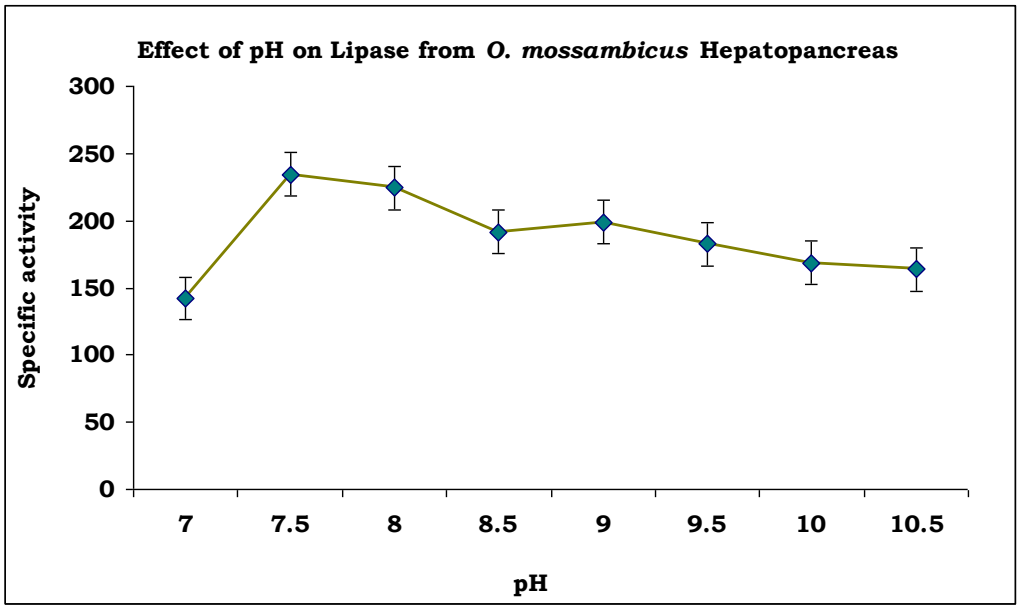


Fig.5.4.2. pH optimum for *O. mossambicus* hepatopancreatic lipase

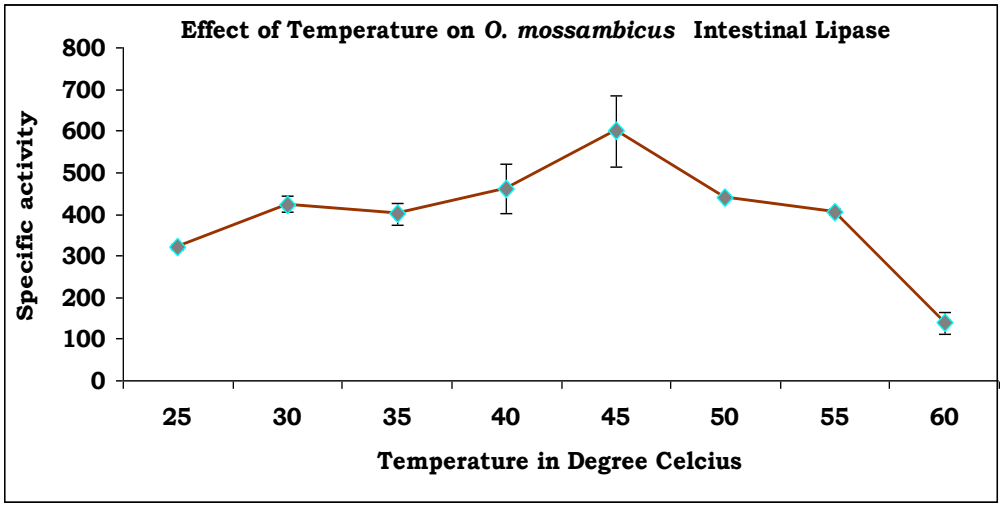


Fig.5.4.3. Optimum temperature curve of *O. mossambicus* intestinal lipase

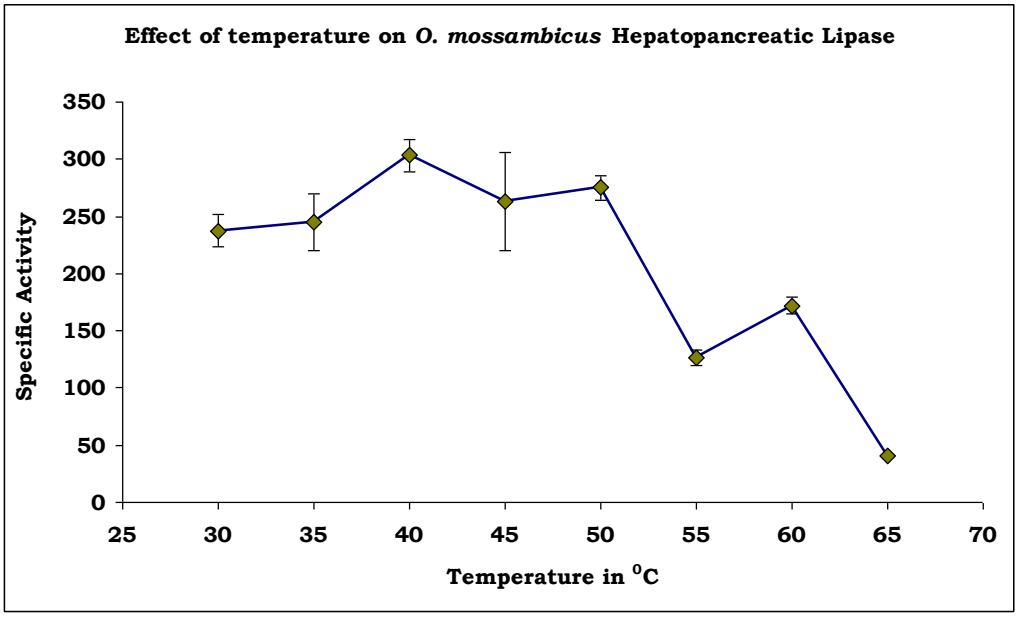


Fig.5.4.4. Optimum temperature curve of *O. mossambicus* hepatopancreatic lipase

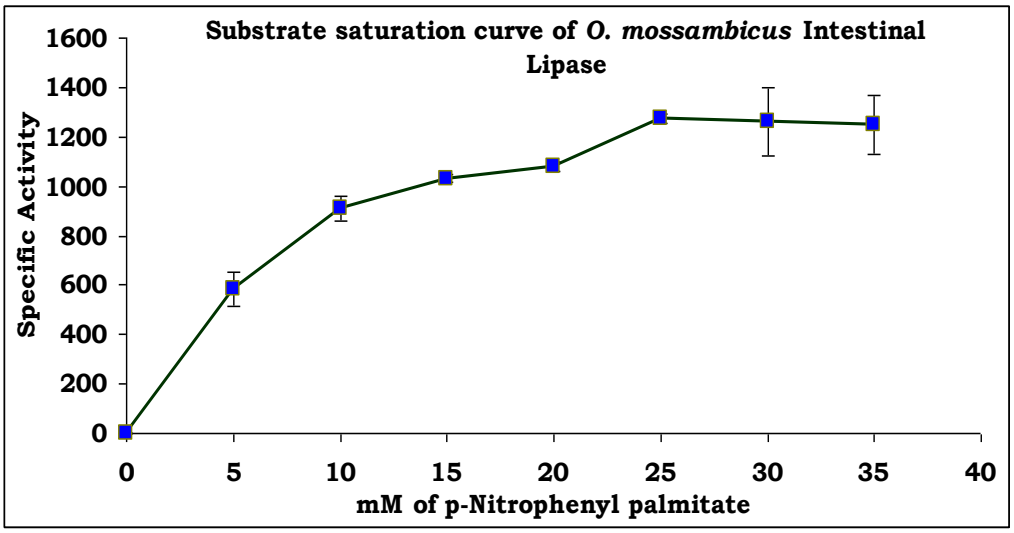


Fig.5.4.5. Substrate saturation curve of *O. mossambicus* intestinal lipase

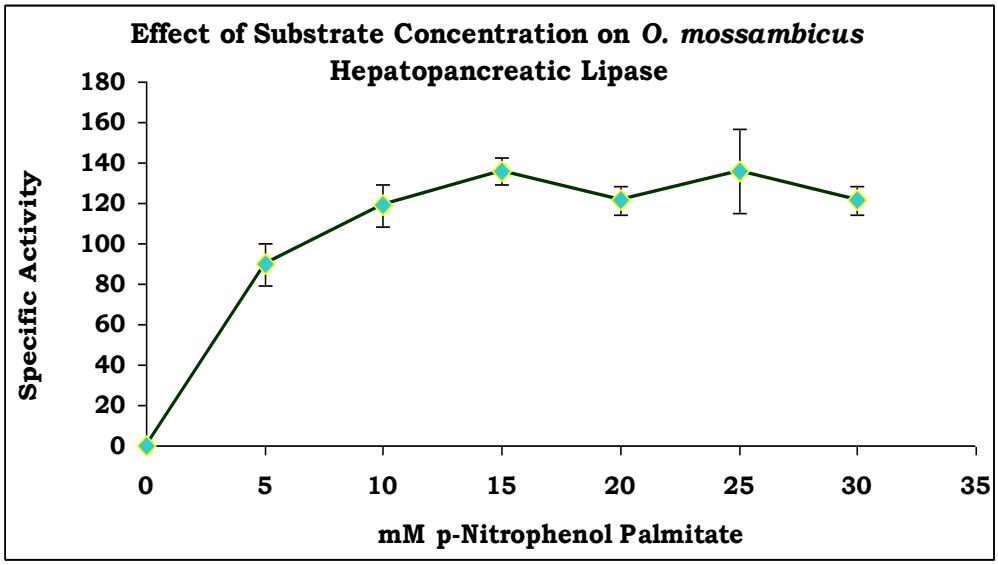


Fig.5.4.6. Substrate saturation curve of *O. mossambicus* hepatopancreatic lipase

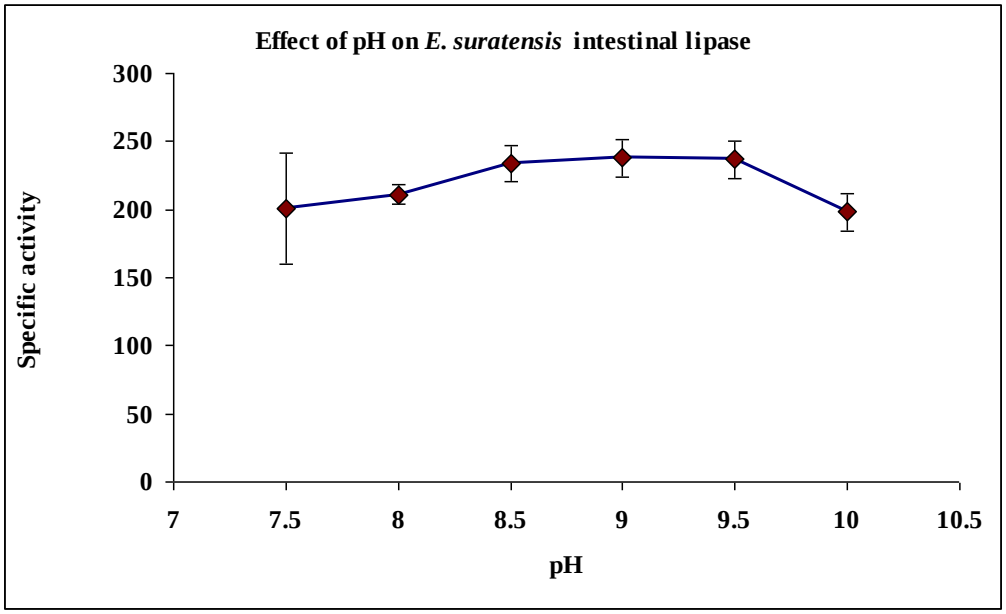


Fig.5.4.7. Optimum pH curve of *E. suratensis* intestinal lipase

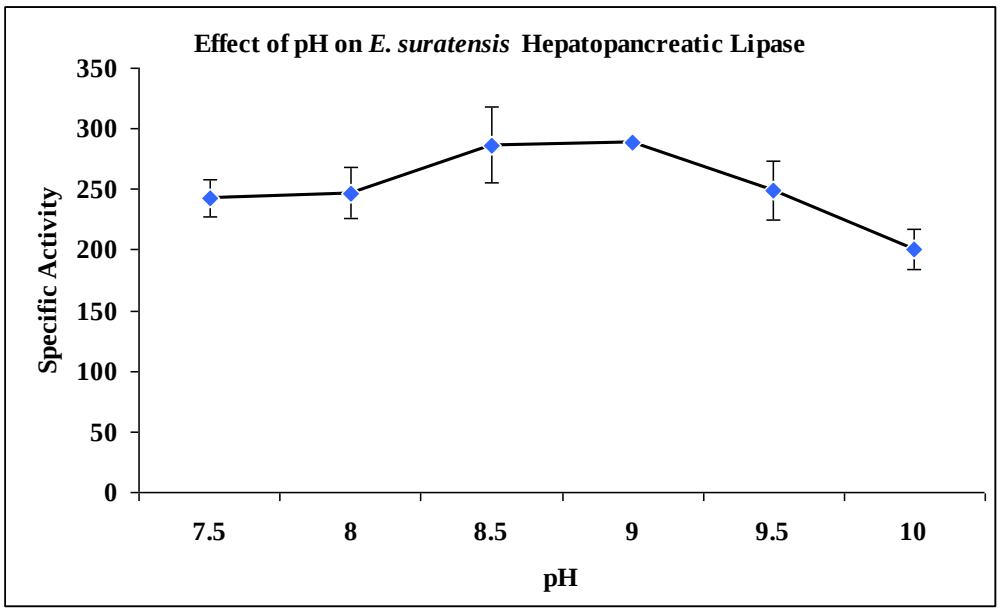


Fig.5.4.8. Effect of pH on *E. suratensis* hepatopancreatic lipase

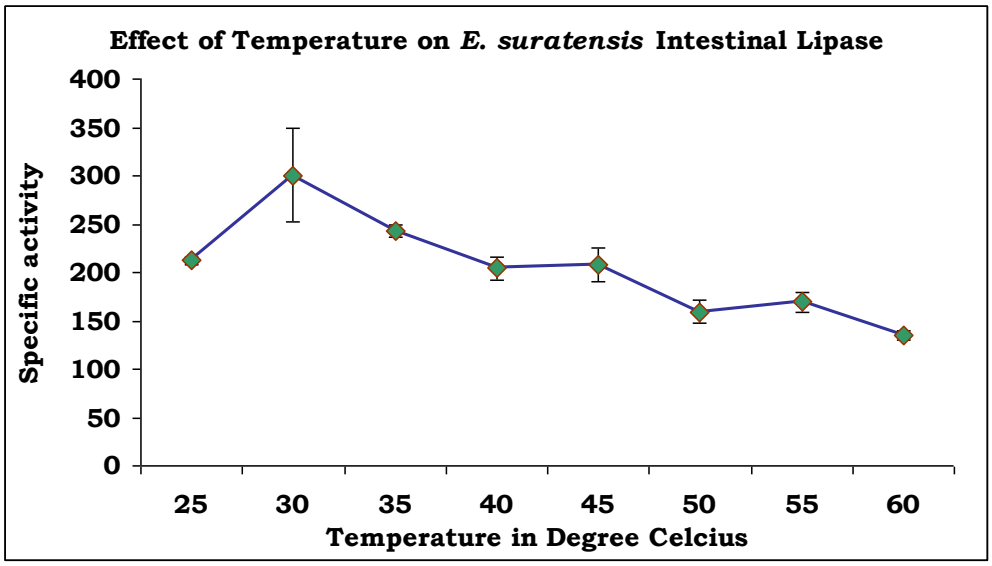


Fig.5.4.9. Optimum temperature curve of *E. suratensis* intestinal lipase

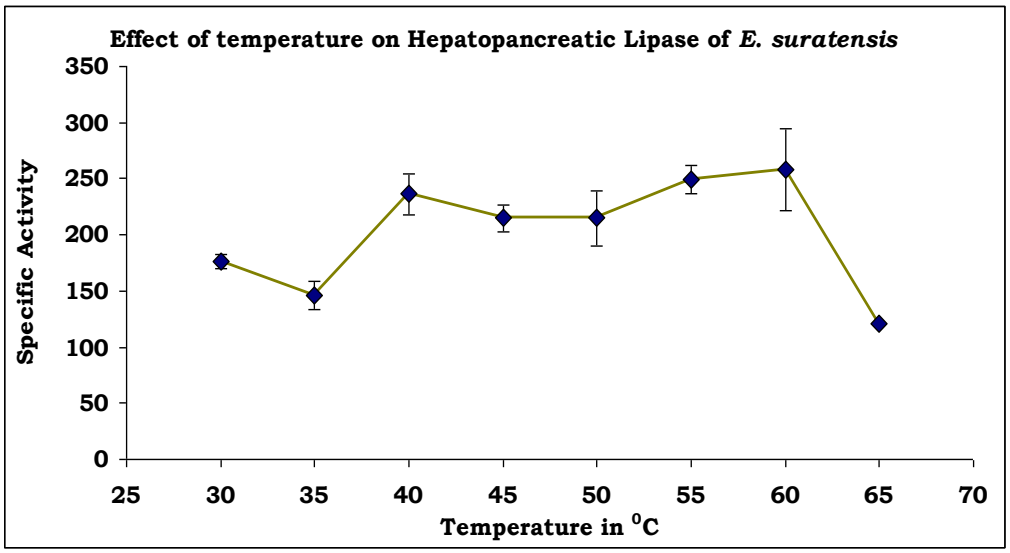


Fig.5.4.10. Optimum temperature curve of *E. suratensis* hepatopancreatic lipase

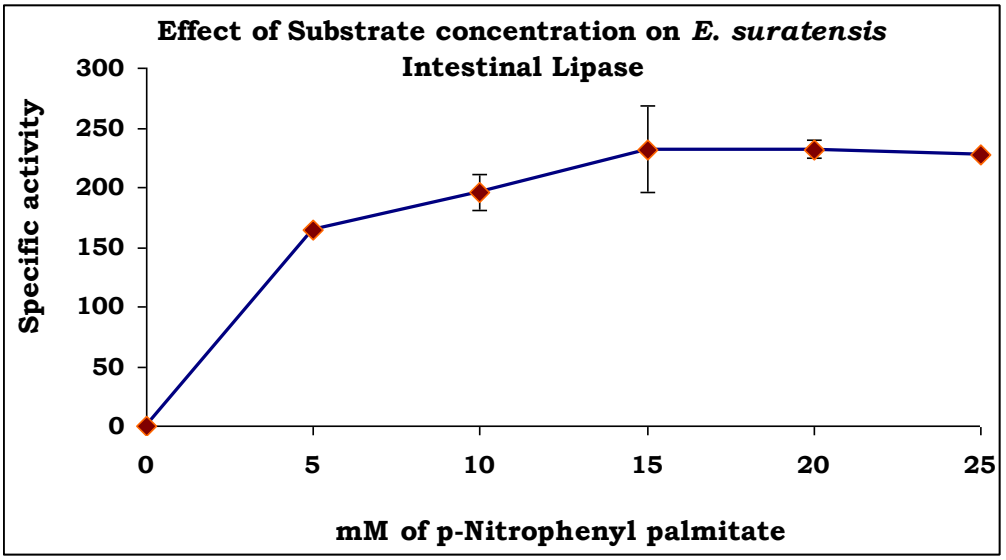


Fig.5.4.11. Substrate saturation curve of *E. suratensis* intestinal lipase

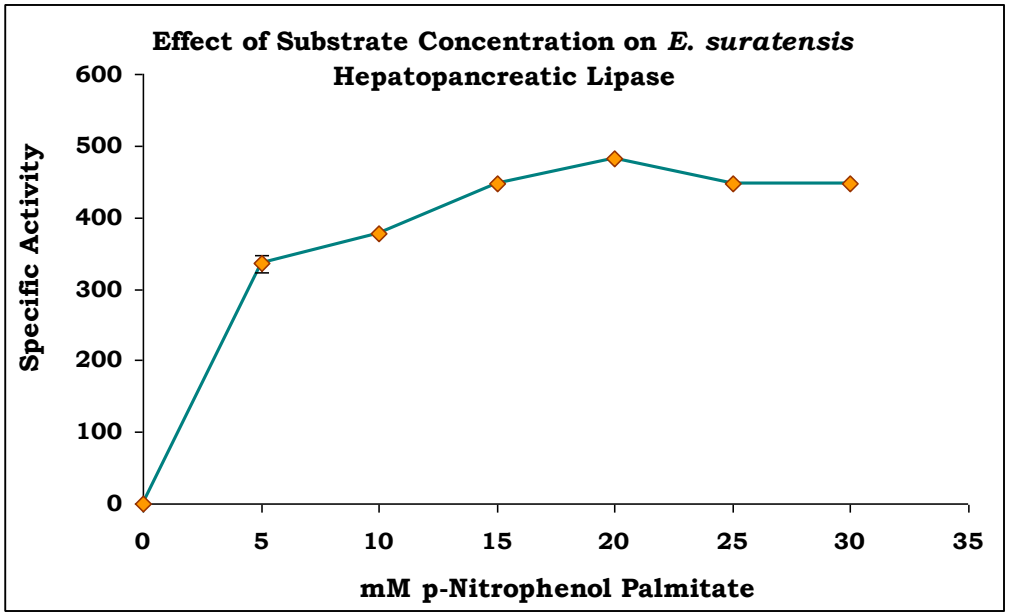


Fig.5.4.12. Substrate saturation curve of *E. suratensis* hepatopancreatic lipase

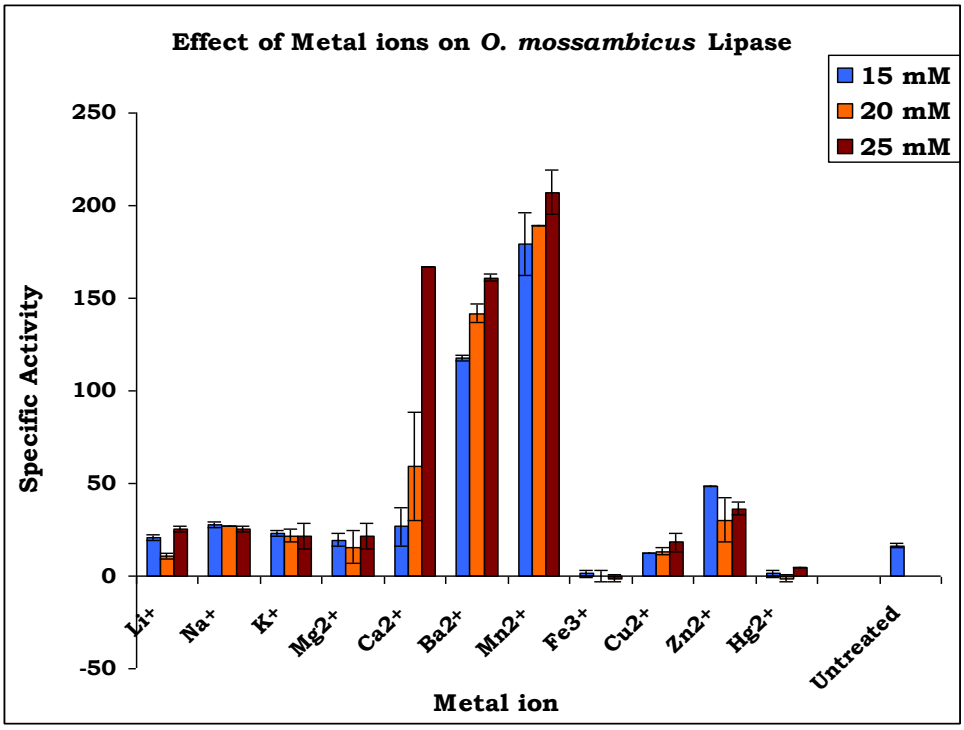


Fig.5.4.13. Effect of metal ions in *O. mossambicus* intestinal lipase activity

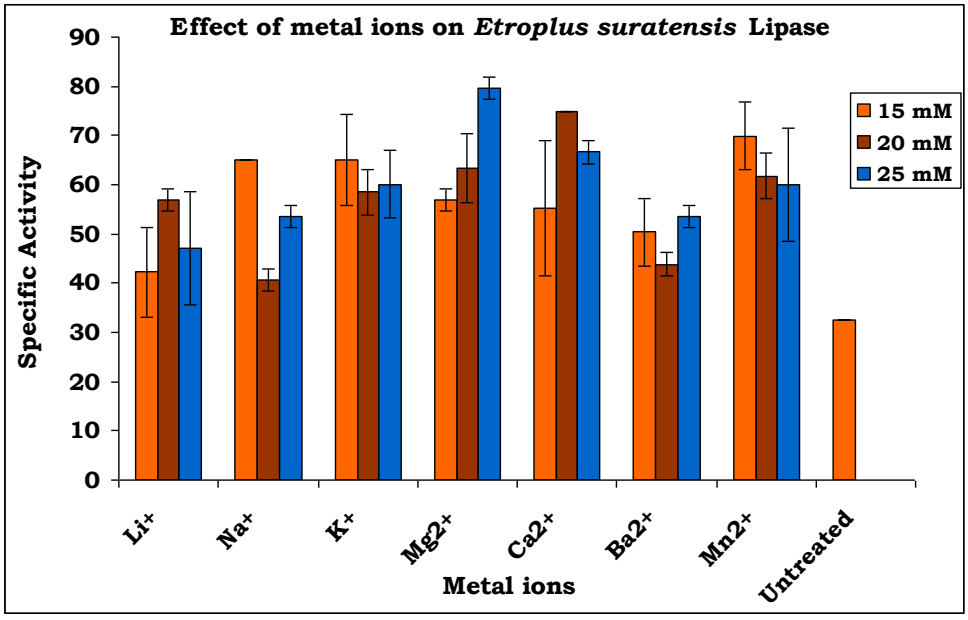


Fig.5.4.14. Effect of metal ions on *E. suratensis* intestinal lipase

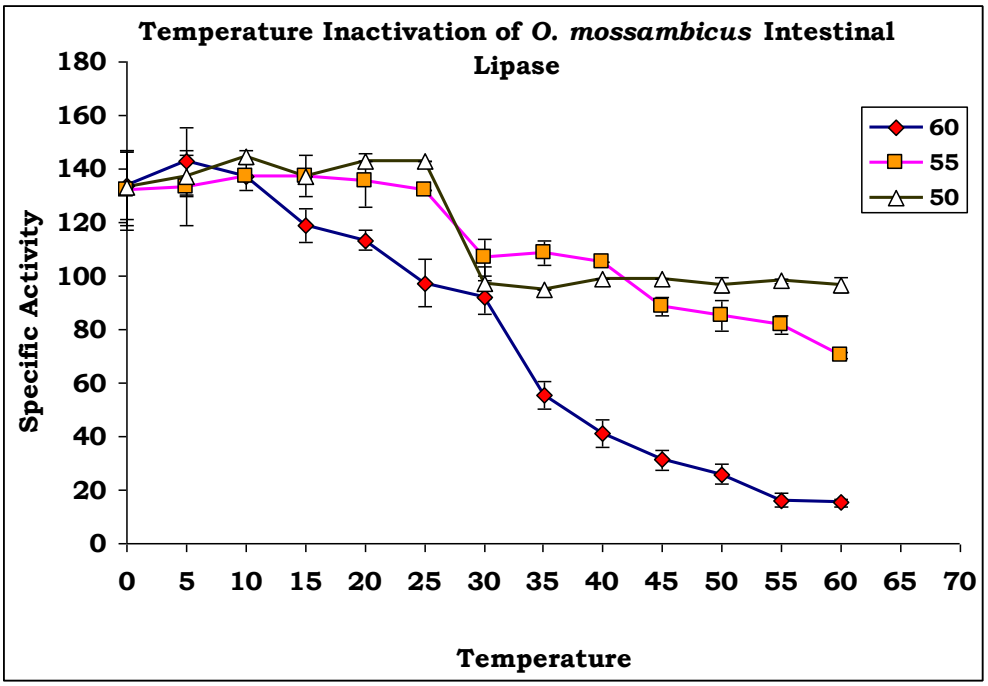


Fig.5.4.15. Thermal inactivation kinetics of *O. mossambicus* intestinal lipase

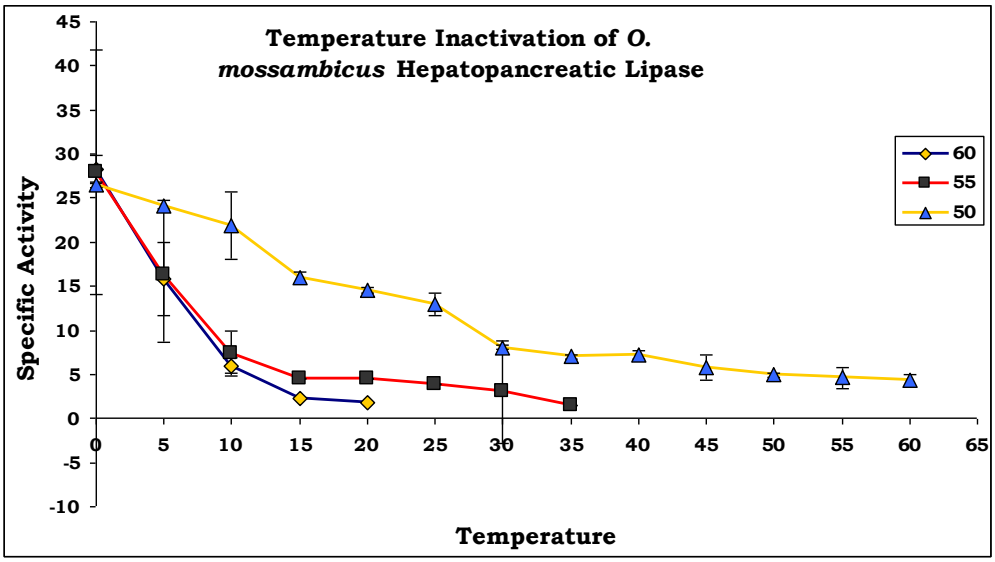


Fig.5.4.16. Thermal inactivation kinetics of *O. mossambicus* hepatopancreatic lipase

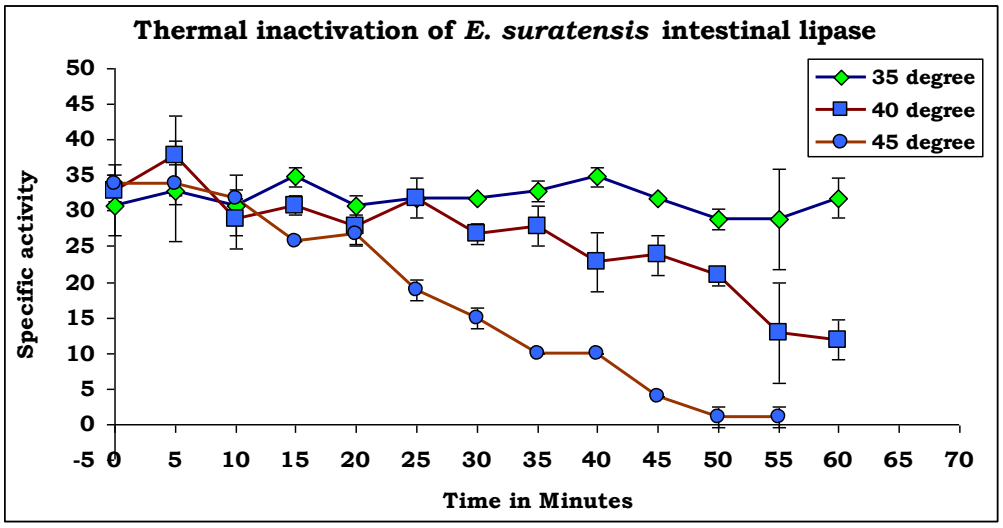


Fig.5.4.17. Thermal inactivation kinetics of *E. suratensis* intestinal lipase

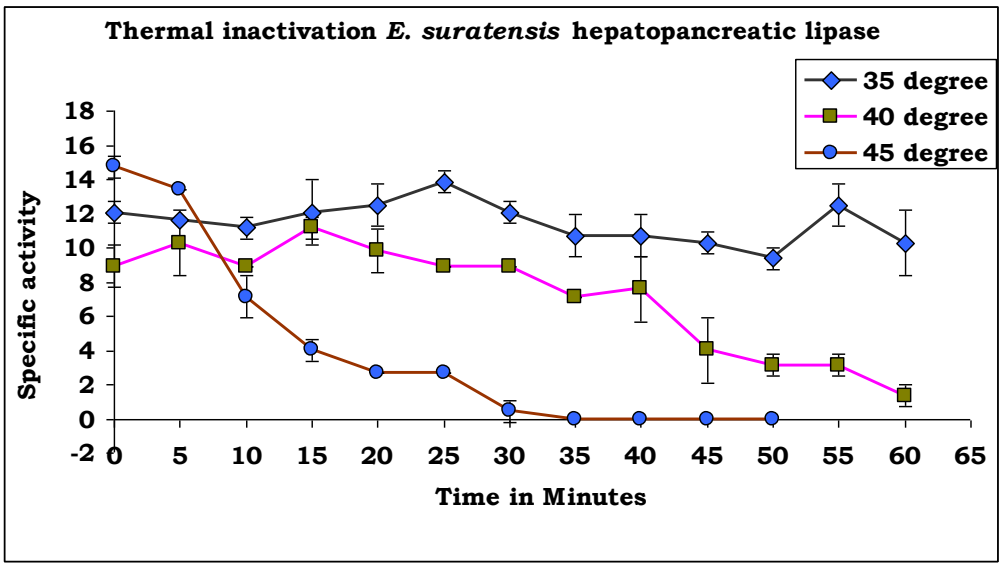


Fig.5.4.18. Thermal inactivation kinetics of *E. suratensis* hepatopancreatic lipase

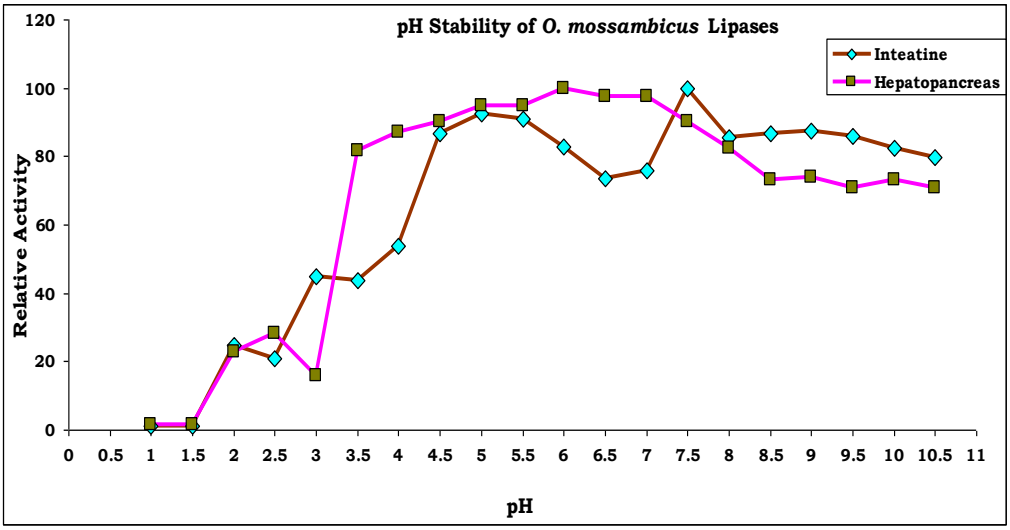


Fig.5.4.19. pH stability curve of *O. mossambicus* lipases

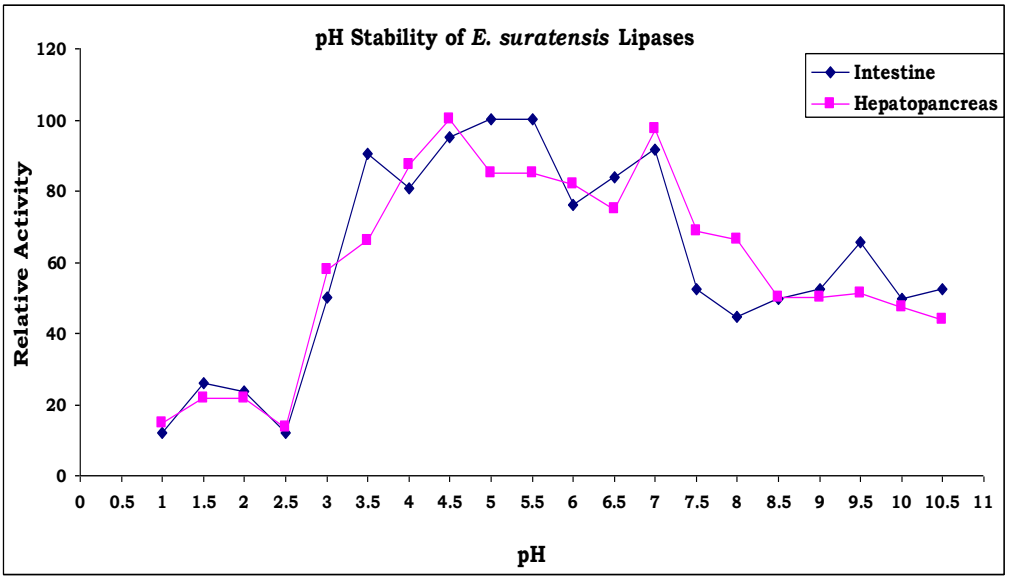


Fig.5.4.20. pH stability curve of *E. suratensis* lipases

5.5. Discussion

Lipids play major roles as sources of metabolic energy for ontogeny, growth, reproduction, movement as well as migration. Fish lipids are rich in

ω-3 fatty acids, highly unsaturated fatty acids (HUFA) that have particularly important roles in animal nutrition, reflecting their roles in critical physiological processes. Poikilotherms, especially fishes, tend to employ a more diverse strategy of lipid deposition than homeotherms (Sheridan, 1990). Lipids are stored among several depot organs, including mesenteric fat, liver and dark muscle. Moreover, while triacylglycerol is the predominant lipid storage form in fish, some species make use of other compounds *(e.g.*, glycerol ether analogs of triacylglycerols) (Sheridan, 1988). Hydrolysis of stored lipid has been demonstrated in the mesenteric fat (Sheridan and Allen, 1984) and dark muscle (Bilinski and Lau, 1969) of fish. Many species of fish like salmonids store comparatively large amounts of lipid as triacylglycerols in the liver (Henderson and Tocher, 1987).

Like mammals the pancreas or hepatopancreas is generally considered to be the main resource of digestive lipase enzymes in fish (Kapoor *et al*., 1975; Fange and Grove, 1979). Digestive lipases may also be secreted by the intestinal mucosa or intestinal segments of many fish species and according to Vonk (1937) the lipase activity in the intestine is due to the possible absorption of the pancreatic enzymes into the intestinal mucosa. In mammalian gut, there are two main lipases, the pancreatic lipase-colipase system (EC 3.1.1.3) and the less specific bile salt-activated lipase (EC 3.1.1.1). The few lipases that have been studied from fish and other aquatic animals include lipases from the leopard shark (Patton *et al*., 1977), rainbow trout (Tocher and Sargent, 1984), Atlantic cod (Lie and Lambersten, 1985; Gjellesvik *et al*., 1992), dog fish (Rasco and Hultin, 1988), sardine (Mukundan *et al*., 1985), anchovy, striped bass and salmon (Leger *et al*., 1977), as well as red sea bream (Iijima *et al*., 1998). Other investigators have reported lipases and lipolytic activities in the digestive glands, liver and adipose tissues of different fish species (Izquierdo *et al*., 2000;

Liang *et al*., 2002). In many marine and freshwater fish intestinal extracts the stimulation by bile salts on lipase activity has been identified (Olsen and Ringo, 1997). Rainbow trout (*Salmo gairdneri*, L) (Tocher and Sargent, 1984), Atlantic salmon (*Salmo salar*), Turbot (Olsen and Ringo, 1997), Cod (Gjellesvik *et al*., 1992) red sea bream (*Pagrus major*) (Iijima *et al*., 1998) are shown to be have high lipase activity in the presence of bile salt. According to Aryee *et al* (2007), bile salts may protect lipases from proteolysis. Bile salts may also facilitate the absorption of free fatty acids by forming mixed micelle with the products to enhance lipase activity (Lowe, 1999; Wang and Lee, 1985). However, evidence for a pancreatic lipase-colipase system in fish is sparse. An enzyme similar to pancreatic lipase has been identified in trout and unlike mammalian pancreatic lipase; the trout enzyme had relatively low specific activity and level of colipase activation (Leger *et al*., 1977). A lipase that hydrolysed triolein in the absence of bile salts was also purified from sardine (*Sardinella longiceps*) hepatopancreas (Mukundan *et al*., 1985). A lipase from hepatopancreas of common carp (*Cyprinus carpio*) was purified by Kayama *et al*., 1979.

Dietary lipid requirements vary among species based on feeding habit, stage of life and habitat (Biswas *et al*., 2009). Specific activity of lipase was lower in acidic conditions than in neutral and alkaline conditions and three optimal pH levels (7, 8 and 11) were detected in different stages of Siamese fighting fish (*Betta splendens*). For this species it is suggested that the most suitable pH to determine lipase activity in was pH 8 and 40°C, regardless of sex and age (Thongprajukaew *et al*., 2010). In addition, lipase specific activity exhibited in this early stage might be related to lipid breakdown from oocyte, or correspond to fatty acid requirements for larval development (Morais *et al*., 2006). Carnivorous fish usually consume fat-rich diets, and high lipase activity could result from the exogenous lipase present in live diets (Morais *et al*., 2007).

The partially purified grey mullet lipase was active within the pH range of 7–10, with an optimum pH of 8.0, and was stable from pH 4 to 10. The enzyme was active within the temperature range of 20–60^{0}C, and exhibited an optimum temperature for the hydrolysis of *p*-nitrophenyl palmitate at 50^{0}C, and was stable between 10 and 50^{0}C, beyond which it lost activity progressively (Aryee *et al*., 2007). Lipase activity of juvenile *Cherax quadricarinatus* digestive gland, using b-naphthyl caprylate as substrate, was optimum between 35 and 45 ^{0}C and decreased above 50 ^{0}C (Lopez-Lopez *et al*., 2003). A similar response for lipase activity was found by Biesot and Capuzzo (1990) in stage I of the lobster *Homarus americanus*, with triolein as substrate, peaking at 45^{0}C and decreasing above 50^{0}C. Ramana-Rao and Surendranath (1991) found optimal lipase activity at 37^{0}C in *Metapenaeus monoceros.* Unlike known digestive lipases, crab digestive lipase displayed its maximal activity on long and short-chain triacylglycerols at a temperature of 60^{0}C (Cherif *et al*., 2007). Lipases were purified from pyloric caeca of two fishes, Chinook salmon (*Oncorhynchus tshawytscha*) and hoki (*Macruronus novaezelandiae*), had the optimum activity at 35^{0}C (Kurtovic *et al*., 2010). However, in the present study the temperature optimum for *O. mossambicus* intestinal lipase is found to be 45^{0}C, whereas it is 40^{0}C for its hepatopancreatic counterpart (**Fig.5.4.3** and **Fig.5.4.4**). Together with the increase in temperature, lipase activity also showed increases. The intestinal lipase of *E. suratensis* exhibited a specific optimum temperature of 30^{0}C but the hepatopancreatic lipase acts effectively over a wide temperature range from 40^{0}C to 60^{0}C with an optimum around 55-60^{0}C (**Fig.5.4.9** and **Fig.5.4.10**). Optimum temperature of the neon flying squid *(Ommastrephes bartramii)* hepatopancreatic lipase reaction was around 25^{0}C. The enzyme was tolerably stable up to 37^{0}C (Sukarno *et al*., 1996). Optimum temperature of sea bream

(*Sparus aurata*) pyloric caeca-duodenal lipase was found at 50^0C, but lipase was stable at temperatures below 40^0C (Nolasco *et al*., 2011).

The pH optimums for *Oreochromis mossambicus* intestinal and hepatopancreatic lipase have been estimated as 9.00 and 7.5 respectively (**Fig.5.4.1** and **Fig.5.4.2**). The intestinal lipase of *E. suratensis* possesses optimum activity in a range of pH from 8.5 to 9.5. However, the hepatopancreatic lipase showed its optimum activity in a short range (8.5 to 9) (**Fig.5.4.7** and **Fig.5.4.8**). Figueiredo *et al* (2001) found lipase activity in adult *C.quadricarinatus* gastric fluid was highest at pH 8, but they did not find lipase activity in the hepatopancreas. This absence of lipase in the hepatopancreas suggests synthesis in the form of a proenzyme. Brockeroff *et al* (1970) found that lipase activity using triolein and tributyrin as substrate in gastric juice of adult *H. americanus* was optimum at pH 6.8. Biesot and Capuzzo (1990) reported higher lipase activity using triolein as substrate at pH 5.5 and lower lipase activity with alkaline pH in hepatopancreas of larval *H. americanus*. Ramana- Rao and Surendranath (1991), studying the effect of kelthane on lipase activity in *Metapenaeus monoceros*, reported three peaks of activity between pH 4.5 and 8.5. In control organisms the neutral lipase activity was higher, but, acidic lipase activity dominated under kelthane toxic stress. Tietz and Shuey (1993) indicated that optimum pH for lipase ranged between pH 7.4 and 10, depending on the type of substrate and buffer used. Enzyme purity could affect the final results (Biesot and Capuzzo, 1990). Lopez-Lopez *et al* (2003) found enzyme activity at acid pH, but the value was low; the highest activity was found between pH 8.5 and 9. The optimal pH of crab digestive lipase activity was found to be around 8 using TC4 as substrate and measured at 60^0C. Interestingly, crab digestive lipase was found to be most stable at higher pH values, and maintains 65% of its activity after 4 h

incubation at pH 10 and loses its activity at pH values lower than 4 (Cherif *et al*., 2007). The neutral nature of the trout liver triacylglycerol lipase, as indicated by pH optimum in between 6.5-7.5 (Harmon *et al*., 1991), is similar to other cytosolic acyl hydrolases (Khoo *et al*., 1976; Fredrikson, 1981), including trout adipose tissue (Sheridan and Allen, 1984). *Oncorhynchus tshawytscha* and *Macruronus novaezelandiae* lipases were more acid stable compared to other fish lipases and had a pH optimum of 8–8.5 (Kurtovic *et al*., 2010). Optimum pH of the neon flying squid *(Ommastrephes bartramii)* hepatopancreatic lipase was around 7.0 and the enzyme was relatively stable between pH 6.0 and 9.0 for 6 h at 25^0C (Sukarno *et al*., 1996). Optimum activity of sea bream (*Sparus aurata*) pyloric caeca-duodenal lipase was found at pH 8.5 (Nolasco *et al*., 2011). In this study the pH optimum of *O. mossambicus* and *E. suratensis* have been identified as 9.00 and 8.5-9.00 respectively **(Fig.5.4.1** and **Fig.5.4.7)**, it is being in agreement with the optimal pH reported for other intestinal enzymes. Similar values have been described for lipases in other fish species like the gilthead sea bream (*Sparus aurata*) (Nolasco *et al*., 2011; Munilla-Moran and Saborido-Rey 1996a; 1996b), rainbow trout (*Oncorhynchus mykiss*), cod (*Gadus morhua*), red sea bream (*Pagrus major*), Pacific blue tuna (*Thunnus orientalis*), grey mullet (*Liza parsia*), Chinook salmon *(Oncorhynchus tshawytscha*) and hoki (*Macruronus novaezelandiae*), or carnivorous teleost fish of Tibet (*Glyptosternum maculatum*) (Tocher and Sargent, 1984; Gjellesvik *et al*., 1989; Iijima *et al*., 1998; de la Parra *et al*., 2007; Islam *et al*., 2008; Kurtovic *et al*., 2010; Xiong *et al*., 2011; respectively). The crude enzymes from viscera of yellowfin tuna and skipjack tuna exhibited the highest activities at pH 10.0 whereas it was at pH 9.0 for those from viscera of tonggol tuna (Prasertsan *et al*., 2008). When the effect of pH on liver lipase activity of *Cyprinus carpio*

was studied, it was found that liver lipase showed a high activity at the pH values between 7.0 and 8.5. In general, the lowest lipase activities were obtained at acidic pH values, specifically at pH 6.0. The pH optimum for liver lipase was to be found 8.0 (Gorgun and Akpınar, 2012).

Hydrolytic activity of lipase was enhanced by Mg^{2+}, Mn^{2+}, NaN_3, and EDTA, but significantly inhibited by Hg^{2+}, and Cu^{2+} (Aryee *et al*., 2007). Lipolytic activity in juvenile *C. quadricarinatus* was partially inhibited by calcium ions and EDTA, and total inhibition was produced by heavy metal ions, with the exception of cobalt and zinc ions. Magnesium ions (Mg^{2+}) did not have any effect on activity (Lopez-Lopez *et al*., 2003). Iijima *et al* (1998) purified and isolated lipase from fish, and found that adding Ca^{2+} did not affect activity, as has been demonstrated in lipases of mammals. Alkali metals like calcium, barium and the transition metal manganese exerted a tremendous increase in intestinal lipase activity with respect to increase in the respective ion concentrations. Conversely, lithium, sodium, potassium, copper and zinc ions do not affect the enzyme activity significantly. Iron and mercury completely inhibits the lipase activity (Fig.5.4.13). The lipases of *Oreochromis niloticus*, and grey mullet which were highly inhibited by heavy metals like Cd^{2+}, Zn^{2+}, and Hg^{2+} (Taniguchi *et al*., 2001; Islam *et al*., 2008). Here, in the case of *Etroplus suratensis* intestinal lipase all most all metal ions showed an enhancing activity significantly except zinc and mercury. Enzyme treated with iron ions exhibited a decreasing trend in activity with increase in concentration of Fe^{3+} (**Fig.5.4.14**). Nolasco *et al* (2011) showed that lipase possesses a low requirement for Na^{+}, since maximum activity was achieved at 50 mM of salt concentration in the reaction mixture. Some ions like Ca^{2+} or Mg^{2+} produced a non-significant effect on the activity until reaching quite high concentrations (10 mM). In contrast, other ions like Pb^{2+}, Fe^{3+}, Zn^{2+}, or Hg^{2+} produced a

negative effect, decreasing the activity below 20% at concentrations of 5 mM or even 1 mM. The effect of Mn^{2+} or Co^{2+} was intermediate between the two described responses.

The thermal inactivation kinetics of *O. mossambicus* intestinal lipase showed that the enzyme is more stable below 50^0C. The enzyme exhibited a slow thermal denaturation within 30 minutes at 50^0C and a drastic denaturation at 55^0C. The intestinal lipase of *O. mossambicus* lost its activity within 50 minutes at 60^0C (**Fig.5.4.15**). The hepatopancreatic lipase of *O. mossambicus* exhibited a fast decline in enzyme activity over the temperature 50^0C. The enzyme has been completely thermally inactivated within 15 minutes at 55 and 60^0C (**Fig.5.4.16**). Lopez-Lopez *et al* (2003) established that the stability of lipase isolated from *C. quadricarinatus* esterases was affected after incubation for 30 min at temperatures from 30 to 70^0C. At 70^0C, the lipolytic activity was completely destroyed. Similar results were found in *Cancer borealis* and *Cancer irroratus*, for which Brun and Wojtowicz (1976) found that esterase activity decreased above 40^0C when extracts were subjected to a temperature gradient for 20 min. A crab digestive lipase was completely inactivated at a temperature higher than 65^0C (Cherif *et al*., 2007). The *Etroplus suratensis* intestinal lipase is stable up to 35^0C and beyond this temperature thermal denaturation of the enzyme starts and inactivation occurs. The enzyme is stable for 50 minutes and for 40 minutes at 40^0C and 45^0C respectively (**Fig.5.4.17**). The lipase from *E. suratensis* hepatopancreas exhibits thermal stability up to 35^0C and the thermal denaturation gradually starts from 30 minute of the experiment at 40^0C. At 45^0C, the enzyme loses its activity drastically and is completely denatured within 35 minutes (**Fig.5.4.18**). The optimum temperatures for lipases from yellowfin tuna (*Thunnus albacares*), skipjack tuna (*Katsuwonus pelamis*) and tonggol tuna

(*Thunnus albacares*) activities is 60^0C, but the extracted enzyme was more stable in the temperature range of 37- 40^0C (Prasertsan *et al*., 2008). These indicate that lipase is well adapted to function at temperatures at which the organisms normally live.

The hepatopancreatic lipase from *O. mossambicus* is more stable in acidic pH range than the intestinal enzyme. The intestinal lipase exhibited its maximum stability at pH-7.5 (100% relative activity). However it is more stable in the alkaline range with about 80% relative activity. The lipase activity was highly stable at pH 7–8, this being in agreement with the pH range found for many fish intestinal digestive enzymes (Iijima *et al*., 1998; Ugolev and Kuzmina, 1993; Kuzmina and Ushakova, 2007), as well to the normal pH values measured in the intestine of this species (Deguara *et al*., 2003). In the present study, the *O. mossambicus* hepatopancreatic lipase exhibited its maximum stability at acidic pH than in alkaline range (**Fig.5.4.19**). The intestinal and hepatopancreatic lipase from *E. suratensis* showed maximum stability in the acidic to neutral pH. Specifically, the intestinal lipase is significantly stable from pH 3.5 to 7.0 and the hepatopancreatic lipase is stable from pH 4.0 to 7.0. Both enzymes retained approximately 50% stability in the alkaline pH range (7.5 to 10.5) (**Fig.5.4.20**). Species with a highly functional stomach, like the oil sardine (*Sardinella longiceps*) or the grey mullet (*Liza parsia*) show a marked reduction in activity at acidic pH as described for lipases in other species (Mukundan *et al*., 1985; Islam *et al*., 2008). Under the physiological conditions existing in the live fish the continuous flow of acid chyme coming from the stomach to the proximal intestine determines that optimal pH for the in vitro activity of the enzyme regardless of the secretion of bicarbonate into the duodenum. Therefore, a low sensitivity to acid pH could be interpreted as a

functional adaptation to perform lipid hydrolysis even under non-optimal conditions. Borlongan (1990) described the presence of intestinal and pancreatic lipases in the milkfish *Chanos chanos*, showing maximum activities at pH 6.8 and 8.0, respectively. The visceral lipases from yellowfin tuna (*Thunnus albacares*), skipjack tuna (*Katsuwonus pelamis*) and tonggol tuna (*Thunnus albacares*) were the most stable at their optima pH after 120 min incubation (Prasertsan *et al*., 2008). In the present study, the detection of two well-defined optimal pH values for both the intestinal and pancreatic lipases suggests a physiological versatility for lipid digestion in this two selected species.

The data about substrate saturation kinetics of fish lipase enzyme is scarce. However, Gorgun and Akpınar (2012) showed that the Km and Vmax of lipase from the liver of *Cyprinus carpio* L. (1758) as 0.17 mM p-NPB and 2.6 μmol/ml.dk, respectively by using p -nitrophenyl butyrate (p-NPB) as substrate. Kinetic analysis indicated that *Vmax* and *Km* of rainbow trout, *Oncorhynchus mykiss* is 0.016 nmol/h/mg protein and 0.28 mM triolein. Another study focused on liver lipase of *O. mykiss* determined *Km* and Vmax values as 0.12 mM and 0.40 U/mg, respectively, using p-nitrophenyl acetate (p-NPA) as substrate (Metin and Akpınar, 2000). Aryee *et al* (2007) indicated that esters of short chain aliphatic acids with p- nitro phenol were poor substrates for the lipase purified from the viscera of *M. cephalus* but medium and long chain p-nitrophenyl esters were showed better performance as substrate. *Km* and *V*max, for the *p*-NPP hydrolase reaction were calculated as 0.22mM and 20 μmol min^{-1} mg^{-1}, respectively. Based on these, the corresponding catalytic efficiency (*Vmax/Km*) was determined as 90.91μmol min^{-1} mg^{-1} mM^{-1}. However, in our experiments with crude enzyme preparations, at around 25mM, the intestinal lipase of *O. mossambicus* is

completely saturated with p-nitrophenyl palmitate substrate. But the hepatopancreatic lipase has been saturated by 15mM p nitrophenyl palmitate (**Fig.5.4.5** and **Fig.5.4.6**). Both intestinal and hepatopancreatic lipase of *E. suratensis* showed a substrate saturation at 15mM of p-Nitrophenyl palmitate (**Fig.5.4.11** and **Fig.5.4.12**). The high values of substrate saturation could be due to higher enzyme concentration as well as higher digestive capabilities of the selected tropical fishes.

As a summary, present study reveals that *O. mossambicus* lipase has higher temperature and pH optima than that of *E. suratensis*. The lipase from *O. mossambicus* digestive organs is much more stable than the lipase from *E. suratensis* at different pH values and temperatures. Lipids are the energy rich biomolecules and the higher ability to utilize lipid components in the diet will help fishes to adapt, survive and to establish in their respective habitats.

Chapter 6

FUNCTIONAL ZONATION OF DIFFERENT DIGESTIVE ENZYMES OF *ETROPLUS SURATENSIS* AND *OREOCHROMIS MOSSAMBICUS*

Contents

A comparative study on distribution of digestive enzymes and digestive indices was done using *E. suratensis* and *O. mossambicus*. The present study investigated the acid protease, amylase, lipase and total alkaline protease activity along the gastrointestinal tract of *E. suratensis* and compared with that of *O. mossambicus* in order to increase our knowledge on the digestive physiology of these Cichlid species and gain information concerning its nutrition.

6.1. Introduction

Etroplus suratensis, (Pearl spot, In Malayalam: *Karimeen*), the 'upper-middle class' fish has got a profile uplift as the official state fish of the state of Kerala, South India and the year 2010-11 was observed as 'The Year of Karimeen'. The measure is expected to boost the production of *Etroplus suratensis*. The knowledge about digestive physiology of *E. suratensis* is very limited. Tilapias are one of the most important freshwater finfish cultured in the world and they represent approximately 6% of total farmed fish production (FAO, 2004). *Oreochromis mossambicus* is one of the hardiest fish of aquaculture farms. Once introduced into a habitat, they generally establish themselves very quickly. The presence of native fishes in the river systems had

dwindled wherever the presence of tilapia was found in large numbers (Welcomme and Vidthayanom, 2003). However, being predominantly phytoplankton feeders, in many water bodies they act as omnivores and even carnivores. Competition between the introduced species and native species is frequently cited as a cause of potential difficulty in aquaculture. This might be due to its early maturity, continuous breeding, high survival rate and its carnivorous behavior on eggs and juveniles of fish and other aquatic fauna. The digestive enzymes play an important role in the development and growth of fishes. The developmental phase of major morphological events is associated with the digestive system. Regional differentiation and gastric gland formation as well as the enzymology of the developing alimentary canal have been studied in a number of marine species (Pittman *et al*., 1990; Bisbal and Bengtson, 1995; Baglole *et al*., 1997) and freshwater species (Govoni *et al*., 1986) in euryhaline teleosts such as *O. mossambicus* (Lo and Weng, 2006).

The gastro intestinal tract of teleost fishes is functionally diverged in to different zones as that of mammals and this functional zonation has a major role in digestion (Harpaz and Uni, 1999), absorption (Bakke-McKellep *et al*., 2000) and osmoregulation by water and ionic balance (Varsamos *et al*., 2005 ; Martin Grosell., 2006). The ability of the fish to utilize ingested nutrients depends on the activities of digestive enzymes present in various locations along the digestive tract. In certain fish species, investigations on digestive secretions have been employed to define the requirements of dietary protein (Twining *et al*., 1983) and carbohydrates (Spannhof and Plantikow, 1983). Proteases of several species have been studied in order to develop a cost effective diet for intensive farming (Clark *et al*., 1985; Alarcon *et al*., 1998; Glass *et al*., 1989) and matching of an artificial diet to the nutritional needs of the fish (Furne *et al*., 2005). Rate of proteolytic digestion and absorption of

essential amino acids could also be determined with proper knowledge of functional activities of proteases (Eshel *et al*., 1993). Hofer and Kock (1989) suggested that the profile of digestive enzymes reflects the ability of a species to use different nutrients. The understanding of the functional properties and optimal conditions for hydrolysis of nutrients by digestive enzymes in fish will facilitate a more precise measurement of nutrient digestibility by a particular species. Alpha-amylase is a key enzyme for carbohydrate digestion. It is probably secreted by the pancreas into the intestine and the pyloric caeca. By the action of amylase, polysaccharides such as starch and glycogen are hydrolyzed into maltose, maltotriose, branched oligosccharides (limit dextrin) and some glucose (Jobling, 1995). Reports on the digestive secretions with lipase activity in fish are limited. Leger *et al* (1979) purified a lipase and colipase in rainbow trout. Lie and Lambertsen (1985) have described digestive lipase activity in Atlantic cod. Borlongan (1990) reported optimal activity for pancreatic and intestinal lipase of milkfish, at two pH values. Generally, carnivorous fish have higher digestive lipase activity than do omnivorous or herbivorous fish (Chakrabarti *et al*., 1995; Opuszynski and Shireman, 1995; Tengjaroenkul *et al*., 2000).

Digestive tissues are notoriously plastic in their responses to dietary change (Starck, 1999). Several researchers identified variations in gut morphology in response to fasting, increases in food intake and changes in diet (Starck., 1999; Naya *et al*., 2007; Olsson *et al*., 2007) and patterns consistent with adaptive plasticity in intestine morphology have been demonstrated in rodents (Naya *et al*., 2008). Initially natural selection could amplify small morphological changes initiated by plasticity and, on the other hand plasticity could retard evolution by shielding genotypes from selection (West-Eberhard, 2003; Crispo, 2007). Thus, gut morphology associated with dietary shifts has

received increasing interest, in the context of explaining divergence and adaptive radiation (through "genetic accommodation"), recently (Ledon-Rettig *et al*., 2008). The phylogenetic reconstruction in several cichlid groups has been proposed by the characterization of coiling pattern and intestinal length (Zihler, 1982; Yamaoka, 1985). Thus, diet is a strong predictor of both intra- and interspecific variation in the intestinal length, indicating that fish adjust their phenotype to balance nutritional needs against energetic costs (Wagner *et al*., 2009). Therefore, it is necessary to analyze some morphometric parameters of the digestive tract, such as: intestinal coefficient, digestive somatic index, and hepatosomatic index of the selected teleosts. The results should contribute to a better understanding about the gastrointestinal tract morphology, which can be used as indicators of the feeding habits. Thus, studies on digestive secretions in fish can elucidate certain aspects of its nutritive physiology and help to resolve nutritional problems, such as the matching of an artificial diet to the nutritive capabilities of fish and formulation of cost effective feed. Most importantly, in India especially in the state of Kerala, the native species were under menace due to alien species invasion and flourishment (Bijukumar, 2000).

6.1.1. Objectives of the study

The aim of the present work was to study the acid protease, amylase, lipase and total alkaline protease digestive enzymes of *E. suratensis* and compare it to *O. mossambicus* in order to increase our knowledge on the digestive physiology of this *cichlid* species and gain information concerning its nutrition.

6.2. Material and Methods

6.2.1. Fish and Preparation of Crude Enzyme Extract

Experimental fishes of almost similar size (5-10cm) were collected from the Fisheries Station, Kerala University of Fisheries and Ocean Studies, Puthuvyppu. The fishes were acclimated to laboratory condition for a week. A commercial diet with known proximate composition was given adlibitum. The fish were starved for approximately 12 h prior to sampling and subsequently killed by cold shock and dissected immediately. The digestive tract of each fish was divided in to stomach, anterior, middle and posterior intestine. Since intestines of tilapia and pearlspot lacked visually distinct regions, they were divided into three segments of equal length and designated as anterior, middle and posterior intestine. The hepatopancreas was also taken as a digestive organ. The stomach contents were squeezed out and rinsed with cold distilled water. Ten percent (w/v) tissues homogenate was prepared in cold Tris–HCl 50 mM buffer pH 7.4 using an electric homogenizer (KEMI Model No: KHH 1), in ice cold condition. The homogenate was then centrifuged at 4^0C at 10,000*g* for 5 min. The supernatant containing the enzymes was stored at -20^0C until the analysis.

6.2.2. Methods

The tissue homogenates were purified as described in second chapter and the soluble protein content of enzyme extract was measured according to Lowry *et al*., 1951.

Estimation of digestive enzyme activities

The digestive enzymes like Pepsin, Amylase, Total alkaline proteases and Lipase were estimated as described in the above chapters.

Indices Calculations

The digestive indices were calculated as following

Digestive somatic index (DSI)= (Digestive tract weight/Body weight)X 100

Hepatosomatic index (HSI) = (Hepatopancreas weight/Body weight) X 100

Intestinal coefficient (IQ) = Digestive tract length/Total fish length

Intestinal to Standard Length ratio = Total gut length/ Standard length

6.3. Results

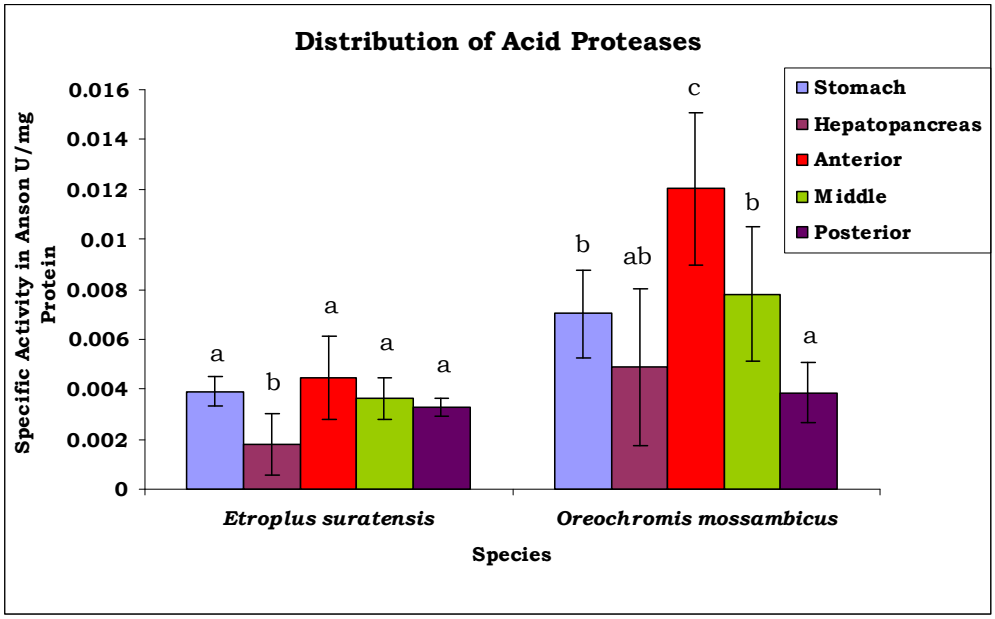


Fig.6.3. 1 Distribution of acid protease along the digestive segments of *E. suratensis* and *O. mossambicus*

The acid proteases like pepsin act only at low pH and the presence of such protease activity was observed along the gastro intestinal tract. The presence of acid protease extends all along the digestive tract. The activity decreases gradually towards the posterior part of the intestine. Each bar diagram represents mean ± standard deviation. On each set of bars values with

different lower case letters vary significantly (P<0.05) in each tissue on the two different species (One-way ANOVA).

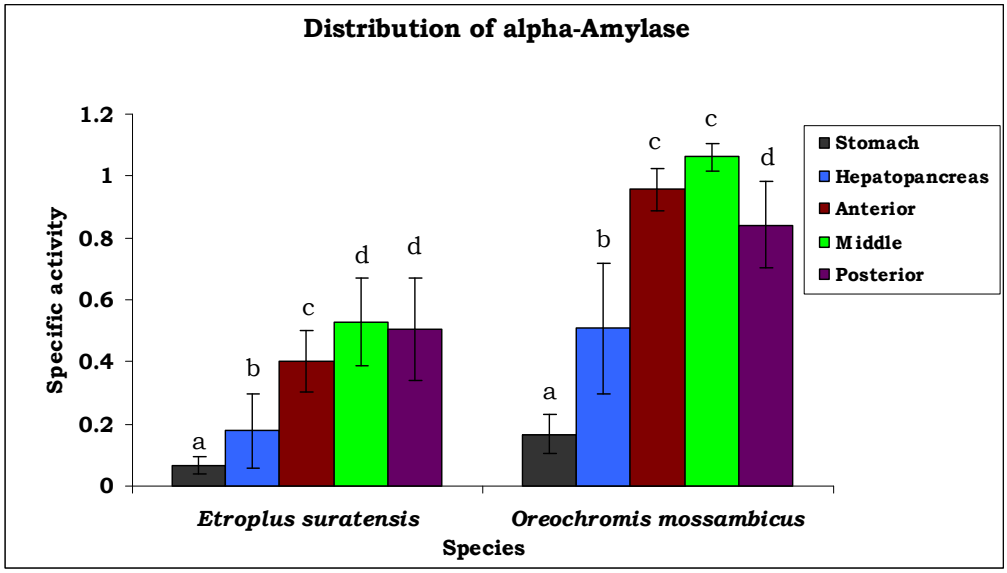


Fig. 6.3.2 Distribution of alpha-amylase along the digestive segments of *E. suratensis* and *O. mossambicus*

Comparatively low alpha amylase activity was shown by the *E. suratensis* and the enzyme was distributed almost equally throughout the intestinal segments in both the species. However, the middle intestine showed slightly higher activity. The neutralization of chyme by bile may extend to and get completed at the middle intestinal segment and it may be the reason for a slightly higher alpha amylase activity in that segment. Each bar diagram represents mean±standard deviation. On each set of bars values with different lower case letters vary significantly (P<0.05) in each tissue on the two different species (One-way ANOVA).

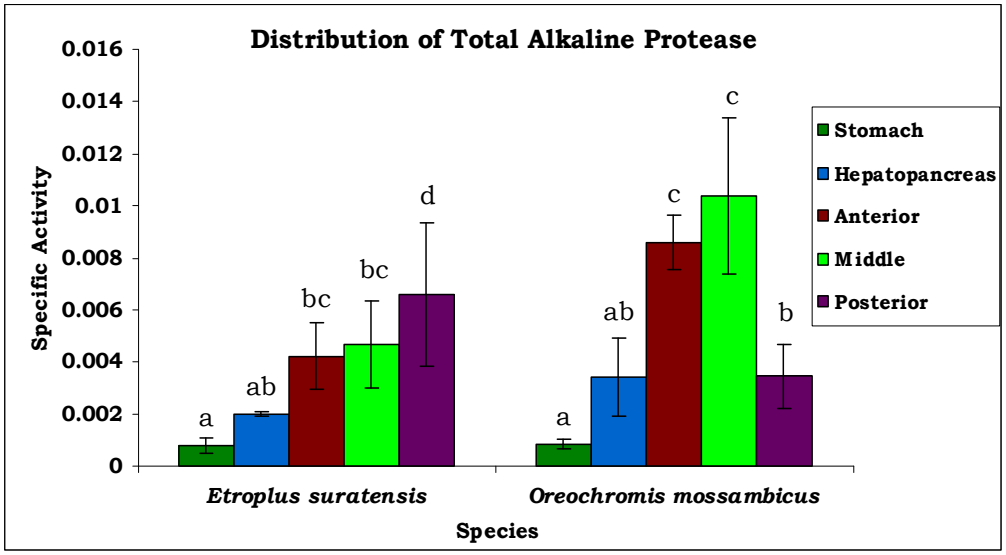


Fig.6.3.3 Distribution of total alkaline protease along the digestive segments of *E. suratensis* and *O. mossambicus*

Very low alkaline protease activity was found in the stomach of both the fishes. This indicates the union of pancreatic duct to the gastro intestinal tract is after the stomach portion along with the bile duct. The enzyme activity diminishes intensively at the posterior portion in *O. mossambicus* but in *E. suratensis* the activity remains all along the digestive tract. Each bar diagram represents mean ± standard deviation. On each set of bars values with different lower case letters vary significantly ($P<0.05$) in each tissue on the two different species (One-way ANOVA).

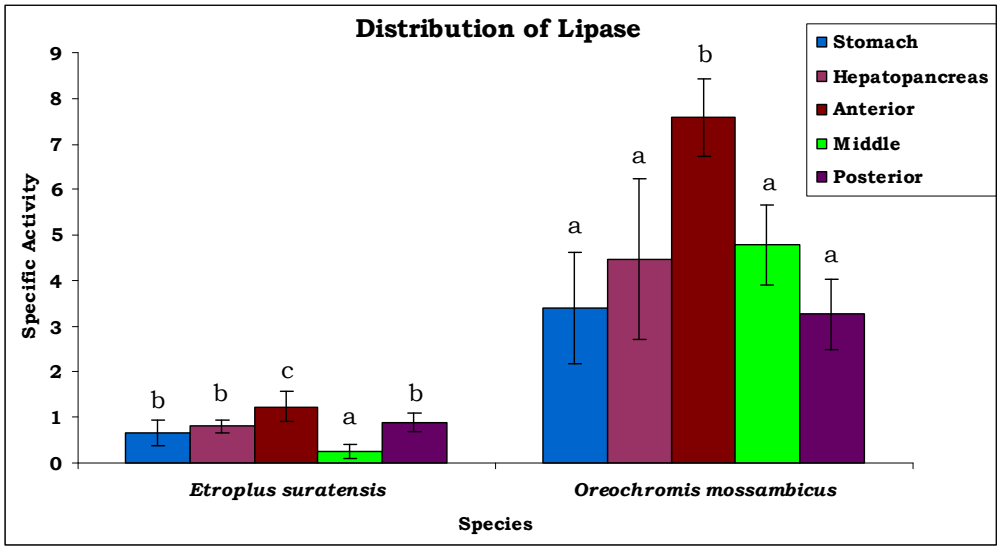


Fig.6.3.4 Distribution of lipase along the digestive segments of *E. suratensis* and *O. mossambicus*

The present study showed that lipase is one of the prominent digestive enzymes in *O. mossambicus* with a remarkable specific activity through out the digestive tract. Low activity of stomach lipase was shown by *E. suratensis*. The high profile of lipase in *O. mossambicus* in comparison with *E. suratensis* has been proven Tilapia can thus efficiently digest various lipids in the available feed of plant or animal origin more effectively than pearlspot. Each bar diagram represents mean ± standard deviation. On each set of bars values with different lower case letters vary significantly ($P<0.05$) in each tissue on the two different species (One-way ANOVA).

Table 6.1. Length-Weight relationships and digestive indices of *Etroplus suratensis* and *Oreochromis mossambicus*

	TL	SL	TW	COR	TGL	TGW	HW	SW	DSI	HIS	IC	Gut Vs SL Ratio
E. suratensis	11.950±1.31	9.333±1.0	42.167±14.89	0.99	41.167±5.74	0.37±0.06	0.613±0.42	0.165±0.03	0.956±0.307[a]	1.332±0.475[a]	3.445±0.303[a]	4.411±0.391[a]
O. mossambicus	11.283±0.64	8.833±0.41	21.19±2.58	0.73	65.50±21.64	0.977±0.24	0.305±0.14	0.133±0.05	4.584±0.874[b]	1.475±0.701[a]	5.755±1.61[b]	7.353±2.11[b]

TL- Total Length, SL- Standard Length, TW- Total Weight, COR-Correlation between Total length and Total weight, TGL- Total Gut Length, TGW- Total Gut Weight, HW- Hepatopancreas Weight, SW- Stomach Weight, IC- Intestinal coefficient. Length was measured in centimeter and weight in grams and they were reported as mean ± Standard deviation (SD). Digestive somatic index (DSI), Hepato-somatic index (HIS), Intestinal coefficient and Gut Vs Standard Length Ratio were reported as mean ± SD. On each columns values with different lower case letters vary significantly (P<0.05) in each tissue on the two different species (One-way ANOVA)

6.4. Discussion

Proper digestion and absorption of nutrients requires contact of food with digestive enzymes for a certain period and the food should be subjected to grinding, mixing, and advance movements characteristic of the digestive tract. A longer stay of the food in the digestive tract could compensate for lower enzyme activity (Sanz *et al*., 1987). Distribution and intestinal digestive enzyme activity along and within the intestinal tract varies with feeding habit and intestinal morphology (Hofer and Shiemer, 1981; Kuzmina, 1984; Kuzmina and Smirnova, 1992; Sabapathy and Teo, 1993; Kolkovski, 2001; Gawlicka., *et al*, 2002). In Chinook salmon, weight gain was found to be positively correlated with the ability of the digestive enzymes to hydrolyze diets (Haard *et al*., 1996).

In this study, it is found that proteases are very prominent in both stomach and intestinal segments and they are widely distributed though out the alimentary canal. Various studies on other fish digestive secretions have shown the occurrence of acid proteases that have high activity in the acidic region in the stomach and alkaline proteases acting actively in alkaline pH region in the intestine (Clark *et al*., 1985; Martinez and Serra, 1989; Chong *et al*, 2002a; Xiong *et al.*, 2011).

Proteolytic activities at low pH have also been reported in species with prominent stomach region and a high pepsin secretion such as eel, tilapia, salmon, sea bass and trout (Jonas *et al*., 1983; Twining *et al*., 1983; Torrissen, 1984; Sabapathy and Teo, 1993; Yamada *et al*., 1993; Xiong *et al.*, 2011). Pepsins, a member of the aspartic endopeptidase family, have been identified in several species (Haard, 1986). Most fish species contain two or three major pepsins with an optimum hemoglobin digestion at pH between 2 and 4

(Gildberg, 1983). Protein digestion in the vertebrate stomach occurs as a result of the synergistic action of pepsins and hydrochloric acid. Pepsin has been identified as the first proteolytic enzyme acting in fish digestive tract as a major acidic protease (Sabapathy and Teo, 1993; Tengjaroenkul *et al*., 2000). In our examination, the acid protease like pepsin is found to be present in all segment of the alimentary tract. This is in accordance with the findings of Odedeyi and Fagbenro (2010). But in the natural physiology of digestion, the acid protease will not be active in the intestinal segments due to the neutralization of acidic condition by the action of bile. Tilapia require a highly acidic medium to enable biochemical digestion of protein due to thin stomach walls when compared to fishes with muscular stomach like African catfish which relies more on mechanical breakdown of nutrients and possesses lower pepsin secretion (Maier and Tullis, 1984; Uys and Hecht, 1987).

After gastric digestion, the neutral and basic proteases of intestinal and pancreatic origin complete the digestion. We found a large quantity of basic protease enzyme activity in both species. Enzymes such as trypsin, chymotrypsin, collagenase, elastase, and carboxypeptidase have been characterised in different types of fish (Eshel *et al*., 1993; Chiu and Pan, 2002; Chong *et al*., 2002a). High protease activity by intestinal extract has been shown by different researchers in various species. Species like carp (Jonas *et al*., 1983), rainbow trout and Atlantic salmon (Torrissen, 1984), halibut and turbot (Glass *et al*., 1989), striped and European sea bass (Eshel *et al*., 1993), seabream and dentex (Alarcon *et al*., 1998), goldfish (Hidalgo *et al*., 1999) and discus fish (Chong *et al*., 2002a) possess optimal activity at two alkaline pH range proved the presence of two major group of alkaline proteases with different optimum pH.

In comparison to proteases, knowledge about carbohydrases and lipases is still lacking in many species despite reports on important role of these enzymes (Hidalgo *et al*., 1999; Fernandez *et al*., 2001). Various workers have demonstrated that amylase activity is greater in omnivorous and herbivorous fish than in carnivorous fish (Fange and Grove, 1979; Ugolev *et al*., 1983; Hidalgo *et al*., 1999). Low or moderate amylase activities have been reported in other carnivorous species (Sabapathy and Teo, 1993; Munilla-Mora´n and Saborido-Rey, 1996b). Comparatively a lower activity of amylase was detected in the stomach in this study and researchers suggested that presence of amylase in stomach could be due to some exogenous contamination from intestinal activity (Uys and Hecht, 1987; Xiong *et al*., 2011). In the natural environment, carbohydrates are indeed more predominant than protein. Thus, there is possibility of carbohydrate digestion beginning from the stomach (Munilla-Mora´n and Saborido-Rey, 1996b).

With respect to lipase activity, the presence of lipases in carnivorous fishes could be due to higher consumption of fat-rich food (Chakrabarti *et al*., 1995). This lipase activity is greater than that in the omnivorous or herbivorous fish (Opuszynski and Shireman, 1995; Tengjaroenkul *et al*., 2000). Lipolytic activity in fish is generally greatest in the proximal part of the intestine and the pyloric caeca if present, but can extend into the lower parts of the intestine with the activity decreasing progressively. Exceptions do occur as in turbot (*Scophthalmus maximus*) and plaice (*Pleuronectes platessa*), which possess a higher lipolytic activity in the distal part of the intestine than other digestive segments (Koven *et al*., 1994a; Olsen and Ringo, 1997). This may be an adaptation to a short digestive tract with few pyloric caeca as in turbot, although plaice have numerous pyloric caeca. Low lipolytic activity has also been found in the stomach of several fishes but the physiological significance

of gastric lipolytic activity in fish is unclear (Olsen and Ringo, 1997). The pancreas or hepatopancreas is generally considered as the major source of digestive lipase enzymes in fish as it is in mammals (Kapoor *et al*., 1975; Fange and Grove, 1979). However, several studies have found high lipase activity in mucous layers or intestinal segments of many fish species, although these lipases may actually be of pancreatic origin, resulting from adsorption of the pancreatic enzyme into the intestinal mucosa (Fange and Grove, 1979; Smith, 1989a). Lipase activity was determined in intestinal segments even after 48 and 24 hours starvation in African catfish (*Clarius gariepinus*) and grass carp (*Ctenopharyngodon idella*), respectively (Ghosh, 1976; Das and Tripathi, 1991). The substrate specificities support the prospect that intestinal cells can secrete lipolytic enzymes actively (Borlongon, 1990; Uematsu *et al*., 1992; Koven *et al*., 1994a). The lipolytic activity found in stomach is different from that of pancreatic origin, suggesting that the stomach is also a source of lipases, and the intestinal flora also contributes lipolytic activities in the digestive tract of fish (Olsen and Ringo, 1997). In the present study anterior and middle part of both the fishes showed the most intense activity of lipase and it is in agreement with the results obtained by Tengjaroenkul *et al* (2000).

In higher vertebrates, digestive enzymes are restricted to distinct gut sections, showing a clear functional zonation. This arrangement has not been clearly observed for most fishes, in which case they are usually distributed throughout the tract. Most of the zones of the digestive tract possess and facilitate all the principal digestive enzyme activities. Thus, it may be assumed that during the early phase of evolution of digestive system there was no specific functional zonation of the digestive tract. The digestive system of confined aquatic teleost fishes has no specific strategy with regard to the food and feeding habit, because of their incomplete segregation of food niche as

well as their evolutionary adaptation (Chakrabarthi *et al.*, 1995). In conclusion, the comparative study of the enzymatic content in the digestive tract, with acid and alkaline protease, amylase, and lipase activity, in *E. suratensis* and *O. mossambicus* shows not only that *O. mossambicus* would digest the dietary proteins and fats as any other carnivore would, but also that the amylase pool would enable this fish to digest carbohydrates at herbivore levels.

In teleosts, the gastrointestinal tract morphology generally shows specific variations with respect to diet, feeding habit phylogeny, body shape, and features that reflect functional differentiation (Noaillac-Depeyre and Gas, 1974; Kapoor *et al.*, 1975; Anderson, 1986; Abaurrea *et al.*, 1993). Data on digestive indices including total length, standard length, total weight, total gut length, total gut weight, hepatopancreas weight, stomach weight, digestive somatic index (DSI), hepato-somatic index (HIS) and intestinal coefficient were reported in **Table 6.1**. *O. mossambicus* has a higher index for DSI, HIS, IQ and gut Vs standard length ratio than that of *E. suratensis* indicating higher digestive and metabolic capability. The fast growth and attainment of maturity of *O. mossambicus* can be explained from the difference in digestive indices. In addition, there is a correlation between the structures of the digestive apparatus and the feeding habit of fishes (Buddington *et al.*, 1987; Fugi *et al.*, 2001; Ward-Campbell *et al.*, 2005). The morphological structures give insights on the feeding ecology of a species, since these peculiarities suggest how a fish is able to feed (Ward-Campbell *et al.*, 2005). Moreover, the diet influences the intestinal morphology (Dabrowski and Kaushik, 1985) and the structural variability of the gastrointestinal tract (Buddington *et al.*, 1987). The intestinal coefficient can be used to estimate the feeding habit of fishes (Becker *et al.*, 2010). It is commonly emphasized that herbivore and

detritivore fish species tend to have longer, thinner and narrower intestines than carnivores (Fange and Grove, 1979; Junger *et al*., 1989, Fugi *et al*., 2001), and the intestine of omnivores species has an intermediate length (Ward-Campbell *et al*., 2005). Here, when compared, *O. mossambicus* exhibits the omnivore gut characteristics like long, highly coiled intestine but *E. suratensis* has a short intestine. De Silva *et al* (1983) reported distinct difference in the mean relative intestinal length between populations from coastal lagoon and inland reservoirs in correlation with their respective feeding habit. In lagoon it fed mainly on mollusks and in the freshwater reservoirs on macrophytes.

This knowledge will be helpful to understand the competitive feeding strategies of native and alien species. The survival and establishment of species like tilapia in native water bodies are mainly due to their vigorous digestive capacity, their by faster growth and early maturity. This will ultimately lead to quick breeding and increase in their population. The knowledge about the digestive enzymes and digestive capabilities would be advantageous for diet manufacturing, as carbohydrates could be added at a greater proportion than protein and thereby save on feed manufacturing costs. In turn, the higher proportion of protease enzymes and amylase with respect to lipase in pearlspot may help to reduce the fat content in feed thereby increase the shelf life of artificial feed for this species. Further studies at the physiological level, with different type of diets, with varying environmental factors like salinity, temperature, pH, dissolved oxygen etc., will provide progressively more information about the digestive particularities of these species of fish with such importance, both scientific and commercial.

Pepsin-like acid proteases that act at low pH have been identified all along the digestive tract of both the fishes. Comparatively low alpha amylase

activity is shown by the *E. suratensis* and the enzyme is distributed almost equally throughout the intestinal segments in both the species. Very low alkaline protease activity is found in the stomach of both the fishes and in *O. mossambicus*, the enzyme activity diminishes extensively towards the posterior portion of the intestine whereas in *E. suratensis* the activity increases towards the posterior part. The present study showed that lipase is one of the prominent digestive enzymes in *O. mossambicus* with a remarkable specific activity throughout the digestive tract than that of *E. suratensis*. It has been noted that *O. mossambicus* has a higher values for digestive somatic index, hepato somatic index, intestinal coefficient and gut *Vs* standard length ratio than that of *E. suratensis* indicating its higher digestive and metabolic capabilities.

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Chapter 7

EFFECT OF PROBIOTICS ON DIGESTIVE ENZYMES

Contents

The essentiality of any aquaculture practice is to ensure better growth and survival of cultured fishes for economic feasibility. Optimum fish health can be achieved by various treatments and manipulations in the culture system. The present chapter deals with the effect of probiotics on digestive enzymes of the selected fishes with respect to their growth and survival during probiotic administration. The chapter is divided into sections as in the second chapter.

7.1. Introduction

The growth of aquaculture as an industry has progressed over the past decades. Aquaculture is the farming of aquatic organisms by intervention in the rearing process to enhance production. The contribution of aquaculture to world food production and supply of raw materials for industrial and pharmaceutical use, and aquatic organisms for stocking or ornamental trade has increased dramatically in recent decades. The report "World Aquaculture 2012" states that global production of fish from aquaculture grew more than 30% between 2006 and 2011, from 47.3 million tons to 63.6 million tons. It also forecasts that in the coming years more than 50% of the world's food fish consumption will come from aquaculture and it is expected to overtake capture fisheries as a source of edible fish (FAO, 2012). The need for increased disease resistance, growth, and feed efficiency has brought about the use of probiotics in aquaculture practices. Probiotics, according to the

currently adopted definition by Food and Agricultural Organization/World Health Organization, are live microorganisms that when administered in adequate amounts confer a health benefit on the host (FAO and WHO, 2001). Due to the problem of antibiotic resistance and ensuing reluctance of antibiotics administration, the use of probiotics in larval culture is becoming increasingly popular. Probiotics, live microbes that may serve as dietary supplements to improve fish growth and immune responses, have received some attention in aquaculture (Gatesoupe, 1999; Irianto and Austin, 2002).

7.2. Objectives of the study

The aim of the present work is to study about the modulations in pepsin like acid protease, amylase, lipase and total alkaline protease digestive enzymes of *E. suratensis* and *O. mossambicus* during probiotic administration. Present study also estimates the growth performance of these selected species in order to increase our knowledge on alterations in the hydrolytic enzymes and digestive physiology by probiotic treatments.

7.3. Review of Literature

The term probiotics is constructed from the Latin word pro (for) and the Greek word bios (life) (Zivkovic, 1999) and was created in the 1950s by Kollath (1953). Elie Metchnikoff's work at the beginning of this century is regarded as the first research conducted on probiotics (Fuller, 1992). He described them as microbes ingested with the aim of promoting good health. This same definition was modified to 'organisms and substances, which contribute to intestinal microbial balance to a live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance' (Fuller, 1989). This definition was broadened by Havenaar and Huisin't Veld (1992) to a 'mono- or mixed-culture of live

microorganisms which benefits man or animals by improving the properties of the indigenous microflora'. These definitions were originally applied to farm animals, ruminants, poultry and pigs, since the first studies were carried out in these species. Gatesoupe (1999) defines probiotics as microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be kept alive, with the aim of improving health. Gram *et al* (1999) broadened the definition by removing the restriction to the improvement to the intestine: a live microbial supplement, which beneficially affects the host animal by improving its microbial balance. Biological control has been described as the utilization of natural enemies to reduce the damage caused by noxious organisms to tolerable levels (Debach and Rosen, 1991) or more precisely, the control or regulation of pest populations by natural enemies (Smith, 1919). The microbial community inside the gut of some animals confers some degree of resistance to or protection against disease (Fox, 1988). The most widely used definition of probiotics is given by Fuller (1989) as "a live microbial feed supplement which beneficially affects the host animal by improving intestinal balance". Strictly speaking, a probiotic ought not to be classified as a biological control agent, since a probiotic microorganism does not necessarily attack the noxious pathogenic agents. It need not necessarily be a natural enemy of the pathogen, but it merely prevents damage to the host caused by the pathogen, usually through competition but at most, it may produce substances that inhibit the growth or attachment of the harmful micro organism. Neither view classifies probiotics as growth promoters since their action is not confined to improved growth but is associated with a general improvement in health. Researchers proved that the use of proper probionts would lead to a positive effect on survival and growth by altering the digestive and metabolic enzyme profiles and by inducing specific immune responses in the host organism. The intensive use of

antibiotics to prevent and control the bacterial diseases in aquaculture has led to an increase in antibiotic-resistant bacteria (Alderman and Hastings, 1998; Teuber, 2001). The abuse of antimicrobials can result in the development of resistant strains of bacteria (Weston, 1996). Such resistance can be readily transferred to other strains, either following alterations to the existing genome or by transfer of genetic material between cells through plasmids or bacteriophages (Towner, 1995). This is even more likely if chemotherapeutics have been used prophylactically in the culture of penaeid shrimps (Brown, 1989). Therefore, several alternative strategies to the use of antimicrobials have been proposed, such as the use of probiotics as biological control agents. The use of non-pathogenic, indigenous bacteria as biocontrol rhizosphere or probiotics to fish and mammals to prevent microbial diseases has, during the last decade received increasing attention.

Considerable attention has been given to alteration of the gut microbiota to boost health in humans and other animals in recent years through the use of probiotics and prebiotics (termed biotics). The goal of these dietary supplements is similar, but the manner in which they alter the gut microbial community is varied. Furthermore, some of the probiotic and prebiotic products are similar in composition, containing inactivated microbes or microbial components, which have led to some confusion over what exactly constitutes a "probiotic" or a "prebiotic" (Welker and Lim, 2011). However, living microorganisms, by the most of the researchers, will be considered as probionts. Prebiotics, on the other hand, are any non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (Ringo et al., 2010).

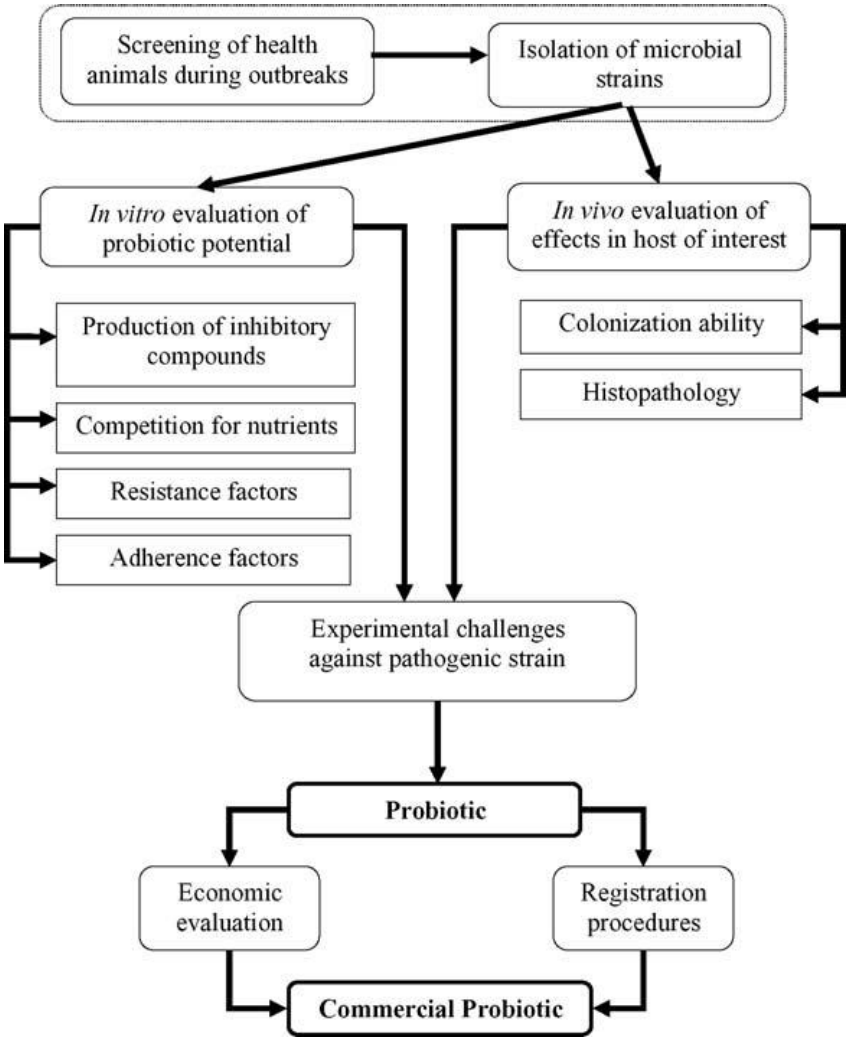

Fig.7.1.1 Diagram for selection of probiotics as biocontrol agents in aquaculture (Adopted from Balcazar *et al*., 2006)

The suggested probiotic modes of action in fish include: production of inhibitory compounds, competition for chemicals or available energy, competition for adhesion sites, inhibition of virulence gene expression or disruption of quorum sensing, improvement of water quality, enhancement of the immune response, source of macro and/or micronutrients and enzymatic contribution to digestion (Sugita *et al*., 1996; 1997; 1998; Vershuere *et al*., 2000; Olafsen, 2001; Vine *et al*., 2004; Gatesoupe 2008; Gòmez and Balcàzar,

2008; Ringø, 2008; Tinh *et al*., 2008). Probiotics used in animals include *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Streptococcus*, *Pediococcus*, *Enterococcus* and yeasts such as *Saccharomyces cerevisiae* and *Saccharomyces boulardii* (Fuller, 1992). In addition, tolerance against high temperature stress was observed in Rainbow trout (*Onchorhynchus mykiss*) fry on probiotic (*Bacillus*) treatment (Ahmadvand *et al*., 2012). The administration of lactic acid bacterial isolates as probiotics lowered water pH and increased digestive enzyme activity of crab *Portunus Pelagicus* particularly protease and amylase (Talpur *et al*., 2012).

Prebiotics

The use of probiotics in many cases might be difficult in commercial aquaculture because of the low viability of the bacteria after pelleting and during storage or leaching from the feed particle in rearing water, as well as due to problems related with feed handling and preparation. As an alternative, prebiotics have been assessed in an attempt to overcome difficulties associated with probiotic applications. A prebiotic is defined as a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth or the activity of specific health promoting bacteria that can improve the host health (Gibson and Roberfroid, 1995). Prebiotics mainly consist of oligosaccharides promoting beneficial bacterial growth within the GI tract (Yazawa *et al*., 1978; Gibson *et al*., 2004). According to Gibson *et al* (2004), only three oligosaccharides were classified as prebiotics: inulin, transgalacto oligosaccharide (TOS) and lactulose. A more recent study includes fructo oligosaccharides (FOS) in the list of prebiotics (Soleimani *et al*., 2012).

Synbiotics

Synbiotics, the combined application of probiotics and prebiotics, is based on the principle of providing a probiont with a competitive advantage (a fermentable energy source) over competing endogenous populations; Thus, effectively improving the survival and implantation of the live microbial dietary supplement in the gastrointestinal tract of the host (Gibson and Roberfroid, 1995).

Probiotics can be supplemented even at larval and early fry stages (Muroga *et al*., 1987). A number of studies suggest that probiotic supplementations can reduce the cost of culture by improving feed utilization efficiency and there by enhanced growth of fish (Bogut *et al*., 1998; Ghosh *et al*., 2003; Carnevali *et al*., 2006; Wang and Xu, 2006; Mazurkiewicz *et al*., 2007; Kesarcodi-Watson *et al*., 2008). A number of micro organisms such as *lactococcus* (Hagi *et al*., 2004; Sugita *et al*., 2009), *Bacillus* sp. (Kumar *et al*., 2006; 2008), *Lactobacillus* (Ramakrishnan *et al*., 2008) and *Saccharomyces cerevisiae* (Ramakrishnan *et al*., 2008) have been screened and practiced as probiotics. Majority of these probiotics are nonpathogenic and non-toxic and can survive in the gut and remain potent for long period under storage and field conditions (Ramakrishnan *et al*., 2008). Ringo *et al*., (2006) has confirmed that gut microbiota of fish are sensitive to dietary changes. The effect of dietary components on the gut microbiota is important to investigate as the gastro intestinal tract is one of the major routes of infection in fish (Ringo *et al*., 2004; Birkbeck and Ringo, 2005). Distribution and activity of intestinal digestive enzymes along the intestinal tract varies with feeding habit and intestinal morphology (Kuzmina, 1984; Sabapathy and Teo, 1993; Kolkovski, 2001; Gawlicka *et al*., 2002). Haard *et al*., (1996) demonstrated that in Chinook salmon, weight gain was found to be positively correlated with

the ability of the digestive enzymes to hydrolyze diets. Several researchers identified variations in gut morphology in response to fasting, increases in food intake and changes in diet (Starck, 1999; Olsson *et al*., 2007; Wagner *et al*., 2009). Thus, diets are a strong predictor of both intra- and inter specific variation in the intestinal length, indicating that fish adjust their phenotype to balance nutritional needs against energetic costs. Therefore, it is necessary to analyze some growth parameters of the fish such as weight gain, feed conversion ratio (FCR) and specific growth rate (SGR) of the selected teleosts.

7.4. Materials and Methods

Etroplus suratensis and *Oreochromis mossambicus* of almost equal size (5-6 cm) were collected from Fisheries station, Kerala University of Fisheries and Ocean Studies, Puthuvyppu, Cochin, Kerala. They were acclimated to laboratory conditions for two weeks. The fishes were fed on commercial feed (Godrej Grower) 5% fish weight/ day. The proximate composition of the feed has been showed in the **Table 7.4.1**.

Table 7.4.1. Proximate composition (in g/100g) of the experimental feed (Godrej-Higashimaru Grower Pellets)

Size	2.2 x 3 mm
Moisture	7.41
Dry weight	92.59
Crude protein*	34.45
Ether extract*	7.5
Crude fibre*	3.2
Organic matter**	79
Ash*	13.59
Acid insoluble ash	12.69

* Calculated on the basis of dry weight
** Organic matter = dry weight- ash

Total length, Standard length and live weight were taken before and after the feeding experiment. Probiotic species *Micrococcus MCCB 104* and *Bacillus MCCB 101* culture preparations were obtained from National Center for Aquatic Animal Health, CUSAT. In aquaculture, probiotics can be administered either as a food supplement or as an additive to the water (Moriarty, 1998). In the present study, we administrated these probiotics incorporated with feed because in fresh water, fishes will not drink considerably to maintain water homeostasis. Three types of test feed preparations were formulated along with a control.

Test 1- *Micrococcus* alone

Test 2- *Bacillus* alone

Test 3- *Micrococcus* and *Bacillus* together

Each experimental group was fed with experimental diet with specific bacterial culture (10^3 cells/ animal / day). Prior to feeding the diet preparations were air dried to avoid the leach out of bacterial cells. A control group was maintained by feeding experimental feed without probiotics. 14 days' and 28 days' experiments were designed. Each experimental group consisted of 12 fishes and a duplicate has been done. After the feeding trials the fishes were kept on fasting for 12 hours and sacrificed. The fishes were dissected on a glass plate kept in ice and the homogenates were prepared as explained in chapter 2. The enzyme assays were conducted as illustrated in the respective chapters.

$$\text{Feed Conversion Ratio } (\text{FCR}) = \frac{\text{Feed Intake } (\text{Weight of Feed})}{\text{Weight Gain}}$$

$$\text{Specific Growth Rate } (\text{SGR}) = \frac{\ln \text{W2} - \ln \text{W1}}{\text{t2} - \text{t1}}$$

$$\%\text{Survival} = \frac{\text{Final number of fishes}}{\text{Initial Number of Fishes}} \times 100$$

Statistical Analysis

The biochemical data from the probiotic experiments in this chapter is analyzed statistically by using SPSS software version 13 and the charts were plotted using MS Excel of Windows 7. Two-way ANOVA was used to compare variables among control and treatments, and if significant (fixed at $P<0.05$) differences were found, these data were analysed by post hoc test by multiple comparison using Tukey's test to determine which individual groups were significantly different from control. All the data are presented as mean ± standard deviation (SD) of the mean of at least six observations. The results were subjected to test for homogeneity using Tukey's post hoc analysis and depicted by lower case letters. Same lower case letters indicate that the results of effect of probiotic treatments obtained belong to same homogeneous subset and difference in homogeneity has been expressed by different lower case letters. Similarly the test for homogeneity of the results for days of feeding experiments were designated by integers as subscript and different integers indicates that the results belong to different homogeneity subsets.

7.5. Results

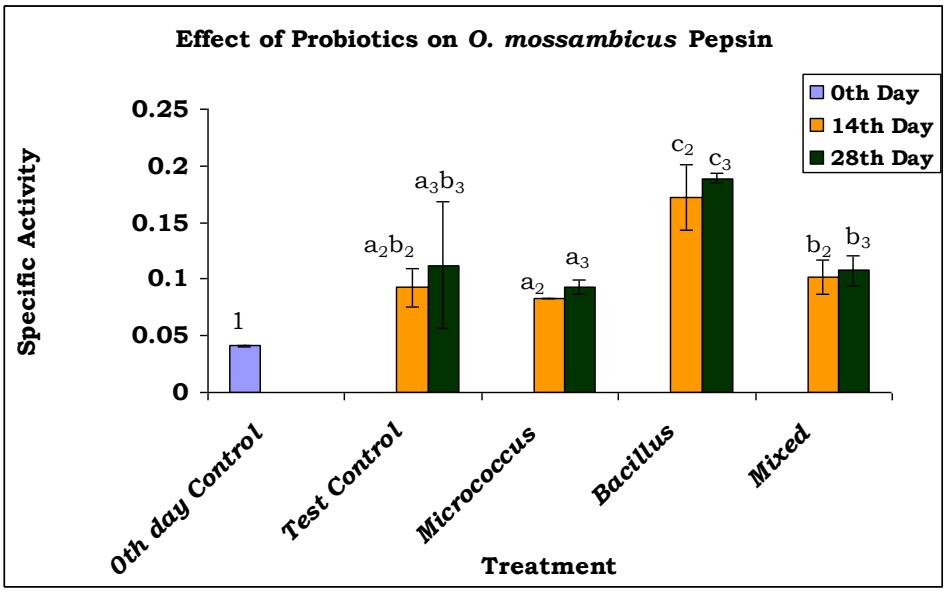

Fig.7.5.1. Effect of probiotics on *O. mossambicus* Pepsin

The results obtained were analyzed statistically using Two-Way ANOVA of the raw data, followed by multiple comparisons using Tukey's method. Two Factor ANOVA table indicated that *O. mossambicus* pepsin levels varied significantly (P<0.005) between probiotics. Between experimental periods a marked significant difference (P<0.005) was noted. In the case of interaction effects of both the probiotics as well as period of exposure, there is no significant difference (P<0.005).

Table.7.5.1. ANOVA for changes in the activity of *O. mossambicus* pepsin in relation to probiotics and experimental periods. Tests of Between-Subjects Effects

Source of Variation	SS	df	MS	F	P-Value
Between Probiotics	0.083	3	0.028	243.076	0.000
Between Period	0.005	2	0.002	21.143	0.000
Probiotics * Period	0.000	3	0.000	1.333	NS
Error	0.007	63	0.000		
Total	0.095	71			

SS- Sum of Squares, df- degrees of freedom, MS- Mean Square, NS-Not significant

To study further statistical relevance, comparing the significant difference in enzyme activity induced by different probiotics and experimental periods with respect to control, multiple comparisons using Tukey's post hoc test was conducted.

Table.7.5.2. Multiple Comparisons of factors such as probiotic treatments and experimental

(I) Treatment	(J) Treatment	P-Value
Control	*Micrococcus* alone	NS
	Bacillus alone	0.000
	Micrococcus and *Bacillus*	0.053
Micrococcus alone	Control	NS
	Bacillus alone	0.000
	Micrococcus and *Bacillus*	0.000
Bacillus alone	Control	0.000
	Micrococcus alone	0.000
	Micrococcus and *Bacillus*	0.000
Micrococcus and *Bacillus*	Control	0.053
	Micrococcus	0.000
	Bacillus alone	0.000
(I) Period	(J) Period	P-Value
0^{th} Day	14th Day	0.000
	28th Day	0.000
14^{th} Day	0th Day	0.000
	28th Day	0.000
28^{th} Day	0th Day	0.000
	14th Day	0.000

The multiple comparison tests using Tukey's method shows that the administration of *Bacillus* and mixed culture of both *Bacillus* and *Micrococcus* significantly ($P<0.005$) affect the *O. mossambicus* pepsin activity irrespective of the period of administration. The comparisons of enzyme activity between days of feeding trials show that enzyme activity is significantly enhanced by the probiotic treatment than that of control.

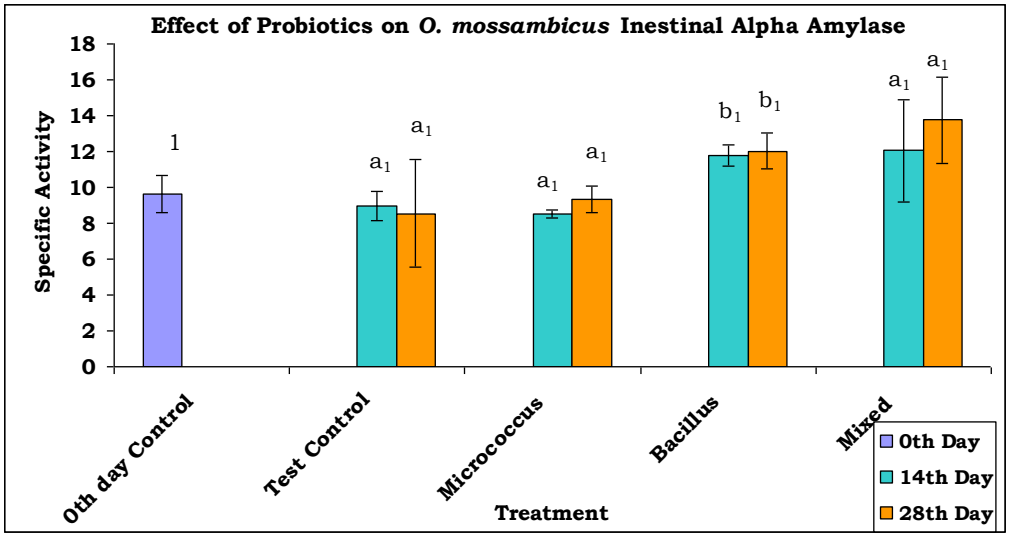

Fig.7.5.2. Effect of probiotics on *O. mossambicus* intestinal alpha amylase

Table.7.5.3. ANOVA for changes in the activity of *O. mossambicus* intestinal amylase in relation to probiotics and experimental periods. Tests of Between-Subjects Effects

Source of Variation	Sum of Squares	df	Mean Square	F	P-Value
Between Probiotics	211.929	3	70.643	24.060	0.000
Between Period	9.557	2	4.778	1.627	NS
Probiotics * Period	9.782	3	3.261	1.111	NS
Error	184.974	63	2.936		
Total	416.242	71			

SS- Sum of Squares, df- degrees of freedom, MS- Mean Square, NS-Not significant

The Two Factor ANOVA table indicated that *O. mossambicus* intestinal alpha amylase levels varied significantly (P<0.005) between probiotics. Between experimental periods, no significant difference (P<0.005) was noted. In the case of interaction effects of both the probiotics as well as period of exposure, there is no significant difference (P<0.005).

Table.7.5.4. Multiple Comparisons of factors such as probiotic treatments and experimental

(I) Treatment	(J) Treatment	P-Value
Control	*Micrococcus* alone	NS
	Bacillus alone	0.000
	Micrococcus and *Bacillus*	0.000
Micrococcus alone	Control	NS
	Bacillus alone	0.000
	Micrococcus and *Bacillus*	0.000
Bacillus alone	Control	0.000
	Micrococcus alone	0.000
	Micrococcus and *Bacillus*	NS
Micrococcus and *Bacillus*	Control	0.000
	Micrococcus	0.000
	Bacillus alone	NS
(I) Period	(J) Period	P-Value
0th Day	14th Day	NS
	28th Day	NS
14th Day	0th Day	NS
	28th Day	NS
28th Day	0th Day	NS
	14th Day	NS

Comparing the difference in enzyme activity induced by probiotics and experimental periods with respect to control using multiple comparisons using Tukey's post hoc analysis revealed that *Bacillus* alone and mixed culture of both *Bacillus* and *Micrococcus* exhibited significant ($P<0.005$) enhancement in the *O. mossambicus* intestinal alpha amylase activity. However, the period of probiotic administration does not affect the enzyme activity significantly.

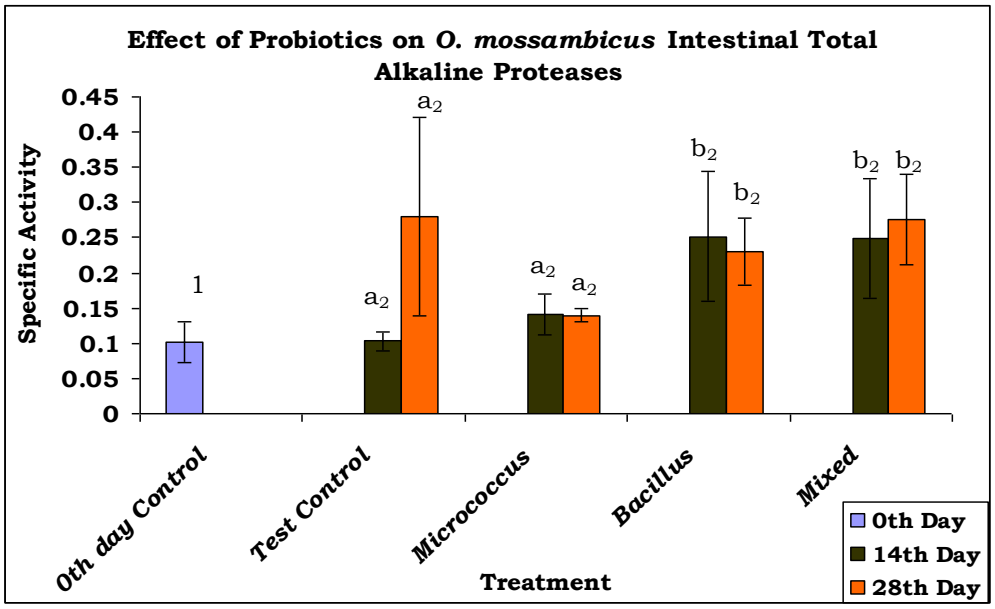

Fig.7.5.3. Effect of probiotics on *O. mossambicus* intestinal Total Alkaline Protease

Table.7.5.5. ANOVA for changes in the activity of *O. mossambicus* intestinal Total Alkaline Protease in relation to probiotics and experimental periods. Tests of Between-Subjects Effects

Source of Variation	SS	df	MS	F	P-Value
Between Probiotics	0.142	3	0.047	9.638	0.000
Between Period	0.074	2	0.037	7.544	0.001
Probiotics * Period	0.097	3	0.032	6.545	0.001
Error	0.310	63	0.005		
Total	0.623	71			

SS- Sum of Squares, df- degrees of freedom, MS- Mean Square, NS-Not significant

Comparison of the effect of probiotics and different exposure periods by using two way analysis of variance showed that there is an overall significant ($P<0.005$) increase in the *O. mossambicus* intestinal total alkaline protease activity. There are significant changes in enzyme activity with respect to the probiotic administration and the period of administration.

Table.7.5.6. Multiple Comparisons of factors such as probiotic treatments and experimental periods

(I) Treatment	(J) Treatment	P-Value
Control	*Micrococcus* alone	NS
	Bacillus alone	0.005
	Micrococcus and *Bacillus*	0.000
Micrococcus alone	Control	NS
	Bacillus alone	0.001
	Micrococcus and *Bacillus*	0.000
Bacillus alone	Control	0.005
	Micrococcus alone	0.001
	Micrococcus and *Bacillus*	NS
Micrococcus and *Bacillus*	Control	0.000
	Micrococcus	0.000
	Bacillus alone	NS
(I) Period	(J) Period	P-Value
0^{th} Day	14^{th} Day	0.010
	28^{th} Day	0.000
14^{th} Day	0^{th} Day	0.010
	28^{th} Day	0.035
28^{th} Day	0^{th} Day	0.000
	14^{th} Day	0.035

The statistical analysis of results using Two-Way ANOVA followed by multiple comparisons using Tukey's method revealed that the *Bacillus* and mixed probiotic cocktail of both *Micrococcus* and *Bacillus* significantly ($P<0.005$) enhances the *O. mossambicus* intestinal total alkaline protease activity. The periods of probiotic administration, 14 days and 28 days, affect

significantly the enzyme activity irrespective of the nature of probiotic administrated.

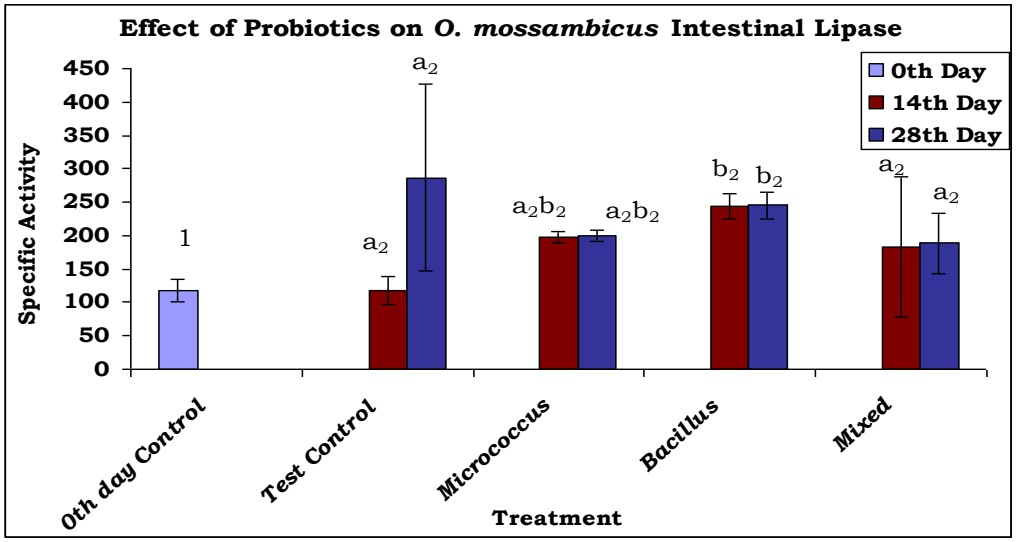

Fig.7.5.4. Effect of probiotics on *O. mossambicus* intestinal Lipase

Table.7.5.7. ANOVA for changes in the activity of *O. mossambicus* intestinal lipase in relation to probiotics and experimental periods. Tests of Between-Subjects Effects

Source of Variation	Sum of Squares	df	Mean Square	F	P-Value
Between Probiotics	31446.888	3	10482.296	2.554	NS
Between Period	70425.920	2	35212.960	8.578	0.001
Probiotics * Period	83613.952	3	27871.317	6.790	0.000
Error	258604.010	63	4104.826		
Total	444090.80	71			

SS- Sum of Squares, df- degrees of freedom, MS- Mean Square, NS-Not significant

The two-way ANOVA shows that the administration of *Bacillus* and mixed culture of both *Bacillus* and *Micrococcus* does not significantly affect the *O. mossambicus* intestinal lipase. However, during prolonged exposure (14 days as well as 28 days) the intestinal lipase activity has been significantly

(P<0.005) enhanced. This may be due to the enhancement of enzyme production by resident intestinal microbial flora through the establishment of probiotic species.

Table.7.5.8. Multiple Comparisons of factors such as probiotic treatments and experimental periods

(I) Treatment	(J) Treatment	P-Value
Control	*Micrococcus* alone	NS
	Bacillus alone	0.006
	Micrococcus and *Bacillus*	NS
Micrococcus alone	Control	NS
	Bacillus alone	NS
	Micrococcus and *Bacillus*	NS
Bacillus alone	Control	0.006
	Micrococcus alone	NS
	Micrococcus and *Bacillus*	NS
Micrococcus and *Bacillus*	Control	NS
	Micrococcus	NS
	Bacillus alone	NS
(I) Period	(J) Period	P-Value
0^{th} Day	14^{th} Day	0.024
	28^{th} Day	0.000
14^{th} Day	0^{th} Day	0.024
	28^{th} Day	0.018
28^{th} Day	0^{th} Day	0.000
	14^{th} Day	0.018

The multiple comparison of different treatments along with the period of probiotic administration revealed that only *Bacillus* species significantly (P<0.005) enhances the *O. mossambicus* intestinal lipase with respect to that of control. The comparison between periods of administration established that there is significant (P<0.005) difference in enzyme activity with that of control.

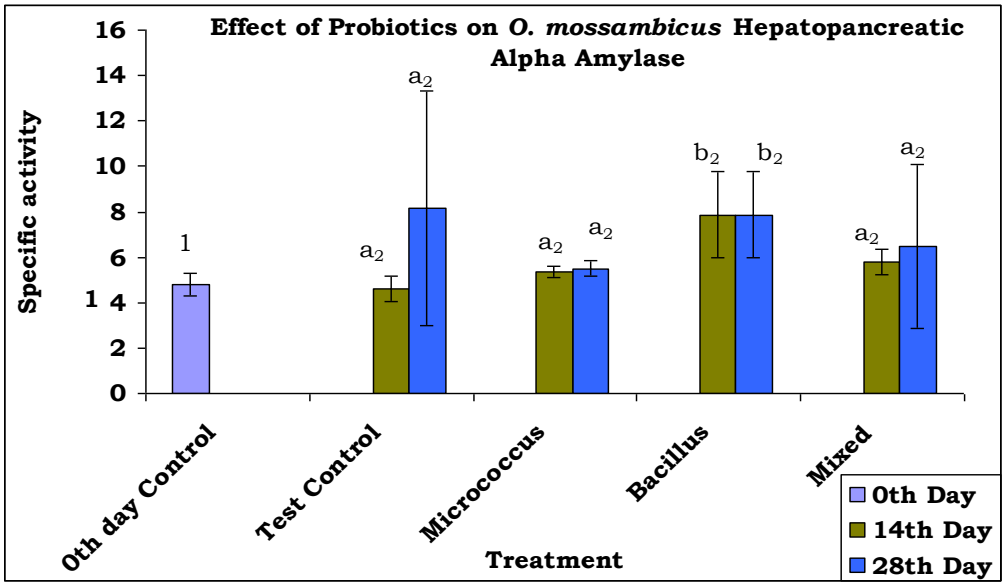

Fig.7.5.5. Effect of probiotics on *O. mossambicus* hepatopancreatic alpha amylase

Table.7.5.9. ANOVA for changes in the activity of *O. mossambicus* hepatopancreatic alpha amylase in relation to probiotics and experimental periods. Tests of Between-Subjects Effects

Source of Variation	Sum of Squares	df	Mean Square	F	P-Value
Between Probiotics	50.921	3	16.974	9.490	0.000
Between Period	36.803	2	18.402	10.289	0.000
Probiotics * Period	33.119	3	11.040	6.172	0.001
Error	112.679	63	1.789		
Total	233.522	71			

SS- Sum of Squares, df- degrees of freedom, MS- Mean Square, NS-Not significant

The two-way ANOVA of the experimental results displayed that both probiotic treatments and days of exposure enhances the *O. mossambicus* hepatopancreatic alpha amylase activity significantly (P<0.005). All the analysis of variance between subjects such as probiotics, periods and both probiotics and days of experiments were showed significant (P<0.005) changes.

Table.7.5.10. Multiple Comparisons of factors such as probiotic treatments and experimental periods

(I) Treatment	(J) Treatment	P-Value
Control	*Micrococcus* alone	NS
	Bacillus alone	0.000
	Micrococcus and *Bacillus*	NS
Micrococcus alone	Control	NS
	Bacillus alone	0.000
	Micrococcus and *Bacillus*	NS
Bacillus alone	Control	0.000
	Micrococcus alone	0.000
	Micrococcus and *Bacillus*	0.003
Micrococcus and *Bacillus*	Control	NS
	Micrococcus	NS
	Bacillus alone	0.003
(I) Period	(J) Period	P-Value
0^{th} Day	14^{th} Day	0.039
	28^{th} Day	0.000
14^{th} Day	0^{th} Day	0.039
	28^{th} Day	0.005
28^{th} Day	0^{th} Day	0.000
	14^{th} Day	0.005

The comparison between factors such as each probiotics and different experimental periods exhibited that among probiotics only *Bacillus* showed significant (P<0.005) difference with control and other probiotic groups, while all the period of administration showed significant (P<0.005) enhancement on *Oreochromis mossambicus* hepatopancreatic alpha amylase activity.

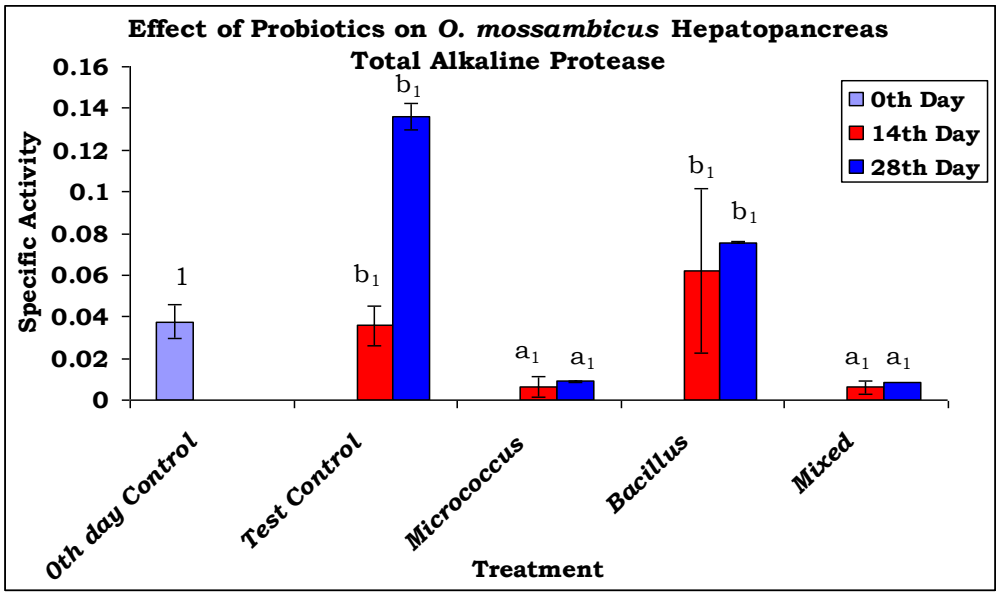

Fig.7.5.6. Effect of probiotics on *O. mossambicus* hepatopancreatic total alkaline protease.

Table.7.5.11. ANOVA for changes in the activity of *O. mossambicus* hepatopancreatic total alkaline protease in relation to probiotics and experimental periods. Tests of Between-Subjects Effects

Source of Variation	SS	df	MS	F	P-Value
Between Probiotics	0.081	3	0.027	9.767	0.000
Between Period	0.026	2	0.013	4.806	0.011
Probiotics * Period	0.027	3	0.009	3.260	0.027
Error	0.173	63	0.003		
Total	0.307	71			

SS- Sum of Squares, df- degrees of freedom, MS- Mean Square, NS-Not significant

The Two Factor ANOVA table indicated that *O. mossambicus* hepatopancreatic total alkaline protease levels varied significantly (P<0.005) between probiotics. Between experimental periods a marked significant difference (P<0.005) was noted. In the case of interaction effects of both the probiotics as well as period of exposure, there is significant difference (P<0.005).

Table.7.5.12. Multiple Comparisons of factors such as probiotic treatments and experimental periods

(I) Treatment	(J) Treatment	P-Value
Control	*Micrococcus* alone	0.003
	Bacillus alone	NS
	Micrococcus and *Bacillus*	0.003
Micrococcus alone	Control	0.003
	Bacillus alone	0.008
	Micrococcus and *Bacillus*	NS
Bacillus alone	Control	NS
	Micrococcus alone	0.008
	Micrococcus and *Bacillus*	0.008
Micrococcus and *Bacillus*	Control	0.003
	Micrococcus alone	NS
	Bacillus alone	0.008
(I) Period	(J) Period	P-Value
0^{th} Day	14^{th} Day	NS
	28^{th} Day	NS
14^{th} Day	0^{th} Day	NS
	28^{th} Day	NS
28^{th} Day	0^{th} Day	NS
	14^{th} Day	NS

The *O. mossambicus* hepatopancreatic total alkaline protease showed significant (P<0.005) difference only with *Micrococcus* administrated group with that of control during analysis using Tukey's multiple comparison after two-factor ANOVA. The multiple comparison tests revealed that *Bacillus* alone as well as the mixed administration of both *Micrococcus* and *Bacillus* together have no significant (P<0.005) difference in *O. mossambicus* hepatopancreatic total alkaline protease activity. The comparison between experimental period displayed that there is no significant (P<0.005) difference between periods of exposure.

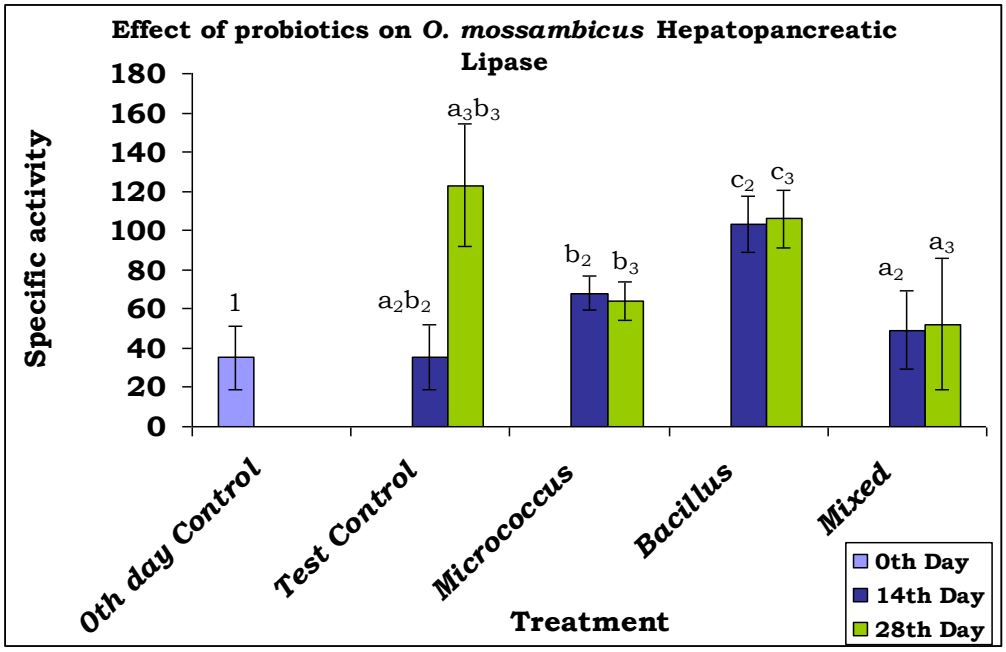

Fig.7.5.7. Effect of probiotics on *O. mossambicus* hepatopancreatic lipase

Table.7.5.13. ANOVA for changes in the activity of *O. mossambicus* hepatopancreatic lipase in relation to probiotics and experimental periods. Tests of Between-Subjects Effects

Source of Variation	SS	df	MS	F	P-Value
Between Probiotics	25093.857	3	8364.619	29.146	0.000
Between Period	18381.091	2	9190.545	32.024	0.000
Probiotics * Period	22859.250	3	7619.750	26.551	0.000
Error	18080.160	63	286.987		
Total	84414.36	71			

SS- Sum of Squares, df- degrees of freedom, MS- Mean Square, NS-Not significant

The statistical analysis for changes in the activity of *O. mossambicus* hepatopancreatic lipase in relation to probiotics and experimental periods by two-way ANOVA found that there are significant ($P<0.005$) differences between the activities among probiotic group. Further analysis based on treatment periods conforms the differences in activities are significant at a confidence limit of $P<0.005$. The analysis between probiotics and periods of the study establishes marked significant ($P<0.005$) difference among the treatment groups.

The Tukey's multiple comparison tests (Table.7.5.14) showed that the activity of *O. mossambicus* hepatopancreatic lipase in *Bacillus* treated group of experiments were significantly ($P<0.005$) different from control and the other treated groups. Subsequent analysis with various periods revealed the differences in *O. mossambicus* hepatopancreatic lipase activities between all the stages of study are significant ($P<0.005$).

Table.7.5.14. Multiple Comparisons of factors such as probiotic treatments and experimental periods

(I) Treatment	(J) Treatment	P-Value
Control	*Micrococcus* alone	NS
	Bacillus alone	0.000
	Micrococcus and *Bacillus*	NS
Micrococcus alone	Control	NS
	Bacillus alone	0.000
	Micrococcus and *Bacillus*	NS
Bacillus alone	Control	0.000
	Micrococcus alone	0.000
	Micrococcus and *Bacillus*	0.000
Micrococcus and *Bacillus*	Control	NS
	Micrococcus	NS
	Bacillus alone	0.000
(I) Period	(J) Period	P-Value
0^{th} Day	14^{th} Day	0.000
	28^{th} Day	0.000
14^{th} Day	0^{th} Day	0.000
	28^{th} Day	0.000
28^{th} Day	0^{th} Day	0.000
	14^{th} Day	0.000

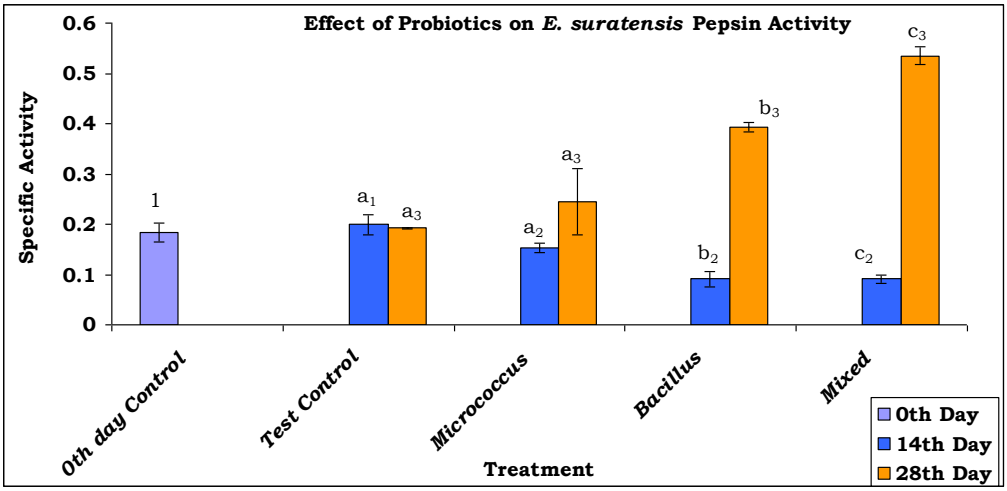

Fig.7.5.8. Effect of probiotics on *E. suratensis* pepsin

Table.7.5.15. ANOVA for changes in the activity of *E. suratensis* pepsin in relation to probiotics and experimental periods. Tests of Between-Subjects Effects

Source of Variation	SS	df	MS	F	P-Value
Between Probiotics	0.153	3	0.051	101.209	0.000
Between Period	0.615	2	0.308	610.038	0.000
Probiotics * Period	0.469	3	0.156	309.651	0.000
Error	0.032	63	0.001		
Total	1.269	71			

SS- Sum of Squares, df- degrees of freedom, MS- Mean Square, NS-Not significant

E. suratensis pepsin showed a significant (P<0.005) difference among probiotic treatments. The two-way ANOVA table also depicts the significant (P<0.005) changes in enzyme activity among the periods of study. There are considerable alterations in enzyme activity between the probiotic treatments and the days of exposure has been noted during the statistical analysis and the results showed that the changes were significant (P<0.005).

Table.7.5.16. Multiple Comparisons of factors such as probiotic treatments and experimental periods

(I) Treatment	(J) Treatment	P-Value
Control	*Micrococcus* alone	NS
	Bacillus alone	0.000
	Micrococcus and *Bacillus*	0.000
Micrococcus alone	Control	NS
	Bacillus alone	0.000
	Micrococcus and *Bacillus*	0.000
Bacillus alone	Control	0.000
	Micrococcus alone	0.000
	Micrococcus and *Bacillus*	0.000
Micrococcus and *Bacillus*	Control	0.000
	Micrococcus	0.000
	Bacillus alone	0.000
(I) Period	(J) Period	P-Value
0^{th} Day	14^{th} Day	0.000
	28^{th} Day	0.000
14^{th} Day	0^{th} Day	0.000
	28^{th} Day	0.000
28^{th} Day	0^{th} Day	0.000
	14^{th} Day	0.000

The post hoc analysis of the experimental results with Tukey's method explains that the changes in *E. suratensis* pepsin activity is significant ($P<0.005$) in the *Bacillus* treated groups with respect to that of control. In addition, significant ($P<0.005$) alterations in enzyme activity are shown by the mixed probiotics treatments with both *Micrococcus* and *Bacillus* strains. However the *Micrococcus* treated groups never exhibited a significant ($P<0.005$) change in their pepsin like acid proteases-mediated hydrolysis of hemoglobin.

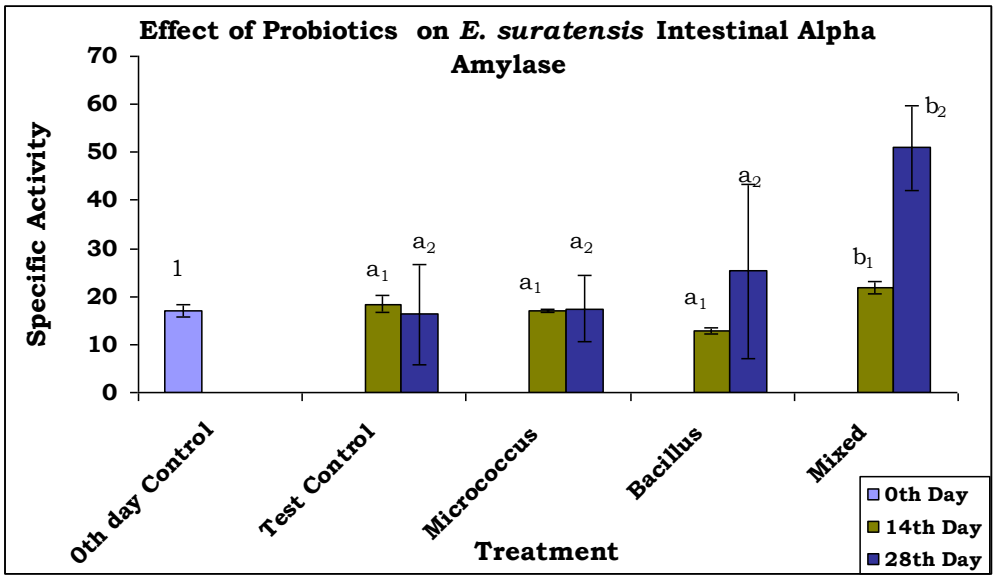

Fig.7.5.9. Effect of probiotics on *E. suratensis* intestinal alpha amylase

Table.7.5.17. ANOVA for changes in the activity of *E. suratensis* intestinal alpha amylase in relation to probiotics and experimental periods. Tests of Between-Subjects Effects

Source of Variation	SS	df	MS	F	P-Value
Between Probiotics	4176.069	3	1392.023	25.630	0.000
Between Period	1580.066	2	790.033	14.546	0.000
Probiotics * Period	2446.467	3	815.489	15.015	0.000
Error	3421.694	63	54.313		
Total	11624.3	71			

SS- Sum of Squares, df- degrees of freedom, MS- Mean Square, NS-Not significant

All the statistical analysis between subjects like probiotic groups, days of exposure and between probiotics and period showed similar results. The two-way ANOVA outcome showed that there are considerable changes in *E. suratensis* intestinal alpha amylase activity among probiotic treated groups and the changes were significant at a confidence level of 95%. The analysis between periods of feeding trials exhibited significant ($P<0.005$) differences.

Table.7.5.18. Multiple Comparisons of factors such as probiotic treatments and experimental periods

(I) Treatment	(J) Treatment	P-Value
Control	*Micrococcus* alone	NS
	Bacillus alone	NS
	Micrococcus and *Bacillus*	0.000
Micrococcus alone	Control	NS
	Bacillus alone	NS
	Micrococcus and *Bacillus*	0.000
Bacillus alone	Control	NS
	Micrococcus alone	NS
	Micrococcus and *Bacillus*	0.000
Micrococcus and *Bacillus*	Control	0.000
	Micrococcus	0.000
	Bacillus alone	0.000
(I) Period	(J) Period	P-Value
0^{th} Day	14^{th} Day	NS
	28^{th} Day	0.002
14^{th} Day	0^{th} Day	NS
	28^{th} Day	0.000
28^{th} Day	0^{th} Day	0.002
	14^{th} Day	0.000

Despite of the results obtained by two-way ANOVA, the multiple comparison analysis showed that there is significant enhancement in intestinal alpha amylase activity of *E. suratensis* only within the group which is treated with both *Micrococcus* and *Bacillus* probiotics. The mixed culture fed group showed significant difference ($P<0.005$) in their alpha amylase activities with other probiotic treatments specifically *Micrococcus* alone and *Bacillus* alone

groups. The comparative study between the days of feeding experiment revealed that the 28 days experimental group only differ significantly ($P<0.005$) in the intestinal alpha amylase activity.

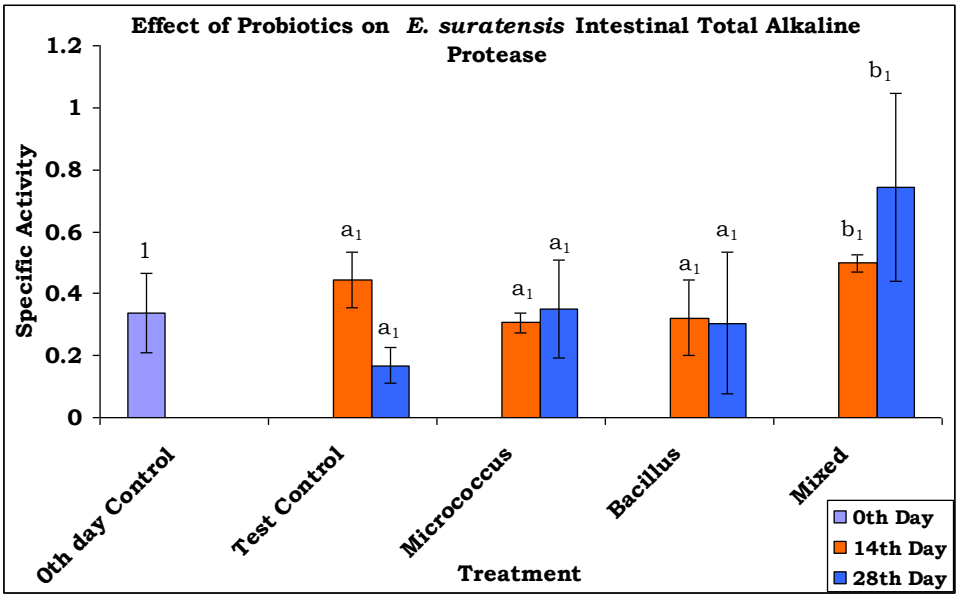

Fig.7.5.10. Effect of probiotics on *E. suratensis* intestinal total alkaline protease

Table.7.5.19. ANOVA for changes in the activity of *E. suratensis* intestinal total alkaline protease in relation to probiotics and experimental periods. Tests of Between-Subjects Effects

Source of Variation	SS	df	MS	F	P-Value
Between Probiotics	1.111	3	0.370	18.239	0.000
Between Period	.005	2	0.003	0.130	NS
Probiotics * Period	.551	3	0.184	9.048	0.000
Error	1.279	63	0.020		
Total	2.946	71			

SS- Sum of Squares, df- degrees of freedom, MS- Mean Square, NS-Not significant

The *E. suratensis* intestinal total alkaline protease activity difference during the probiotic feeding studies were analyzed statistically by using two-

factor ANOVA and the results indicate that differences in activity between different treatment groups and experimental periods. However the total alkaline protease activity does not exhibit significant (P<0.005) difference between days of feeding studies, while a significant (P<0.005) difference has been observed in the treatments with various probiotics. The ANOVA table also showed significant (P<0.005) variation between probiotics and days of feeding trials.

Table.7.5.20. Multiple Comparisons of factors such as probiotic treatments and experimental periods

(I) Treatment	(J) Treatment	P-Value
Control	*Micrococcus* alone	NS
	Bacillus alone	NS
	Micrococcus and *Bacillus*	0.000
Micrococcus alone	Control	NS
	Bacillus alone	NS
	Micrococcus and *Bacillus*	0.000
Bacillus alone	Control	NS
	Micrococcus alone	NS
	Micrococcus and *Bacillus*	0.000
Micrococcus and *Bacillus*	Control	0.000
	Micrococcus	0.000
	Bacillus alone	0.000
(I) Period	(J) Period	P-Value
0^{th} Day	14^{th} Day	NS
	28^{th} Day	NS
14^{th} Day	0^{th} Day	NS
	28^{th} Day	NS
28^{th} Day	0^{th} Day	NS
	14^{th} Day	NS

The multiple comparison table obtained after the post hoc analysis by Tukey's method revealed that the mixed culture with both *Micrococcus* and *Bacillus* species have significant (P<0.005) variations in their total alkaline protease activity with other probiotic treatments such as *Micrococcus* alone and *Bacillus* alone. On the other hand, the multiple comparison analysis between periods of feeding experiments does not show any significant (P<0.005) difference.

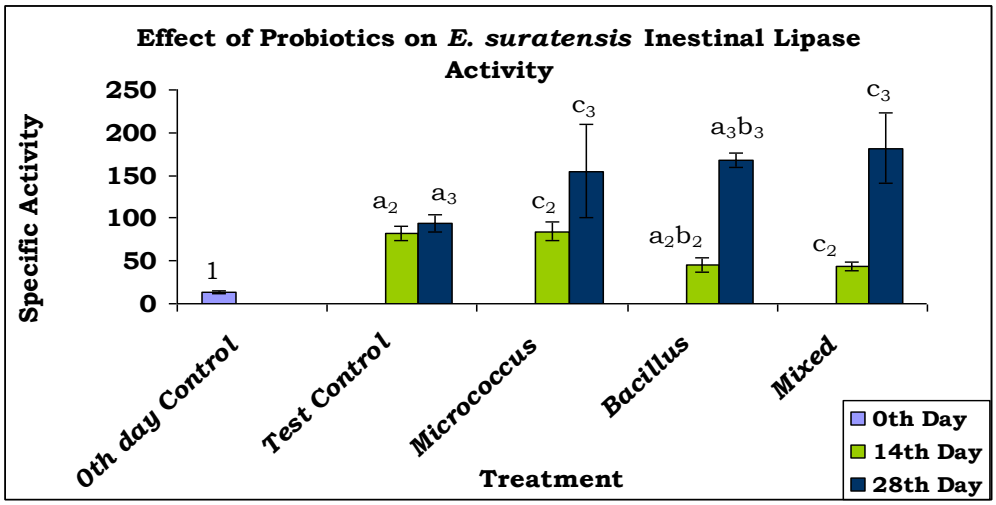

Fig.7.5.11. Effect of probiotics on *E. suratensis* intestinal lipase

Table.7.5.21. ANOVA for changes in the activity of *E. suratensis* intestinal lipase in relation to probiotics and experimental periods. Tests of Between-Subjects Effects

Source of Variation	SS	df	MS	F	P-Value
Between Probiotics	9092.066	3	3030.689	1.239	0.303
Between Period	145769.233	2	72884.616	29.801	0.000
Probiotics * Period	39368.566	3	13122.855	5.366	0.002
Error	154080.902	63	2445.729		
Total	348310.8	71			

SS- Sum of Squares, df- degrees of freedom, MS- Mean Square, NS-Not significant

The two-way analysis of variance (ANOVA) for changes in the activity of *E. suratensis* intestinal lipase in relation to probiotics and experimental periods revealed that the *E. suratensis* intestinal lipase activity differs significantly (P<0.005) with in the days of feeding studies. The analysis between probiotic treatments does not show significant (P<0.005) difference in their intestinal lipase activity. The examination of significance for the extent of variation by including both probiotic treatments and period of study showed that there is significant (P<0.005) difference among their intestinal lipase activity.

Table.7.5.22. Multiple Comparisons of factors such as probiotic treatments and experimental periods

(I) Treatment	(J) Treatment	P-Value
Control	*Micrococcus* alone	0.004
	Bacillus alone	0.043
	Micrococcus and *Bacillus*	0.015
Micrococcus alone	Control	0.004
	Bacillus alone	NS
	Micrococcus and *Bacillus*	NS
Bacillus alone	Control	0.043
	Micrococcus alone	NS
	Micrococcus and *Bacillus*	NS
Micrococcus and *Bacillus*	Control	0.015
	Micrococcus	NS
	Bacillus alone	NS
(I) Period	(J) Period	P-Value
0^{th} Day	14^{th} Day	0.035
	28^{th} Day	0.000
14^{th} Day	0^{th} Day	0.035
	28^{th} Day	0.000
28^{th} Day	0^{th} Day	0.000
	14^{th} Day	0.000

To understand the significance of inter specific variations in the *E. suratensis* intestinal lipase activity between each experiments, a post hoc multiple comparison analysis by Tukey's method was done. The results revealed that treatments like *Micrococcus* alone, *Bacillus* alone and mixed culture of both *Micrococcus* and *Bacillus* showed significant ($P<0.005$) difference with control in their intestinal lipase activity. The multiple comparisons between days of feeding experiments exhibited a significant ($P<0.005$) variation between the data on the day on which the experiment started (0^{th} day), 14 days and 28 days.

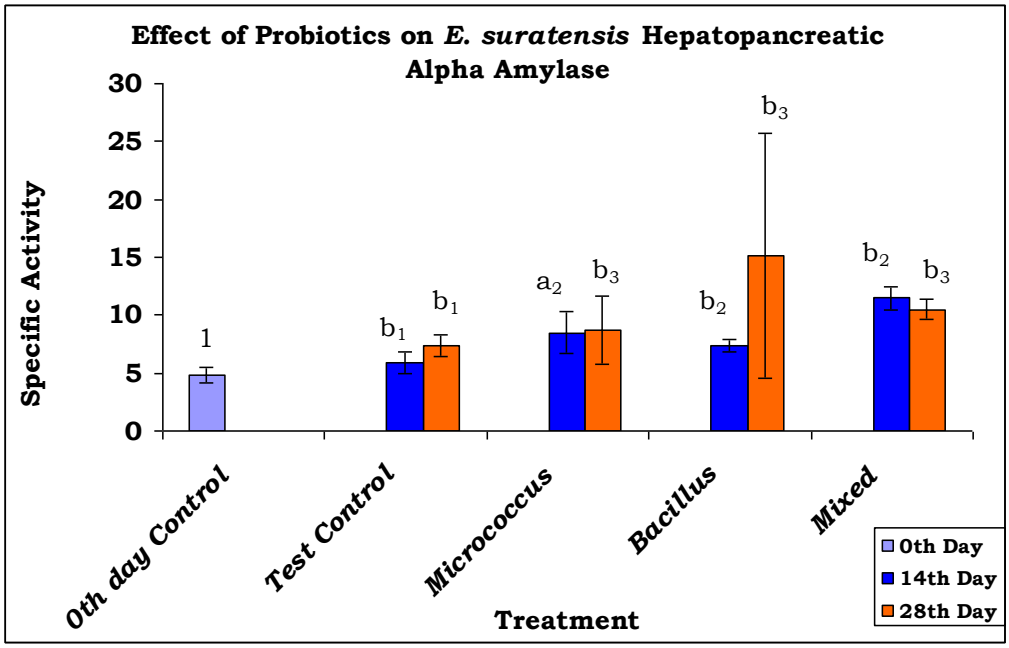

Fig.7.5.12. Effect of probiotics on *E. suratensis* hepatopancreatic alpha amylase

Table.7.5.23. ANOVA for changes in the activity of *E. suratensis* hepatopancreaticl alpha amylase in relation to probiotics and experimental periods. Tests of Between-Subjects Effects

Source of Variation	SS	df	MS	F	P-Value
Between Probiotics	229.184	3	76.395	6.257	0.001
Between Period	89.604	2	44.802	3.670	0.031
Probiotics * Period	183.269	3	61.090	5.004	0.004
Error	769.153	63	12.209		
Total	1271.21	71			

SS- Sum of Squares, df- degrees of freedom, MS- Mean Square, NS-Not significant

The results obtained after the feeding experiments with probiotics, the *E. suratensis* hepatopancreatic alpha amylase specific activities, have been subjected to two-way ANOVA analysis. The ANOVA table revealed that there is significant difference in enzyme activities amidst various probiotic treatments. Similarly the analysis of variation between the days of feeding trials displayed significant differences in their amylase activity. The variations between probiotic treatments and days of feeding trials too exhibited different alpha amylase activity significantly.

The multiple comparison tests by using Tukey's post hoc analysis (Table.7.5.24) unravelled that there is significant ($P<0.005$) difference in *E. suratensis* hepatopancreatic alpha amylase between the *Bacillus* alone and mixed culture treatments with both *Micrococcus* and *Bacillus* species. However, administration of '*Micrococcus* alone' does not show any significant ($P<0.005$) difference in amylase activity with that of control group. The comparison within the periods revealed that all of them (0^{th} day, 14^{th} day and 28^{th} day) exhibit significant ($P<0.005$) variations.

Table.7.5.24. Multiple Comparisons of factors such as probiotic treatments and experimental periods

(I) Treatment	(J) Treatment	P-Value
Control	*Micrococcus* alone	0.116
	Bacillus alone	0.000
	Micrococcus and *Bacillus*	0.000
Micrococcus alone	Control	0.116
	Bacillus alone	0.139
	Micrococcus and *Bacillus*	0.219
Bacillus alone	Control	0.000
	Micrococcus alone	0.139
	Micrococcus and *Bacillus*	0.995
Micrococcus and *Bacillus*	Control	0.000
	Micrococcus alone	0.219
	Bacillus alone	0.995
(I) Period	(J) Period	P-Value
0^{th} Day	14^{th} Day	0.034
	28^{th} Day	0.000
14^{th} Day	0^{th} Day	0.034
	28^{th} Day	0.048
28^{th} Day	0^{th} Day	0.000
	14^{th} Day	0.048

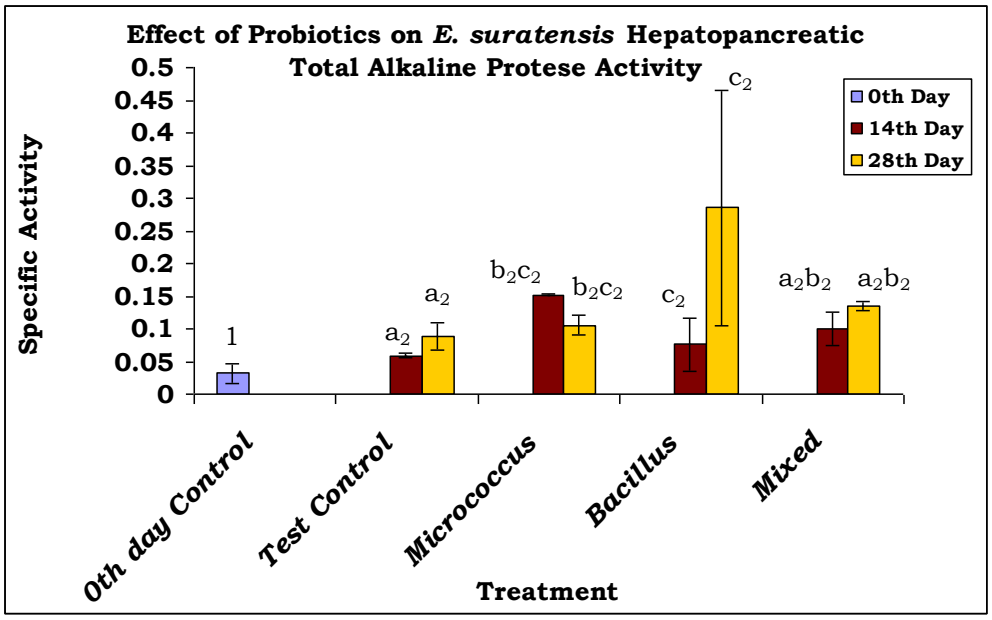

Fig.7.5.13. Effect of probiotics on *E. suratensis* hepatopancreatic total alkaline protease

Table.7.5.25. ANOVA for changes in the activity of *E. suratensis* hepatopancreatic total alkaline protease in relation to probiotics and experimental periods. Tests of Between-Subjects Effects

Source of Variation	SS	df	MS	F	P-Value
Between Probiotics	0.093	3	0.031	6.413	0.001
Between Period	0.060	2	0.030	6.219	0.003
Probiotics * Period	0.140	3	0.047	9.630	0.000
Error	0.306	63	0.005		
Total	0.599	71			

SS- Sum of Squares, df- degrees of freedom, MS- Mean Square, NS-Not significant

Total alkaline protease specific activity has been subjected to two-way ANOVA and the results showed significant ($P<0.005$) variations among probiotic treated groups. The table also indicates that the variations of *E. suratensis* hepatopancreatic total alkaline protease activity within the days of

feeding trials are significant (P<0.005). Variations in enzyme activity between probiotic treatment groups and period of study exhibited prominence at 95% confidence level.

Table.7.5.26. Multiple Comparisons of factors such as probiotic treatments and experimental periods

(I) Treatment	(J) Treatment	P-Value
Control	*Micrococcus* alone	0.016
	Bacillus alone	0.000
	Micrococcus and *Bacillus*	NS
Micrococcus alone	Control	0.016
	Bacillus alone	NS
	Micrococcus and *Bacillus*	NS
Bacillus alone	Control	0.000
	Micrococcus alone	NS
	Micrococcus and *Bacillus*	NS
Micrococcus and *Bacillus*	Control	NS
	Micrococcus	NS
	Bacillus alone	NS
(I) Period	(J) Period	P-Value
0^{th} Day	14^{th} Day	NS
	28^{th} Day	0.000
14^{th} Day	0^{th} Day	NS
	28^{th} Day	0.005
28^{th} Day	0^{th} Day	0.000
	14^{th} Day	0.005

The '*Micrococcus* alone' and '*Bacillus* alone' groups showed significant (P<0.005) difference when analyzed by Tukey's multiple comparison method. The mixed culture group with both *Micrococcus* and

Bacillus species does not exhibit any significant difference (P<0.005) with the hepatopancreatic total alkaline protease of control groups. The multiple comparisons with days of exposure studies revealed that the significant (P<0.005) difference only in the 28 days' experiment group. The 14 days' experiments do not show any significant (P<0.005) difference in the *E. suratensis* hepatopancreatic total alkaline protease activity with that of control.

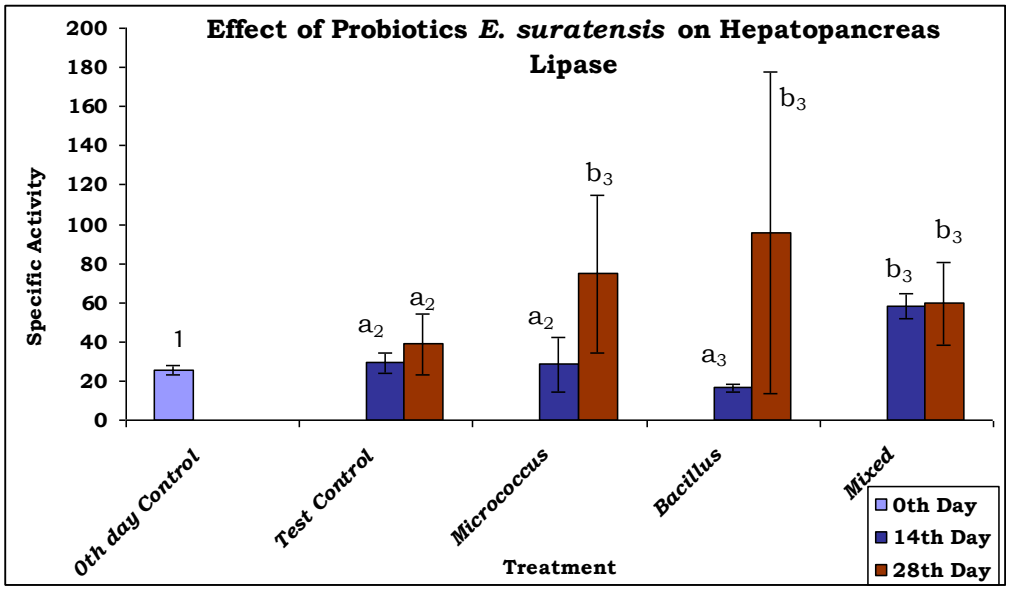

Fig.7.5.14. Effect of probiotics on *E. suratensis* hepatopancreatic lipase

Table.7.5.27. ANOVA for changes in the activity of *E. suratensis* hepatopancreatic lipase in relation to probiotics and experimental periods. Tests of Between-Subjects Effects

Source of Variation	SS	df	MS	F	P-Value
Between Probiotics	5948.744	3	1982.915	2.230	0.093
Between Period	19099.576	2	9549.788	10.739	0.000
Probiotics * Period	15532.561	3	5177.520	5.822	0.001
Error	56021.891	63	889.236		
Total	96602.772	71			

SS- Sum of Squares, df- degrees of freedom, MS- Mean Square, NS-Not significant

The two-way ANOVA of *E. suratensis* hepatopancreatic lipase results showed a considerable variation among the days of feeding experiments and it is significant at a confidence level of 95%. However, the analysis of variance does not show any significant (P<0.005) difference amidst probiotic groups. Nevertheless significant (P<0.005) variations in enzyme activity have been observed while considering both probiotic treatments and period of administration together.

Table.7.5.28. Multiple Comparisons of factors such as probiotic treatments and experimental periods

(I) Treatment	(J) Treatment	P-Value
Control	*Micrococcus* alone	NS
	Bacillus alone	NS
	Micrococcus and *Bacillus*	0.029
Micrococcus alone	Control	NS
	Bacillus alone	NS
	Micrococcus and *Bacillus*	NS
Bacillus alone	Control	NS
	Micrococcus alone	NS
	Micrococcus and *Bacillus*	NS
Micrococcus and *Bacillus*	Control	0.029
	Micrococcus	NS
	Bacillus alone	NS
(I) Period	(J) Period	P-Value
0^{th} Day	14^{th} Day	NS
	28^{th} Day	0.002
14^{th} Day	0^{th} Day	NS
	28^{th} Day	0.000
28^{th} Day	0^{th} Day	0.002
	14^{th} Day	0.000

The only group which is able to produce significant (P<0.005) difference in *E. suratensis* hepatopancreatic lipase activity is the mixed culture treatment with both *Micrococcus* and *Bacillus* species during the multiple comparison analysis by Tukey's post hoc method. The individually administrated groups of *Micrococcus* alone and *Bacillus* alone do not possess significant (P<0.005) differences in enzyme activity with that of control group. While considering the difference among the days of feeding, only 28 days trials gave significant (P<0.005) results. The 14 days feeding groups do not differ in enzyme activity with that of control group.

Table.7.5.29. Growth performance of *Etroplus suratensis*

	Initial Weight (g)	14th day Weight (g)	28th day Weight (g)	FCR 14th day	FCR 28th day	SGR 14th day (% day^{-1})	SGR 28th day (% day^{-1})	Survival rate (%)	
								14th day	28th day
Control	41.33±0.61	45.41±0.64	48.94±1.19	7.09±0.056	7.63±0.48	0.0067±4.99E-05	0.00603±0.00035	100	77.778
Micrococcus	94.23±0.62	104.47±1.54	113.51±1.40	6.47±0.54	6.85±0.23	0.0074±0.00058	0.00665±0.00021	91.667	83.333
Bacillus	89.72±1.01	97.11±1.48	110.24±2.02	8.51±0.44	6.13±0.23	0.0057±0.00028	0.00736±0.00025	100	90.909
Micrococcus and *Bacillus*	88.76±0.19	97.07±0.35	110.44±1.87	7.48±0.12	5.75±0.43	0.0064±0.000101	0.00780±0.00053	100	75.00

Table.7.5.30. Growth performance of *Oreochromis mossambicus*

	Initial Weight (g)	14th day Weight (g)	28th day Weight (g)	FCR 14th day	FCR 28th day	SGR 14th day (% day^{-1})	SGR 28th day (% day^{-1})	Survival rate (%)	
								14th day	28th day
Control	43.67±0.44	48.68±0.51	54.185±1.65	6.10±0.025	5.85±0.61	0.008±2.99E-05	0.008±0.00073	100	100
Micrococcus	84.26±0.60	96.13±0.92	100.72±1.08	4.98±0.098	7.22±0.79	0.009±0.0002	0.006±0.00064	100	100
Bacillus	77.76±0.22	87.99±1.26	94.27±0.67	5.35±0.528	6.60±0.37	0.009±0.0008	0.007±0.00035	100	98
Micrococcus and *Bacillus*	74.24±0.79	84.14±0.14	91.71±1.79	5.27±0.557	6.02±0.96	0.009±0.0009	0.008±0.00108	100	96

7.6. Discussion

Scientific developments in recent years have opened new frontiers and enabled a better understanding of the gastrointestinal tract (GIT) as a complex and delicately balanced ecosystem and there is general agreement on the important role of the gastro-intestinal microflora in the health status of men and animals (Holzapfel *et al*., 1998). There are several reports available about the influence of probiotics on digestive enzyme activity in fish (Tovar *et al*. 2002; Tovar-Ramirez *et al*., 2004; El-Haroun *et al*., 2006; Wache *et al*., 2006; Ghosh *et al*., 2008; Suzer *et al*., 2008; Saenz de Rodriganez *et al*., 2009). During the early stages of development, manipulation of the larval digestive system seems possible through the addition of probiotics either through the culture water or as live food (Vine *et al*., 2005). In aquatic animals, the digestive tract and the surrounding water are important. In natural populations of aquatic animals, the micro flora of the gut might reflect that of the aquatic environment. In this scenario a number of preparations of probiotics are commercially available and have been introduced to fish, shrimp and molluscan farming as feed additives, or are incorporated in pond water (Wang *et al*., 2005). The deterioration in normal gut micro flora may be the result of aquatic pollution by antibiotics and other chemical substances. In aquaculture practices to date, probiotics are considered valid alternatives to antibiotics and in particular, in fish larviculture, to prevent high mortality (Talpur *et al*., 2012). The efficiency of feed utilization in fish is influenced by digestive enzymes. The characterization of these enzymes provides some information regarding the digestive capacity of fish to hydrolyze carbohydrate, protein and lipid ingredients of feed (Lemieux *et al*., 1999). However, information regarding the extracellular enzymes produced by intestinal bacteria and their biochemical significance is limited (Bairagi *et al*., 2002).

In the present study, even though treatment with different probiotic species affect the activities of various digestive enzymes in the two different fishes *Etroplus suratensis* and *Oreochromis mossambicus* differently, we obtained significant enhancement in the specific activity of digestive enzymes. For our study, two microbial species were selected *Micrococcus MCCB 104* and *Bacillus MCCB 101*. Among them, *Micrococcus* (gram positive cocci) occur in a wide range of environments, including water, dust, and soil. The genus *Bacillus* is present as gram positive rods that form a single endospore (spore) and represent a peculiar case among the bacteria used as probiotics. *Bacillus* spp., includes *B. subtilis, B. cereus, B. coagulans, B. clausii, B. megaterium* and *B. licheniformis*, are reported to be used as probiotics (Oggioni *et al*., 2003). Many researchers suggested that different probiotics act differently to enhance the growth and nutrient utilization of various fish species (Noh *et al*., 1994; Bogut *et al*., 1998; Mohapatra *et al*., 2012). We also obtained similar observations in our study. Very little probiotic research has been conducted on the effect of probiotics on tilapia, but of the research that has been performed, in the last few years (Welker and Lim, 2011). We found that in *Oreochromis mossambicus*, the pepsin as well as intestinal digestive enzymes such as amylase, total alkaline protease, lipase and their ehepatopancreatic counter parts showed a significant elevation in their specific activity. Jafaryan *et al* (2008) also reported that the probiotic *Bacillus* supplemented diet significantly increased the weight, length and SGR of fish when compared to the control diet without probiotic supplementation. Amylase, lipase, and protease production was enhanced in tilapia fed on diets containing *Bacillus subtilis* and an unidentified "photosynthetic bacteria" (Honsheng, 2010). However, mixed diet with both *Bacillus* and *Micrococcus* too showed an increase in specific activities of intestinal alpha amylase and

total alkaline proteases of *O. mossambicus* on both 14 days and 28 days feeding experiments **(Fig.7.5.1 to Fig.7.5.7)**. The hepatopancreatic enzymes were highly influenced by the *Bacillus* species in *O. mossambicus*. In most cases, the mechanism for improved growth performance is not known or reported. An enhanced activity of lipolytic enzymes has been found in *Bacillus* fed groups. Majority of probiotics are capable of secreting lipase, which triggers production and assimilation of essential fatty acids resulting in higher growth and immunity in fish. Feed supplementation of essential fatty acid not only boosts the immunity but also triggers the growth (Sharma *et al*., 2009). The present data also confirm this hypothesis. The data clearly indicate that addition of probiotics significantly increases lipase activity irrespective of fish species or combination of probiotics administrated, which corroborates the findings of Yanbo and Zirong (2006). It is difficult to draw concrete conclusions and provide specific recommendations on the effects of dietary probiotics on growth performance of tilapia given that the studies vary widely with regard to fish age and size, stocking density, diet composition, dietary probiont concentration, feed allowances, feeding duration, and of course, type and source of probiont. However, early studies suggest that dietary probiotic supplementation may have beneficial effects when used as growth promoters in tilapia. The microorganisms and their exo-enzymes have a significant role in the digestion process (Munilla-Moran *et al*., 1990) by increasing the total enzyme activity of the gut (Ding *et al*., 2004, Ziaei-Nejad et *al*., 2006) and stimulating the production of endogenous enzymes (Ochoa-Salano and Olmos-Soto, 2006; Wang, 2007) which in turn can increase the food digestibility and thereby, the nutrient utilization. The exogenous enzymes have a broader pH range than endogenous enzymes that prolongs the digestion period and may allow better hydrolysis of substrates. As pointed out by several authors the

activities of digestive enzymes (amylase, protease and lipase) could be improved by administration of probiotics to the diet (Ziaei-Nejad *et al*., 2006; Taoka *et al*., 2007; Wang, 2007; Gomez *et al*., 2008).

Etroplus suratensis, on other hand, is a less studied promising aquaculture species. The probiotic induced survival, growth, immunity and in the alterations of digestive enzyme activities need extensive study. The present study showed that the intestinal digestive hydrolases of *Etroplus suratensis* were profoundly enhanced mainly by *Bacillus* and mixed cultures of both *Bacillus* and *Micrococcus* species **(Fig. 7.5.8 to Fig.7.5.14)**. It is reported that the digestive organs are very sensitive to food composition and cause immediate changes in the activities of the digestive enzymes (Bolasina *et al*., 2006; Shan *et al*., 2008), which is finally reflected in fish health and growth. The hepatopancreatic versions of major digestive enzymes in *E. suratensis* were influenced variously by different probiotic treatments. Amylase and lipase are the major enzymes related to carbohydrate and fat digestion, respectively. From our results, the alpha amylase activity of hepatopancreas has been elevated significantly by mixed culture during 14 days' experiments while on 28 days' trials both *Bacillus* and mixed culture showed an enhancing effect even though there is only slight difference among their specific activity values **(Fig.7.5.12)**. The intestinal lipase enzyme activity has been significantly enhanced by *Micrococcus* during 14 days, feeding trials and by *Micrococcus* alone, *Bacillus* alone as well as *Micrococcus* and *Bacillus* together. The total alkaline proteases also showed variations in the results during probiotic administration. In 14 days' feeding experiments, the group treated with '*Micrococcus* alone' showed an increases in total alkaline protease activity, while during 28 days' trials, the groups treated with '*Bacillus* alone' dominated in enhancement of total alkaline protease activity

(Fig.7.5.13). This change may be due to the replacement of microbial flora by novel strains in the intestine and more research has to be done to specify whether *Bacillus* species is more effective as probiotic than the other. The enzyme lipase has been effectively influenced by mixed culture of both *Micrococcus* and *Bacillus* during all the experimental trials when compared to the control. The groups treated with '*Micrococcus* alone' and '*Bacillus* alone' did not possess any significant difference in their hepatopancreatic lipase activity during the statistical analysis on the effect of probiotics on *E. suratensis* **(Fig.7.5.14)**. It suggests that the higher protease and lipase activities obtained in the probiotic-supplemented fish are mainly the outcome of stimulation by probiotic itself or exogenous enzyme produced by the probiotics. This might have improved the nutrient digestibility leading to better growth performance and feed conversion efficiency in fish. Similar observations have also been reported for other fishes in which the nutrient digestibility increased considerably with the use of probiotic-supplemented diet (Lara-Flores *et al*., 2003; Yanbo and Zirong, 2006). Probiotics and prebiotics offer potential alternatives by providing benefits to the host primarily via the direct or indirect modulation of the gut microbiota. Suggested modes of action resulting from increased favorable bacteria (e.g. lactic acid bacteria and certain *Bacillus* spp.) in the gastrointestinal (GI) tract include the production of antagonistic molecules, competition with potential pathogens, inhibition of virulence gene expression, enhancing the immune response, improved gastric morphology and aids digestive function. The application of probiotics and prebiotics may therefore result in elevated health status, improved disease resistance, growth performance and body composition, reduced malformations and improved gut morphology and

microbial balance. Merrifield *et al* (2010) demonstrated successful proof of these concepts and a foundation for applications in salmonid aquaculture.

The FCR of *Etroplus suratensis* has been increased by *Bacillus* alone as well as the mixed culture of both *Micrococcus* and *Bacillus* species compared to the control group during 14 days' feeding experiments **(Table.7.5.29)**. However the SGR of *E. suratensis* in '*Bacillus* alone 'and mixed culture groups are slightly lower than that of control during 14 days' trial. SGR of both the treatments has been increased than that of the control during 28 days' and this is because of the fact that the utilized nutrients have to be converted to proteins and other metabolites and this will need specific duration of time. The SGR of all treated groups is higher than that of control in 28 days' treatment. Many researchers have reported that the probiotic supplemented diets and control basal diet revealed the same results in growth parameters, that is there is no significant differences in FCR, SGR and other growth indices like gastro somatic index (GSI) (Diab *et al*., 2002; Ahmadvand *et al*., 2012). Significant differences for specific growth rate (SGR) and survival were recorded in shrimp, *Litopenaeus vannamei*, fed on probiotic diet as compared with the control; however, no significant differences were recorded for food conversion ratio (FCR) among all the experimental groups for a period of eight weeks (Zokaeifar *et al*., 2012). Apart from altering the growth rate some probiotics affects the viability against high temperature and high salinity stress (Ahmadvand *et al*., 2012). Numerous microbes have been identified as probiotics for aquaculture programs, many of which differ markedly in their mode of action. There are, however, some common mechanisms of action that have been reported for the majority of probiotic strains. Probiotics help in feed conversion efficiency and live weight gain (Al-Dohail *et al*., 2009; Saenz de Rodriguez *et al*., 2009) and confer protection against pathogens by inhibiting

pathogen adhesion to intestinal mucosa through competitive exclusion for adhesion sites (Buffie and Pamer, 2013; Collado *et al*., 2006), production of organic acids (formic acid, acetic acid, lactic acid), hydrogen peroxide and several other compounds such as antibiotics, bacteriocins, siderophores, lysozyme (Yan *et al*., 2002; El-Dakar *et al*., 2007) and also modulate physiological and immunological responses in fish (Khattab *et al*., 2004; Balcazar *et al*., 2006). Some probiotics have been reported to improved water quality (Dalmin *et al*., 2001). Identifying the specific effect(s) produced by probionts that benefit fish can prove difficult. However, such benefits have been documented in many fish species, including tilapia (Welker and Lim, 2011). Nile tilapia fed on the probiotic diet of *L. rhamnosus* had greater villous height in the proximal and middle sections of the intestine (Pirarat *et al*., 2011). In the present study all the probiotic treatments on *O. mossambicus* exhibited a lower FCR than that of control during 14 days' feeding experiments and also displayed a 100% survival throughout the experimental period. But on 28 days' of experimental period all the probiotic treatments presented an increased FCR among which *Micrococcus* showed highest value. However the SGR corresponding 14 days' studies of all probiotic treatments exhibited an increase when compared to that of control. During 28 days' feeding trials, the mixed culture administrated group is the only one which exhibited SGR similar to that of control group. Other groups, '*Micrococcus* alone' and '*Bacillus* alone', showed a decrease in SGR **(Table.7.5.30)**. Noh *et al* (1994) studied the effect of supplementing yeast (*S. cerevisiae*) and bacteria (*Streptococcus faecium*) in the diet of carp and reported the better growth response of fish fed on probiotic-supplemented diets than that of control. But they found better growth and nutrient utilization in a bacterium-supplemented diet than those fed on yeast. Essa *et al* (2010) and EL-Haroun *et al* (2006)

reported similar results during feeding trials with probiotics in Nile tilapia (*O. niloticus).*

The probiotic strains, *Micrococcus MCCB 104* and *Bacillus MCCB 101* are resistant to each other and combination of these microbes were used for various research applications (Pai *et al*., 2010 and Antony *et al*., 2011). A commercial probiotic mixture of *Micrococcus* and *Bacillus sp.* is available in the market under the brand name "Enterotrophotic" by the National Center for Aquatic Animal Health (NCAAH), CUSAT, Kerala, India.

Summarising the results of the present study, the live probiotic microorganisms may be incorporated while formulating the cost-effective nutritionally balanced diet of *E. suratensis* and *O. mossambicus* for better growth performance and nutrient utilization. The observations of the present study suggest that more research is needed in *E. suratensis, O. mossambicus* and other species to determine the mechanisms by which probiotic microorganisms affect gastrointestinal morphology and digestive physiology.

Chapter 8

SUMMARY AND CONCLUSIONS

The metabolism in animals relies on the key digestive function and thereby the availability of nutrients essential for all biological processes. Taken as a whole, the effectiveness of the digestive process significantly depends on the type and function of the digestive enzymes. Thus an investigation on the digestive physiology and biochemical characteristics of specific digestive enzymes is very important. In addition, the study of digestive enzymes is a key tool while investigating the nutritional condition and adaptation of the organism to dietary change. Therefore, information on digestive enzymes can provide elementary knowledge to help understand the nutritional status of an aquatic animal. In the present study, the results of the second chapter show that the broad optima of pH and temperature for maximum pepsin activity in *E. suratensis* would help the fish to digest different proteinaceous constituents in various feeds in comparison with *O. mossambicus*. Similarity in temperature stability of pepsins from both the fishes is observed in this study. The low susceptibility of *O. mossambicus* to the effect of many of the metal ions may help this fish to attain a better digestibility in brackish waters where the metal ion concentrations are much higher in comparison with freshwater habitats.

O. mossambicus alpha-amylase is very active than *E. suratensis* alpha-amylase since the optimum temperature and pH for maximum enzyme activity are comparative to the average ambient conditions of the natural habitat. The present study reveals that the *O. mossambicus* alpha amylase has characteristics like restoration of protein confirmation thereby stability at low

pH values and at higher temperatures and it can be utilised to develop these enzymes as industrial catalysts.

Medical plants have an important role in the management of diabetes mellitus especially in the developing countries where resources are meager. The kinetic studies on the inhibitory property of *Sonneratia caseolaris* fruit extracts in comparison with other plant extracts like *Cerbera odollam and Murraya koenigii* has proved that the purification and clinical trials would help to develop novel anti-diabetic drugs from natural sources. *C. odollam* extracts also showed alpha amylase inhibitory effect at a lesser extent. The concentration of specific inhibitory molecules in the *C. odollam* fruit extracts could be the cause of decreased inhibitory activity. Its inhibitory property can be improved by further purification and the molecules can be identified and characterised for better applications. The mechanism of inhibition by aqueous extracts of *S. caseolaris* fruits is found to be a non competitive-un competitive mixed type and is different from the classical reversible inhibition kinetics.

The present study revealed that the intestinal total alkaline protease of both *E. suratensis* and *O. mossambicus* slightly differ in their pH and temperature optima and exhibit similar extent of specific activities. The low specific activity of hepatopancreatic total alkaline protease in *Etroplus suratensis* indicates that the fish has less ability to hydrolyse proteins in comparison with *O. mossambicus*. The high pH and temperature optima and low susceptibility of crude total alkaline protease in *O. mossambicus* to metal ions might be helpful in effective hydrolysis of protein fragments in the chyme.

Lipids are energy rich biomolecules and the higher ability to utilize lipid components in the diet will help fishes to adapt, survive and establish in

their respective habitats. *O. mossambicus* lipase has higher temperature and pH optima than that of *E. suratensis*. The lipase from *O. mossambicus* digestive organs is more stable than *E. suratensis* at extreme pH and temperatures.

In the present study, the major digestive enzyme activities and digestive indices were compared between *E. suratensis* and *O. mossambicus*. Pepsin-like acid proteases that act on low pH has been identified all along the digestive tract of both the fishes. Comparatively low alpha amylase activity is shown by the *E. suratensis* and the enzyme is distributed almost equally throughout the intestinal segments in both the species. Very low alkaline protease activity is found in the stomach of both the fishes and in *O. mossambicus,* the enzyme activity diminishes extensively towards the posterior portion of the intestine whereas in *E. suratensis* the activity increases towards the posterior part. The present study showed that lipase is one of the prominent digestive enzymes in *O. mossambicus* with a remarkable specific activity throughout the digestive tract than that of *E. suratensis*. It is noted that *O. mossambicus* has a higher values for digestive somatic index, hepato somatic index, intestinal coefficient and gut *Vs* standard length ratio in comparison with *E. suratensis* and indicates its higher digestive and metabolic capabilities. The early maturity and fast growth of *O. mossambicus* can be explained by their enhanced digestive indices. The comparatively low activities of acid protease, amylase, lipase and total alkaline protease of *E. suratensis* revealed its poor digestive capacity compared to *O. mossambicus*.

In aquaculture, exposure to stressful conditions and problems related to diseases and deterioration of environmental conditions often occur and result in serious economic losses. The use of probiotics or beneficial bacteria, which control pathogens through different mechanisms, aiding digestion by

exoenzyme supply and through establishment of beneficial microflora in the digestive tract has got increased attention recently. The results of the present study suggest that live probiotic microorganisms like *Micrococcus MCCB 104* and *Bacillus MCCB 101* may be incorporated while formulating the cost-effective, nutritionally balanced diet of *E. suratensis* and *O. mossambicus* for better growth performance and nutrient utilization. It is necessary to conduct more research in *E. suratensis*, *O. mossambicus* and other species to determine the mechanisms by which probiotic micro organisms affect gastrointestinal morphology and digestive physiology.

In conclusion, the present research on the characteristics of major digestive enzymes revealed that the digestive capacity of *O. mossambicus* is comparatively better than that of *E. suratensis*. This ability in digestion will help *O. mossambicus* in adaptation with different habitats with varying food availability and the maximum utilization of nutrients for their growth and development.

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