CHAPTER - 1

II. ISOLATION, SCREENING AND IDENTIFICATION OF PROTEOLYTIC BACTERIAL STRAINS

2.1. INTRODUCTION

Proteases are the essential constituents of all forms of life on earth including prokaryotes, fungi, plants and animals. It is the major class of enzymes, also known as peptidyl-peptide hydrolases and it is an industrially useful enzyme, which catalyze the hydrolysis of peptide bond from protein molecule. Proteases are also a highly complex group of enzymes that vary in their physio-chemical and catalytic properties. Proteases constitute 50 - 65% of the global industrial enzyme market, most of which are alkaline protease (Banerjee et al., 1999). Microbial proteases constitute about 40% of the total worldwide production of enzymes (Godfrey and West, 1996). The wide applications of this enzyme reported that in the year 2005, the global proteolytic enzyme demand increased dramatically to 1.0-1.2 billion dollars (Godfrey et al., 1996). Their vast diversity and specific range of action have attracted the attention of biotechnologists' world wide. With the advent of new frontiers in biotechnology, the spectrum of protease application has expanded into many new fields, such as clinical, medicinal and analytical...
chemistry. Increasing demand of protease had lead biotechnologists to explore novel sources of proteases (Joo and Chang, 2005).

Microorganisms represent the most common candidates as sources of new enzymes because of their broad biochemical diversity, feasibility of mass culture and alleviation of genetic manipulation. Microorganisms are the preferred sources of these enzymes in fermentation bioprocesses because of their fast growth rate and also that they can be genetically engineered to generate new enzyme with desirable abilities (Rao et al., 1998). Marine microorganisms represent the potential source for exploring novel enzymes. Due to their unique natural habitat, these microorganisms show distinct physiological characteristics, metabolic patterns and nutrient utilization. Therefore the marine microorganisms produce a variety of enzymes and it can be expected to possess unique properties (Jackson and Young, 2001; Marrs et al., 1999). In general, microbial proteases are extracellular in nature and are directly secreted into the fermentation broth by the producer, thus simplifying downstream processing of the enzyme as compared to proteases obtained from plants and animals. Many of microbes belong to bacteria, fungi, yeast and actinomycetes are known to produce alkaline protease of the serine type (Kumar and Takagi, 1999). Extracellular proteases are important for the hydrolysis of external proteins and enable the cell to absorb and use hydrolytic products (Kalisz, 1998).
As in the case of aquatic animals, fish digestive tracts also anchorage many microbes. But their role in the gut physiology is not clearly understood and hence the possibility of a symbiotic gut flora is now being studied in several marine invertebrates. Such floras have been documented in herbivorous sea urchins (Fong and Mann, 1980), copepods (Sochard et al., 1979) penaeid shrimp (Hood et al., 1971) etc.

The gastro intestinal tract of fish can be generally described as a hollow tube into which food enters and it is processed and absorbed by the animal. Within the tract, there is a very large stable population of bacteria that are able to survive the harsh condition of the gastro intestinal tract. These different species of bacteria that live within the lumen of vertebrates and invertebrates are termed the micro flora of gut. The fish digestive tract is colonized by a great number of heterotrophic bacteria such as facultative and obligate anaerobes (Sakata, 1990). Intestinal bacterial community takes part in the metabolic processes required for the vital activities of the bacteria and macroorganisms. The bacterial flora can synthesize enzymes (Sugita et al., 1997a), vitamins (Sugita et al., 1992) and participates in the infection-protective reactions (Jankauskiene and Lenzner, 1995) and the physiological state of the microorganism is closely related to the activities of the intestinal microflora. Only recently diverse microbial communities have been reported from the gut of fishes (Bairagi et al., 2002; Saha et al., 2006).
The gastrointestinal communities of herbivorous terrestrial vertebrates play a critical role in the conversion of plant fiber into fermentation products that are available for the host animal (Van Soest, 1994). Despite this fact, the biodiversity of these communities are poorly understood for all but a few host species (Bjorndal, 1997; Nelson et al., 2003). Gastrointestinal microorganisms appear to play an important microflora of herbivorous fishes have mainly relied on direct microscopic examinations or culture of organisms from the gut contents (Clements, 1997). The microscopic examination of gut contents from two species of the family Kyphosidae revealed high cell counts (K. Cornelii 2.8 X 10^{11} cells g^{-1} dry weight; K. sydneyanus 5 x 10^{11} cells g-1 dry weight) in the posterior region of the intestine where the highest levels of short chain fatty acids (SCFAs) were also detected. The diversity of microbial communities in the two temperate marine species Odax cyanomelas and O. Pullus detected major differences in the morphological composition of the bacterial communities.

The digestive tract of the fish is an open system constantly contacting the surrounding water and the microflora of water plays an important role in the formation of digestive tract of fish in comparison with environment for microorganisms (Saha et al., 2006). The gastrointestinal tract of fish can influence nutrition, growth and disease susceptibility. The microflora may be
essential in fish that feed on recalcitrant material or on material lacking vitamins which the microflora can synthesize (Trust and Sparrow, 1974).

The gut plays a crucial role in nutrition. It is the place where food is digested and nutrients are observed from the diet (Conway et al., 1986). The bacterial attachment to the digestive tract is very important in maintaining a stable population within the gut. Mucus produced by the intestinal wall may serve as a source of nutrients and they may also enhance colonization of bacteria, serving as an attachment site for the bacteria (Westerdahl et al., 1991).

The biological diversity of marine and estuarine species provides a wide array of enzymes with unique properties. In recent years proteases from the gut of fishes received much attention (Chi et al., 2007). It contributes to the development of high added applications or products by using the enzyme aided proteins digestion from different sources including from marine animals. In aquaculture industry, the proteases are the needful enzymes for the preparation of high quality functional feeds through bioconversion of low cost feed materials. Because the aquaculture industry depends worldwide on the availability of low cost and high quality feeds.
An attempt to isolate numerically significant members of the gastrointestinal microflora of herbivorous or detrivorous fish have been largely unsuccessful. Mountfort and co workers described the isolation of an obligate anaerobic pectinolytic *Eubacterium* (Mountfort *et al.*, 1993), an obligate anaerobic alginolytic *Clostridium* sp (Mountfort *et al.*, 1994) and an anaerobic marine fungus *Paecilomyces lilacinus* (Mountfort and Rhodes, 1991) from the gut of the detrivorous Mullet *Aldrichetta forsteri*. Whether these organisms are functionally important materials to the breakdown and utilization of plant material in this detrivore is unclear.

Microflora may serve as a supplementary source of food and microbial activity in the gut may be a source of vitamins or essential amino acids (Dall and Moriarty, 1983) as the nutritional quality of live food varies significantly from batch to batch as a result of changes in the biochemical composition (Watanabe *et al.*, 1983). Some selected organisms have been found possess appreciable levels of micronutrient (Nayar *et al.*, 1998). However, some beneficial bacterial strains are responsible to restore the water quality of the culture system and also to create a conductive natural environment (Nayar *et al.*, 1998).
Most of the molecular studies on gut flora from marine herbivorous fishes are restricted to phylogenetic characterization of individual fishes. These include the largest known heterotrophic bacteria; the *Euploiscium* sp. is a symbiont of surgeon fishes (Angert *et al*., 1996). Molecular analysis of 16s rRNA genes enable the composition of complex microbial communities to be examined (Forney *et al*., 2004). Such methods have been applied to the studies on the gut flora of hosts including humans (Heilig *et al*., 2002) and ruminants (Tajima *et al*., 1999). Data on the metabolic capabilities of specific microbial groups allow broad interpretation of the functional capabilities of closely related organisms identified by phylogenetic inference from clone libraries. Very little comparable work has been on the gastrointestinal communities of marine herbivorous fishes. Ontogenetic and seasonal changes in the hindgut community of the temperate herbivorous *Kyphosid kyphosus* and *Kyphosid sydneyanus* were examined by Moran *et al*. (2004) and recently by Fidopiastis *et al*. (2006). They used 16s rRNA sequences to characterize the predominant bacterial groups in the gastrointestinal tract of another *Kyphosid* and the warm temperate *Hermosilla azurea*. Collectively, the available culture based and molecular data indicate that gastrointestinal microorganisms in marine herbivorous fishes are abundant.
Numerous studies have been made for the isolation and characterization of the gut microbiota of herbivorous fishes (MacCormack and Fraile, 1990; Luczkovich and Stellwag, 1993). The characterization of gut flora is to select for certain organisms and they are inadequate for studying the microbial ecology of a natural system (Amann et al., 1995). Several investigators have used 16S rDNA to infer phylogeny of as yet unculturable microbes (Woese et al., 1990; DeLong et al., 1993). Recently, these techniques have been used to define the microbial populations in the digestive tracts of animals (Wilson and Blitchington, 1996). Fuhrman et al. (1992) have reported that DNA-based analysis of microbial communities can achieve four goals:

(1) Create a list of "what is there",

(2) Quantity of the abundance of members from the list,

(3) Comparable microbial communities from different samples and

(4) Detect potentially important community members.

Though considerable information is available regarding the intestinal microflora of homeotherms and their role in digestion, reports on the bacterial population in the gastrointestinal tract of poikilothersms, including fish and their roles in digestions are scanty. Reports concerning the microbial enzyme production in the gastrointestinal tracts of fishes are now available (Das and
Tripathi, 1991; Saha and Ray, 1998). In functional terms the community of microorganisms in the digestive tract of the fish is analogous to that in mammals, since the cell structure of the intestines and the physiology of digestion in mammals and fishes are similar in some respects (Ringo et al., 1995).

In context with the above, the present chapter was studied with the aim to isolate and identify the protease producing bacterial strains from the gut of estuarine fish *Etroplus suratensis.*
2.2. MATERIAL AND METHODS

2.2.1. Collection of samples

The experimental fish *Etroplus suratensis* (pearl spot) was collected from the Rajakkamangalam estuary, a minor estuary in Southwest coast of India and transported to the laboratory immediately.

2.2.2. Gut microbial analysis

The gut was dissected out from the experimental fish under sterile condition. Then the gut was ground well in saline buffer at 1g per 100ml (1%) and used as the stock. This stock solution was serially diluted and then spread on the marine zobell agar plate. This method is used to isolate pure culture and also for estimating the total viable colonies (TVC).

\[
\text{Number of bacterial population /1 ml sample} = \frac{\text{Total number of colony}}{\text{Volume of the Sample}} \times \text{Dilution factor}
\]

2.2.3. Microscopical identification of bacteria (Holt *et al.*, 1994)

2.2.3.1. Gram's staining

Most of the bacteria can be differentiated by their Gram's reaction due to differences in their cell wall structure. The technique was developed by Hans Christian Gram.
The organisms which stained dark purple with crystal violet (primary stain) and not decolorized by 95% alcohol are Gram positive and those organisms which after being stained with crystal violet lost their colour when treated with alcohol and stained red with safranin (counter stain) are referred as Gram negative.

2.2.3.2. Motility test

Bacterial motility was identified by hanging drop method. Bacterial motility is characterized by the presence of flagella.

2.2.4. Biochemical tests for identification

2.2.4.1. Catalase test

This test is used to determine the ability of an organism to produce the catalase enzyme. During aerobic respiration, the organism produce H$_2$O$_2$ and super oxide and the accumulation of these substances are toxic to the organisms. Hence it produces catalase and degrades H$_2$O$_2$ into oxygen and water.

2.2.4.2. Oxidase test

Oxidise test is used to identify the organisms, which produce the enzyme oxidase. The enzyme oxidase forms the part of electron transport system, possessed by some bacteria. This enzyme oxidizes the oxidase disc,
which contain the reagent N-N Tetramethyl Paraphenylene Diamine Dihydro Chloride to a deep purple colour.

2.2.4.3. **Triple sugar iron test**

Triple sugar iron test (TSI) is used to determine whether a Gram-negative rod uses glucose, lactose or sucrose fermentative and form hydrogen sulfide (H\textsubscript{2}S). TSI contains 10 parts lactose, 10 parts sucrose, 1 part glucose and peptone. Phenol red and ferrous sulphate serve as the indicators of acidification and H\textsubscript{2}S formation respectively. When fermentative organisms use glucose, the entire medium becomes acidic (yellow) within 8 to 12 hours. The butt remains acidic after the recommended 18 to 24 hours of incubation period because of the presence of organic acids, which results from the fermentation of glucose under aerobic conditions in the butt of the tube. The slant however, reverts to the alkaline (red) state because of oxidation of the fermentation products under aerobic conditions of the slant. The changes, is a result of the formation of CO\textsubscript{2} and H\textsubscript{2}O and the oxidation of peptones in the medium of alkaline amines. In addition to glucose, lactose and sucrose, the large amount of fermentation products formed on the slant will neutralize the alkaline amines and render the slant acidic (yellow), provided the reaction is read in 18 - 24 hours. Reaction in TSI should not be read beyond 24 hours of incubation, because aerobic oxidation of the fermentation product from lactose and sucrose does proceed and the slant will eventually revert to the alkaline
state. The formation of CO\textsubscript{2} and H\textsubscript{2} (hydrogen gas) is indicated by the presence of bubbles or cracks in the agar or by separation of the agar from the slides or bottom of the tube.

2.2.4.4. Sugar fermentation test

This test is used to determine the ability of the organisms to degrade and ferment sugar with the production of an acid or acid gas. Fermentation is an aerobic metabolic process in which the final electron acceptor is an organic substrate instead of oxygen. The most commonly produced end product is lactic acid even though many other substances are also produced as the result of different fermentation pathways. Most organisms obtain their energy through a series of orderly and integrated enzymatic reactions leading to bio-oxidation of the substrate. The medium contains nutrient broth, sugar and the pH indicator nutrient broth supports the growth of organisms. The sugars specifically enhance the fermenting capability of the organisms and the pH indicator phenol red at neutral pH and yellow at acidic pH. Following incubation, the fermented carbohydrates produce acidic pH causing changing the colour to yellow.

2.2.4.5. Indole production test

This test is used to determine the ability of an organism to split tryptophan to form the compound indole.
2.2.4.6. Methyl Red / Voges Proskauer (MR-VP) test

MR-VP test is used to determine the ability of an organism to produce stable acid end products from glucose fermentation to overcome. The buffering capacity of the system and to determine the ability of the source of organisms to produce neutral end products (e.g. acetyl methyl carbinol or acetone) from glucose fermentation.

2.2.4.7. Citrate utilization test

This test is used to determine the ability of an organism to use sodium citrate as its only carbon source and inorganic ammonium salts as its only nitrogen source. Bacteria that can grow in this medium turn the bromothymol blue indicator from green to blue.

2.2.4.8. Gelatin hydrolysis test

This test is used to determine the ability of an organism to produce proteolytic enzymes (Gelatinases) that liquify gelatin.

2.2.4.9. Nitrate reduction test

Nitrate reduction test is used to determine the ability of organisms to reduce nitrate. The reduction of nitrate to nitrite is determined by adding sulfanilic acid and alpha-napthyl-amine. The sulfanilic acid and nitrate react to a diazonium salt. The diazonium salt then couples with the alpha-napthylamine to produce a red, water-soluble azo dye.
2.2.4.10. Urea hydrolysis test

This test is used to determine the ability of an organism to produce the enzyme urease, which hydrolyzes urea. Hydrolysis of urea produces ammonia and CO$_2$. The formation of ammonia alkalinizes the medium and the pH shift is detected by the colour change of phenol red from light orange at pH 6.8 to magenta at pH 8.1.

2.2.4.11. Casein hydrolysis test

Casein, the major milk protein is a macromolecule of aminoacids linked by peptide bonds. A molecule of this magnitude is incapable of permeating cell membranes. The protein should undergo stepwise degradation into peptones, polypeptones, dipeptones and finally to aminoacids for cellular nutrition. This is mediated by cellular exoenzymes called proteases and the process is called as peptidization or proteolysis.

2.2.4.12. Hydrogen sulphide production test

Hydrogen sulphide can be produced by the reduction of organic sulphur present in the amino acid cysteine, a component of peptone in the medium. In the presence of cysteine desulfurase, the cysteine looses the sulphur atom, which is then reduced by the addition of hydrogen from water to form hydrogen sulphide.
2.2.4.13. Starch hydrolysis test

Starch is a linear polymer of glucose molecule linked together by glycosidic bonds. Starch as such cannot be transported into the cell for energy production, because of its high molecular weight. To assimilate starch for energy and catabolic reactions, it must be degraded into basic glucose units by starch hydrolyzing enzymes. These enzymes are secreted by the microorganisms into the medium, which degrade starch primarily to glucose. The resulting low molecular weight soluble glucose molecules are now able to pass into the cell for energy production via, glycolysis.

2.2.5. Screening for protease production

Then the bacterial strains were streaked on skim milk agar plates and incubated at 35°C for 24 hours for testing the ability of protease production. After incubation a clear zone around the bacterial growth indicated the protease production.

2.2.6. Strain identification

2.2.6.1. Genomic DNA extraction

The isolated bacterial strain was grown in 2ml Zobell Marine Broth (2216) (Himedia) overnight at 27°C. The culture was spun at 7000 rpm for 3 min. The pellet was resuspended in 400 μl of sucrose TE buffer. Lysozyme was added to a final concentration of 8 mg/ml and incubated for 1h at 37°C.
To this tube, 100 μl of 0.5M EDTA (pH 8.0), 60 μl of 10% SDS and 3 μl of proteinase K from 20 mg/ml were added and incubated at 55°C overnight. The supernatant was extracted twice with phenol: chloroform (1:1) and once with chloroform: isoamylalcohol (24:1) and ethanol precipitated. The DNA pellet was resuspended in sterile distilled water (Sambrook et al., 1989).

2.2.6.2. Amplification of 16S rRNA gene

Bacterial 16S rDNA was amplified from the extracted genomic DNA using the following universal eubacterial 16S rRNA primers: forward primer 5' AGAGTTTGATCCTGGCTCAG 3' and reverse primer 5' ACGGCTACCT TGTTACGACTT 3'. Polymerase chain reaction was performed in a 50 μl reaction mixture containing 2 μl (10 ng) of DNA as the template, each primer at a concentration of 0.5μM, 1.5 mM MgCl₂, and each deoxynucleoside triphosphate at a concentration of 50 μM, as well as 1 U of Taq polymerase and buffer as recommended by the manufacturer (MBI Fermentas). After the initial denaturation for 3 min at 95°C, there were 40 cycles consisting of denaturation at 95°C for 1 min followed by annealing at 55°C for 1 min and extension at 72°C for 2 min and then a final extension step consisting of 5 min at 72°C; Mastercycler Personal (eppendorf, Germany) was used. The amplification of 16S rDNA was confirmed by running the amplification product in 1% agarose gel in 1X TAE.
2.2.6.3. Cloning and sequencing of 16S rRNA gene

The amplified product (1,500-bp) was purified using GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, U.K) according to manufacturer's instruction. The 16S rDNA amplicon was cloned in pTZ57R/T vector according to the manufacturer's instruction (InsT/Aclone™ PCR Product Cloning Kit #K1214, MBI Fermentas). Full length sequencing of the rRNA gene (about 1500 bp) for the isolated bacteria was carried out in Macrogen (Seoul, Korea).

2.2.6.4. Nucleotide sequence analysis

The full-length sequences obtained were matched with previously published sequences available in NCBI using BLAST (Altschul et al., 1997). Multiple sequence analysis was carried out using CLUSTALX (Thompson et al., 1997) and further NJ plot (Perrière and Gouy, 1996) and PhyloDRAW were employed for constructing phylogenetic tree. To validate the reproducibility of the branching pattern bootstrap analyses imply.
2.3. RESULTS

2.3.1. Total viable count

Table 1 provide the data on total viable colonies of the gut of *Etroplus suratensis*. Among the eight different dilutions, the colony counts were more in $10^{-1}$ to $10^{-2}$ dilutions. In other dilutions less number of colonies were noticed and it was found to be reduced with increase in dilution from $10^{-3}$ to $10^{-6}$ (Plate 1). In highest dilutions of $10^{-8}$ the colonies recorded were too few to count (TFTC).

2.3.2. Morphological appearance

Results on the morphological appearance of the suspected colonies of are provided in plate 1. In total ten different groups of colonies with varying morphology were screened and the details are provided below:

Strain no. 1 - circular in form with flat elevation and entire margin.

Strain no. 2 - circular in form with raised elevation and entire margin.

Strain no. 3 - circular in form with convex elevation and entire margin.

Strain no. 4 - filamentous in form with flat elevation and filamentous margin.

Strain no. 5 - filamentous in form with raised elevation and filamentous margin.
Strain no. 6 - large, rhizoid in form with flat elevation and filamentous margin.

Strain no. 7 - irregular in form with flat elevation and filamentous margin.

Strain no. 8 - irregular in form with flat elevation and curled margin.

Strain no. 9 - circular in form with umbonate elevation and entire margin.

Strain no. 10 - large, mucoid, low convex with irregular surface, an edge that is translucent and an oblong shape. It emits a sweet grape-like odour. Greenish-blue pigment diffused in medium.

2.3.3. Screening of protease producing bacteria

In the present study, altogether ten types of morphologically different colonies were isolated from the gut of estuarine fish *Etroplus suratensis*. Then the organisms were isolated and screened for protease producing ability on skim milk agar plates which form a zone due to hydrolysis of casein. Hence these strains were identified as protease producer. Among the screened isolates, the ten different isolates were identified as protease producer and within these strains only two strains were identified as maximum zone formation and those particular strains were subjected to further experimental analysis (Plate 2).
2.3.4. Identification of protease producing bacteria

Based on the morphological, physiological and biochemical characteristics, the protease producing bacterial strains were identified as standard keys of Bergy’s Manual of Determinative Bacteriology. Table 2 shows the biochemical identification of protease producing bacterial strains.

2.3.5. 16S rRNA gene sequence analysis

The 16S rRNA gene of the gut bacterial strains were amplified through PCR using the 16S rDNA universal primers and both the strands were sequenced. The sequences were compared with the 16S rDNA sequences available in the RDP database (http://rdp.cme.msu.edu/). Sequence analysis revealed that the strains were phylogenetically closely related to the genus of respective bacterial strains. BLAST analysis of the 16S rDNA sequence of identified gut isolates showed that the isolate *Bacillus flexus* AP.MSU2 has 95% sequence similarity in the NCBI (National Centre for Biotechnological Information) database (Accession number – FJ52234.1), *Pseudomonas* sp. AP.MSU3 has 97% sequence similarity in the NCBI (National Centre for Biotechnological Information) database (Accession number – FJ52235.1), *Serratia proteomaculans* AP.CMST1 has 95% sequence similarity in the NCBI (National Centre for Biotechnological Information) database (Accession number – FJ52236.1), *Bacillus aquimaris* AP.MSU5 has 100% sequence similarity in the NCBI (National Centre for Biotechnological
Information) database (Accession number – GU594321.1), *Bacillus licheniformis* AP.MSU6 has 95% sequence similarity in the NCBI (National Centre for Biotechnological Information) database (Accession number – HM101168.1) *Lysinibacillus fusiformis* AP.MSU13 has 95% sequence similarity in the NCBI (National Centre for Biotechnological Information) database (Accession number – HM101171.1), *Pseudomonas aeruginosa* AP.MSU14 has 95% sequence similarity in the NCBI (National Centre for Biotechnological Information) database (Accession number – HM101170.1), *Bacillus thuringiensis* AP.CMST has 98% sequence similarity in the NCBI (National Centre for Biotechnological Information) database (Accession number – HM623612.1) *Vibrio mimicus* AP.CMST has 95% sequence similarity in the NCBI (National Centre for Biotechnological Information) database (Accession number – HM582689.1) and Alcanivorax sp. AP.MSU10 has 98% sequence similarity in the NCBI (National Centre for Biotechnological Information) database (Accession number – HM101169.1) have had more similarity score with their respective related strains. Though the isolates had a close similarity and the dendrogram constructed based on their phylogenetic relationship revealed that all the isolates were distinctly placed under separate clusters. The results of the identification (Gene sequence and phylogenetic tree) of the isolates using different methods are compared and depicted in Figures 1-10.
2.4. DISCUSSION

Symbiotic bacteria in animal’s digestive tract often produce complement enzymes for digestion of plant foods as well as synthesize compounds that are assimilated by the host (McBee, 1971; Hungate, 1975). These symbioses are well studied in terrestrial animals, when compared with aquatic organisms. Being rich nutrients, the environment of the digestive tract of fish confers a more favorable growth environment for microorganisms in comparison with surrounding water (Saha et al., 2006). Enzymatic symbiotic gut microfloras have also been reported in Japanese coastal fishes (Stellwag et al., 1995 and Hoshino et al., 1997) and some fresh water fishes (Bairagi et al., 2002; Ray et al., 2007).

The physiological and biochemical activities of the fish intestinal bacterial flora are directly related to fish nutrition. Earlier researchers have reported that proteolytic and protein mineralizing bacteria prevail in the intestine of the fish feeding on food of animal origin (Lubianski et al., 1982). The symbiotic microorganisms of the gastrointestinal tract have been well studied in herbivorous mammals, birds, and reptiles (Stevens, 1988). The microbial populations grow on the food absorbed by the host animal, digestive secretions and fragments sloughed off the mucosal epithelium (Lesel, 1991). Microflora is not only essential but also vitally important component of the digestive tract in animals, including fishes (Clements, 1997; Kuz’ mina, 1996).
In general the bacterial flora of the gastrointestinal tract represents a very important diversified enzymatic potential and it seems logical to think that the enzymatic mass lodged in the digestive tract might interferes in a considerable way with a major part of the metabolism of the host animal (Clements, 1997).

It is well known that the digestive tracts of the aquatic organisms are colonized by the great number of bacterial flora. Each area of the digestive tract will contain distinctive variety of microbial species. The results of the present study indicated that the microorganisms isolated from fish digestive tract are capable of producing proteolytic enzyme. In this study, the total viable count was ranged from 20.0 to 43.0 X 10^4CFU per gram gut of *Etroplus suratensis*. Out of ten suspected colonies screened, two bacterial strains were found to produce protease by forming a clear zone around it in skim milk agar plates.

The present study revealed that the microorganisms are very important for the digestion process of fish. Earlier investigations have suggested that micro organisms have a beneficial effect in the digestive process of fish (Ringo *et al*., 1995). The use of probiotics has a long tradition in animal husbandry (Stavric and Kroneygay, 1995), but it rarely applied in aquaculture. The main strategy in the use of probiotics is to isolate intestinal bacteria with favourable properties from marine animals of the same species (Gildberg *et al*., 1997).
Bacteria may enter in the fish gut through the open mouth and the *Vibrio* sp. or *Aeromonas* sp. borne by live feed organisms and become dominant after the first feeding. Many studies on intestinal microflora of marine and fresh water fish species have been reported (Sugita *et al*., 1985). Bacteria those are able to survive and colonize in the digestive tract are quiet successful at maintaining large stable population with typical number at approximately $10^{-5}$ g/l (Hansen *et al*., 1992).

In the present study, microorganisms isolated from the gut of estuarine fish *Etroplus suratensis* were identified through the 16S rRNA analysis. The molecular phylogenetic studies to identify the microbiota, archaen and prokaryotes found in the gut contents of marine fish species. Sulphate reducing bacteria are abundant components of mouse intestinal microbiota, accounting for 17-23% of the 16S rDNA examined (Deplanure *et al*., 2000).

The PCR primer set is used to generate the 16S rRNA gene sequence and the majority of the bacterial phylotypes identified from the fish gut libraries were most closely associated with the genus of *Clostridium* sp. and *Eubacterium* sp. Gram-positive bacteria are frequently reported to dominate intestinal ecosystems, numerically as well as in terms of species diversity (Nelson *et al*., 2003). Members of the genera *Clostridium* and *Eubacterium* are typically very abundant and are a major proportion of bacteria found in the
rumen and the intestinal tracts of animals and insects (Stevens and Hume 1995; Suau et al., 1999; Sghir et al., 2000).

Fornaj et al. (2004) have reported that the extraction of DNA from all taxa is a critical factor for 16S rDNA clone libraries. They used a direct DNA extraction procedure and the DNA was extracted from whole gut contents that included both freeze-thaw cycles and bead-beating in order to achieve effective basis of space forming. In contract, indirect methods were used by Fidopiastis et al. (2006). The bacterial cells were isolated from gut contents and DNA extraction was performed on the bacterial pellet. While indirect methods are reported to yield DNA of high purity and hence suitability for enzyme reactions such as PCR direct methods resulted in greater DNA yield and may therefore provide a better representation of microbial diversity (Left et al., 1995; Miller et al., 1999). PCR amplification can also create bias by changing the template – product ratio (Suzuki and Giovannoni, 1996). Fidopiastis et al. (2006) used 40 cycles which may have influenced the prepositional representation of their clone libraries.

It is difficult to estimate the contribution of specific bacteria to the function of the whole gut system. It is reasonable to expect that the overall chemical milieu would be strongly influenced by the predominant populations of organisms. Clostridia are mostly polymer degrader using polysaccharides and proteins as substrates and yielding solvents, alcohols and short chain fatty
acids as fermentation products (Andreeson et al., 1989). A bacterial community dominated by \textit{Clostridial} species is therefore consistent with the ratio of short chain fatty acids previously reported in \textit{K. sydneyanus}, \textit{O. pullulus} and \textit{A. anctidens} (Clements et al., 1994; Mount fort et al., 2002). Current genome sequencing and metagenomic projects on clostridia and related species, including the surgeon fish gastrointestinal symbionts of \textit{Epulopiseium} thrown considerable light on the activities of these organisms (Nelson, 2003).

Mount fort et al. (2002) and Skea et al. (2005) have reported that the organisms identified in the hindgut of \textit{K. sydneyanus}, \textit{O. pullulus} and \textit{A. arcticdens} were affiliated with Clostridiales. These phylogenetic results are consistent with previous work on hindgut fermentation in these fish species suggesting that these gastrointestinal communities function to convert a broad range of algal carbohydrates into a form unusable by the host.. Similarly the present study also gut bacterial strains recorded their presence upto $10^{-5}$ dilution. The isolated bacterial strains were identified biochemically as well using 16S rDNA sequencing and were found to contribute protease enzyme to the host fish species.
Table 1. Total number of bacterial counts (CFU/ml) in the gut of *Etropus suratensis*

<table>
<thead>
<tr>
<th>Serial dilution</th>
<th>Plate 1</th>
<th>Plate 1</th>
<th>Plate 1</th>
<th>Mean ± SD</th>
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</thead>
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<td>$10^{-1}$</td>
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<td>-</td>
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<tr>
<td>$10^{-3}$</td>
<td>226</td>
<td>233</td>
<td>221</td>
<td>226.6 ± 4.92</td>
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<tr>
<td>$10^{-4}$</td>
<td>75</td>
<td>81</td>
<td>85</td>
<td>80.3 ± 4.10</td>
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<tr>
<td>$10^{-5}$</td>
<td>45</td>
<td>40</td>
<td>37</td>
<td>40.6 ± 3.29</td>
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<tr>
<td>$10^{-6}$</td>
<td>21</td>
<td>27</td>
<td>33</td>
<td>27 ± 4.89</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>TFTC</td>
<td>TFTC</td>
<td>TFTC</td>
<td>-</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>TFTC</td>
<td>TFTC</td>
<td>TFTC</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 2. Biochemical characteristics of the *Pseudomonas* sp. and *Bacillus flexus*

<table>
<thead>
<tr>
<th>Biochemical tests</th>
<th><em>Pseudomonas</em> sp.</th>
<th><em>Bacillus flexus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram’s staining</td>
<td>Gram negative (−ve)</td>
<td>Gram positive <em>Bacillus flexus</em></td>
</tr>
<tr>
<td>Endospore staining</td>
<td>Rod</td>
<td>Sub-terminal spores</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Carbohydrate fermentation test</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. D-glucose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>b. Mannitol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>c. Lactose</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>d. Sucrose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Indole production</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methyl red test</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Voges-Proskauer test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Citrate utilization test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin hydrolysis</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Casein hydrolysis</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Urease test</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Catalase test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Nitrate utilization test</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

+: Positive Results; -: Negative Results