9. GUT MICROFLORA & ENZYME STUDIES

9.1 INTRODUCTION

A study on gastrointestinal microbiology of an organism would reveal the complex microcosm of the microbial communities and the primary interfacial activities between the host and numerous living components of the digestive tract. Dense bacterial populations are found at different locales in the digestive system of an organism. As a base line study, it is therefore planned to investigate the spatial distribution of different bacterial populations in the alimentary tract of *B. spirata* and their role in digestion of food of animal.

Bacteriological studies of gastropods are rather limited. The literature available on the bacteriology of gastropods include the reports on the microbial population of shell, mantle and the alimentary tract of *Telescopium telecopium* and *Bullia vittata* (Vanajakumar, 1979), gut microflora of *Bursa spinosa* (Petchimuthu, 1985), neritids (Kanagasabai, 1985) *Rapana rapiformis*, (Rajkumar, 1995) and some other gastropods (Velammal, 1987).
The current explosive growth of knowledge has lead to the large scale production, purification and stabilization of enzymes from animals, plants and microorganisms, and their widespread use in industries such as food production, detergents, medicines, textiles and paper manufacture (Wiseman, 1975). Hence the knowledge available on the digestive enzymes such as amylase, protease and lipase will enable an understanding of their activity and their assays will also be helpful in further purification of these enzymes for their wide application.

The digestive enzymes and the extracellular enzymes of associated microbes inside the tract of alimentary canal play an important role in the digestion of food in carnivorous prosobranchs. In general, earlier workers have rarely investigated the role of symbiotic microorganisms in the production of enzymes, that can be helpful in digestion of food in animals. Therefore, comprehensive study on digestive enzymes and their qualitative assessment in gut microflora and their role in digestion of B. spirata has been undertaken in the present study.
9.2 MATERIAL AND METHODS

9.2.1 Enumeration of total viable heterotrophic bacterial counts

The fresh snails were sampled from the habitat and brought to the microbiological laboratory in a bucket containing the same seawater. The snails were analysed for the total heterotrophic bacterial counts harboured in the different regions like foregut, midgut and the hindgut of the digestive tract of the snail.

9.2.2 Preparation of blanks

Four clean 250 ml conical flasks were taken and 100 ml of 75% sterile and filtered seawater were added to each. The conical flasks were plugged with cotton and wrapped with hard paper, which formed the sample blanks. Serial dilution blanks were prepared by distributing 9 ml of aliquots of 75% sterile seawater into series of test tubes and plugged with cotton and wrapped with hard paper. All blanks were autoclaved at 15 lbs pressure for 15 minutes and then used for the study.

9.2.3 Preparation of inoculum and serial dilution

Alive specimens were brought to the laboratory in sterile polythene bags. Sterile seawater was used to wash the snail and also the
dissected digestive regions externally to ensure the surface sterility. Dissection equipment and containers were always sterilized before usage. The animals were dissected in an aseptic test room and care was taken to insure aseptic dissection to avoid contamination of adjacent tissues.

The animals were cut open, 1g of different regions of digestive tract of *B. spirata* such as, foregut, midgut and hindgut were removed aseptically and homogenized separately using a known volume of 75% sterile seawater employing presterilized homogenizer. Then the homogenized samples were transferred to 100 ml of sterile 75% seawater. Further serial dilutions were made using 9 ml of sterile 75% seawater blanks.

### 9.2.4 Plating and enumeration of viable bacterial counts

The viable number of bacterial counts of foregut, midgut and hindgut of *B. spriata* was enumerated using pour plate method. 1ml of aliquots from the appropriate dilution was pipetted out into a sterile Petri dish. Twenty ml of sterile molten nutrient agar were aseptically poured into the petriplates and were rotated in clockwise and anticlockwise directions and allowed to solidify. For each dilution,
replicates were maintained and the inoculated plates were incubated at 37°C for 49 to 72 hours.

Bacterial colonies were counted using bacteriological colony counter after incubation period. The Petri dishes, which contained 30-300 bacterial colonies, were selected for enumeration of bacterial colonies and the bacterial counts are expressed as number of colony forming units per gram (cfu/gm) of the sample analysed.

9.2.5 Generic composition of the bacterial strains

Well-isolated bacterial colonies with varying morphological growth characteristics were selected and restreaked in air-dried nutrient agar plates to check the purity of the bacterial strains. The pure cultures were then restreaked in nutrient agar slants and stored at 4°C for further bio-chemical studies. A total number of 70 isolates were taken and identified upto generic level. The isolated strains were then subjected to various bio-chemical test and the isolated bacterial strains were identified upto the generic level following the scheme of Simudu and Aiso (1962). The Bergy’s manual of determinative bacteriology (1984-99) was also referred for the identification (Chart – 1).
9.2.6 Physiological grouping of bacterial isolates

Bacteria use different types of nutrients and the end product from such metabolism depends on the types of enzymes liberated by them. Bacterial strains isolated from the digestive tract of *B. spirata* were tested for amylolytic, gelatinolytic, caseinolytic and lipolytic activities. For this, the test bacterial strains were streaked on selective agar plates viz., starch agar, gelatin agar, casein agar, and tween-80 agar. The plates were incubated at 37°C for 48 hours.

9.2.7 Test for amylolytic activity

Bacterial cultures were streaked on air-dried starch agar plates and incubated at 37°C for a period of 24 hours. After incubation, the surface of the plate was flooded with Gram’s iodine solution. The presence or absence of halozone around the bacterial outgrowth was recorded as positive or negative amylolytic reaction respectively.

9.2.8 Test for gelatinolytic activity

A loopful of overnight culture of the test bacterial isolate was streaked on sterile air-dried gelatin agar and incubated at 37°C for 24
hours. After incubation period, the gelatin hydrolyzing activity of the isolate was tested using HgCl₂ solution (0.1%). Appearance of halozone around the bacterial streak was recorded as positive reaction for gelatinolysis.

9.2.9 Test for caseinolytic activity

The 24-48 hours old bacterial cultures were taken and short single line streaks were made on the air-dried sterile casein agar plates. The plates were inverted and incubated at 37°C for 24-48 hours. After the incubation period, the plates were examined for the presence or absence of halozones around the streaks.

9.2.10 Test for lipolytic activity

Tween-80 agar plates were prepared and allowed to solidify. After solidification, the plates were inverted and kept for air-drying for 24 hours. Young bacterial test cultures were taken and short streak was made on Tween-80 agar plate. The streaked plates were incubated at 37°C for 2-7 days. Appearance of opaque zone around the bacterial streak is indicative of lipolytic activity of the test bacterial
isolated. The formation of oleic acid around the edge of the halozone was recorded.

9.2.11 Qualitative assessment of digestive enzymes from the digestive tract of *B. spirata*

The gut of *B. spirata* was dissected and divided into three regions viz.:

(i) the foregut comprising buccal complex and esophagus

(ii) the midgut comprising the stomach and midgut gland, and

(iii) the hindgut comprising the intestine and rectum.

For qualitative analysis of the digestive enzymes of *B. spirata*, the digestive enzyme extracts were prepared by homogenizing weighed quantity of the various digestive parts with measured quantity of distilled water, centrifuged and the supernatant was removed and diluted to 20 ml with distilled water. Then the experiment was carried by following the methods of Jeyaraman (1992).

9.2.12 Test for amylase

Two ml of one percentage starch solution was taken in two test tubes and two ml of distilled water was added to the first tube which
served as control and two ml of enzyme extract was added to another test tube which served as the experimental one. These two test tubes were placed in the water bath for 30 minutes. A drop of iodine was added to each test tube. Appearance of blue colour in the control indicating the presence of starch and the solution in the experimental tube gives no colour in combination with iodine indicating the fact that hydrolysed starch in the experimental tube.

9.2.13 Test for Maltase

Two ml of 1% starch solution in two test tubes and two ml of distilled water was added to the control tube and two ml of enzyme extract was poured into the experimental tube. These two test tubes were placed in water bath, maintained at 38°C for about an hour. After an hour, two ml of Benedict’s reagent was added to each test tube and heated gently. In the experimental tube, if the brown colour appears it indicates the presence of monosaccharides (maltase) and there is no change in control.
9.2.14 Test for invertase

One ml of five percentage sucrose solution was taken in two test tubes. Three drops of distilled water were added in the control and three drops of enzyme extract into the experimental tube. These two tubes were placed in the water bath for about an hour at 38\(^{0}\)C. After an hour, one drops of fehlings ‘A’ and one drop of fehlings ‘B’ solutions were added and heated. Presence of yellow or red precipitate in the experimental tube indicates conversion of sucrose into glucose and fructose by invertase.

9.2.15 Test for Protease

Three ml of two percentage albumin was taken in two test tubes, five ml of distilled water was added in control tube and five ml of enzyme extract into the experimental tube. These two tubes were placed in a water bath at 38\(^{0}\)C for an hour. After one hour, half of the content from both the test tubes were transferred to another two test tubes separately and the following tests were conducted.
a) Conjugation Test

One ml of mercuric chloride was added to both the test tubes. If the experimental tube shows no sign of clotting, it indicates the utilization of protein (albumin) by the enzyme protease and in the control clotting takes place.

b) Biurette Test

One ml of ten percentage Sodium hydroxide solution and two drops of copper sulphate were added to the solution. Non-appearance of violet colour in the experimental tube indicates the digestion of protein by protease enzyme, whereas in the control violet colour appears.

9.2.16 Test for Lipase

Two drops of coconut oil and one ml of absolute alcohol were mixed together and heated gently to dissolve the coconut oil. Then an equal volume of distilled water was added. Now the oil was separated as an emulsion. Then five drops of bromothymol blue was added to neutralize the mixture and shaken well. Then it was transferred to two different test tubes. In one test tube two ml of distilled water was added
which served as a control and two ml of enzyme extract was added in the experimental tube. The test tubes were placed in the hot water bath for about one and half an hour at $38^\circ$C. From the same test tube 1ml of the liquid was removed and dissolved in 1ml of chloroform and a pinch of Sudanblack was added. If the colour is noted then it indicates once again the presence of lipase enzyme. If the experimental tube changes to yellow colour it indicates the digestion of lipid by the enzyme lipase. But in the control no change of colour could be noted.

9.3 RESULTS

The occurrence and activity of various digestive enzymes present in the digestive tract of *B. spirata* are given in table 12.

The results of total heterotrophic bacterial counts (THB) and various physiological groups were enumerated from different parts of the alimentary tract of *B. spirata* and are shown in Tables 2 and 3. The THB recorded in the foregut, midgut and hindgut were $5.7 \times 10^4$ (cfu/g), $6.7 \times 10^2$ (cfu/g) and $5.1 \times 10^5$ (cfu/g) respectively table 13 & plate 8.

In the foregut of *B. spirata* the carbohydrases like amylase, maltase and invertase were predominant. It showed a very low
concentration of the enzyme protease. Enzyme lipase was not at all recorded in the foregut. The physiological bacterial populations like amylolytic, gelatinolytic, caseinolytic and lipolytic groups were also identified from the different regions of alimentary tract. Foregut harboured 15.79% of amylolytic, 63.16% of gelatinolytic, 15.79% of caseinolytic and 5.26% of lipolytic bacterial groups table 15.

In the midgut when the enzyme activities were considered the protease and lipase were more active whereas carbohydrazase was feeble. In the midgut 40% of the amylolytic, 6.67% of gelatinolytic, 26.67% of caseinolytic and 20% of lipolytic populations were recorded.

Except a very weak amylase activity other enzymes were believed to be completely absent in the hindgut region. The highest bacterial counts were recorded in the hindgut than the other two locales. The hindgut showed 19.44% of amylolytic, 8.33% of gelatinolytic, 30.56% of caseinolytic and 41.67% of lipolytic bacterial populations.

Totally 70 bacterial strains were isolated from the alimentary tract and identified upto generic level. Gram-negative forms such as *Vibrio, Pseudomonas, Enterobacteriaceae, Flavobacterium* and
Gram positive forms such as *Micrococcus*, *Corynebacterium* and *Bacillus* were recorded.

9.4 DISCUSSION

The pattern of gut microflora distribution in various parts of the digestive system can be summarized as (1) foregut, (2) midgut and (3) hindgut.

It was evident from earlier literature and from the radular structure this experimental animal is a carnivore. The digestion of the prey started in the foregut itself in the carnivorous *B. spirata*. Galli and Giese (1959) reported strong amylolytic activity in *Tegula funebralis*. Microfloral study of *B. spirata* revealed that foregut ranks second next to hindgut. This kind of trend was also reported by various authors in neogastropods like *Bursa spinosa* (Petchimuthu 1985), from five neritids (Kanagasabai 1985) in *Bullia* and *Murex* (Vellammal, 1987), in *Rapana rapiformis* (Rajakumar 1995).

The dominance of carbohydrases in the foregut region of the study animal revealed that the foregut region was considered to be the
site of carbohydrate digestion, even though the animal was a carnivore. These enzymes might help in digestion of carbohydrates which could be present along with fleshy feed. The predominance of amylase activity in the foregut of some gastropods including some carnivores was reported by Yonge (1932) in Strombids, Graham (1939) in Petella, Hoshimoto and Onama (1949) in Teredo, Manmadha Rao (1977), in Melania crenulata and in Clypeomerus sp. And Yellowless (1980) observed sucrolytic enzymes in some gastropods.

As reported earlier midgut harboured low bacterial counts when compared to foregut and hindgut. The presence of low bacterial count in the midgut was also reported in neogastropods Bullia vittata (Vellammal, 1987), Rapana rapiformis (Rajakumar, 1995). Fencal (1970) also reported the same trend as in the case of the present study of high bacterial count in the hindgut and the declining trend towards foregut and then midgut.

The midgut with digestive gland acts as foremost producers of digestive enzymes in B. spirata. The anatomy of the experimental animal also revealed the presence of digestive glands in the midgut.
Payne et al., (1972) concluded that the low bacterial counts in and around the digestive gland was due to the ingestion of bacteria by phagocytes. Hence the low bacterial load recorded in the midgut in the present study is also justifiable. As reported earlier the midgut is the production site of enzymes and showed the presence of the higher activities of the enzymes like protease, lipase and moderate activity of amylase and maltase. This area was probably the site of protein and lipid digestions. The distribution of protease in \textit{B. spirata} was found to be similar as observed in other carnivorous prosobranchs (neogastropods) by Owen (1966).

The presence of very weak lipolytic enzymes were also recorded by many workers (Meanakshi, 1955; Ward, 1966; Balaparameswara Rao, 1975). Weak lipase activity was also found in neritids (Kanagasabai, 1985). Zacks and Welsh (1953) observed the lipolytic activity associated with midgut epithelium in \textit{Venus mercinaria}. Kamala (1983) found out the presence of lipolytic enzymes in the stomach and digestive gland of \textit{Euchelus asper}. A similar trend in the enzyme study was also reported in \textit{Chicoreus ramosus} by Raghunathan and Ayyakkannu (1992).
Of all the regions the hindgut harbours the maximum bacterial load and this trend was also reported earlier by Kanagasabai (1985) from five neritids, Pitchimuthu (1985) in *Bursa spinosa* Vellammal (1987) in *Bulla vittata*, Rajakumar (1995) in *Rapana rapiformis*.

Except a weak activity of amylase other enzyme activities were totally absent in the hindgut region of the experimental animal. The plausible reason for lesser enzyme activity in the hindgut could be due to the fact that the hindgut was considered to be the limited digestive function and the site of absorption as reported by Fretter (1937). By and large, the number of bacteria and the rate of activity of enzymes of the various parts of the gut could be seen to be inversely proportional to each other, if the bacteria became complementary in function and help in digestion of food. Hence the presence of maximum bacterial load in the hindgut might play a prominent role in the digestion of remaining food material if at all available, by producing suitable enzymes for digestion.

The gut microflora not only play an important role in nutrition, but are also of high ecological significance in detritus
ecosystems for nutrient enrichment (Soedigdo et al, 1970). Bacterial flora in the intestinal tract do exist performing the function of degradation of detritus matter for nutrition allowing the animal enzymes to take care of the digestive processes (Ozaki, 1972). Further Payne et al (1972) reported wherever the enzyme production is minimum or nil the bacterial enzymes released by the gut microflora would be of much helpful in digestion. The present study concludes that the gut microflora could not only help enzymes to function normally but also help in digestion in the absence of digestive enzymes.

It is to be stated here that in carnivorous B. spirata also, the bacteria seem to play an active role in digestion as they do in herbivorous mesogastropods. To a very great extent, they complement the activities of enzymes from the host. By and large, the number of bacteria and the rate of activity of enzymes of the various parts of the gut can be seen to be inversely proportional to each other, if the bacteria became complementary in function and help in the digestion of food. Thus the gut microflora of B. spirata has a significant role to play in digestion.
Chart 1: BACTERIAL IDENTIFICATION CHART

Simudu and Aiso, (1962)

**Gram Staining**

- **Positive**
  - Cocci
  - Small rods
  - Rods
  - Spore
    - Positive: *Bacillus*
    - Negative: *Cyanobacterium*

- **Negative**
  - Pencilin sensitive test 2.5 I v/disc
    - Negative (Resistant)
    - Positive (Sensitive)
      - Hugh and Leifson test
      - Pigmentation (Kings media)

**Fermentative**
- Acid and Gas
  - Kovac’s oxidase Test
    - Positive (Black) *Pseudomonas*
    - Negative *Enterobacter*

**Non-fermentative**
- Acid and No Gas
  - *Pseudomonas*
  - *Flavobacterium cytophasa*

**Yellow/Orange**
- Non-Luminescent *Vibrio*
- Luminescent *Photobacterium*
Table 12: Digestive enzymes activity of the extract of different regions of the alimentary tract of *B. spirata*

<table>
<thead>
<tr>
<th>ENZYMES</th>
<th>REGIONS</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>FOREGUT</td>
</tr>
<tr>
<td>I. Carbohydrates</td>
<td></td>
</tr>
<tr>
<td>1. Amylase</td>
<td>+++</td>
</tr>
<tr>
<td>2. Maltase</td>
<td>+++</td>
</tr>
<tr>
<td>3. Invertase</td>
<td>++</td>
</tr>
<tr>
<td>II. Protease</td>
<td>+</td>
</tr>
<tr>
<td>III. Lipase</td>
<td>-</td>
</tr>
</tbody>
</table>

+ = Weak
++ = Moderate
+++ = Prominent
- = Absent
Table 13: Incidence of total heterotrophic bacterial counts (THB) (CFU/g) of the various parts of the alimentary tract of *B. spirata*.

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>BACTERIAL COUNTS (CFU/g)</th>
</tr>
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<tbody>
<tr>
<td>Foregut</td>
<td>$5.7 \times 10^4$</td>
</tr>
<tr>
<td>Midgut</td>
<td>$6.7 \times 10^2$</td>
</tr>
<tr>
<td>Hindgut</td>
<td>$5.1 \times 10^5$</td>
</tr>
</tbody>
</table>
Table 14: Enumeration of various physiological, bacterial counts associated with different regions of alimentary canal of *B. spirata*

<table>
<thead>
<tr>
<th>BACTERIAL TYPE</th>
<th>BACTERIAL COUNTS (cfu x 10^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FOREGUT</td>
</tr>
<tr>
<td>Amylolytic</td>
<td>3 (15.79%)</td>
</tr>
<tr>
<td>Gelatinolytic</td>
<td>12 (63.16%)</td>
</tr>
<tr>
<td>Caseinolytic</td>
<td>3 (15.79%)</td>
</tr>
<tr>
<td>Lipolytic</td>
<td>1 (5.26%)</td>
</tr>
</tbody>
</table>

Values in parenthesis are percentage of bacterial colonies showing the activity.
PLATE 8: THB in different locales of gut of *Babylonia spirata*