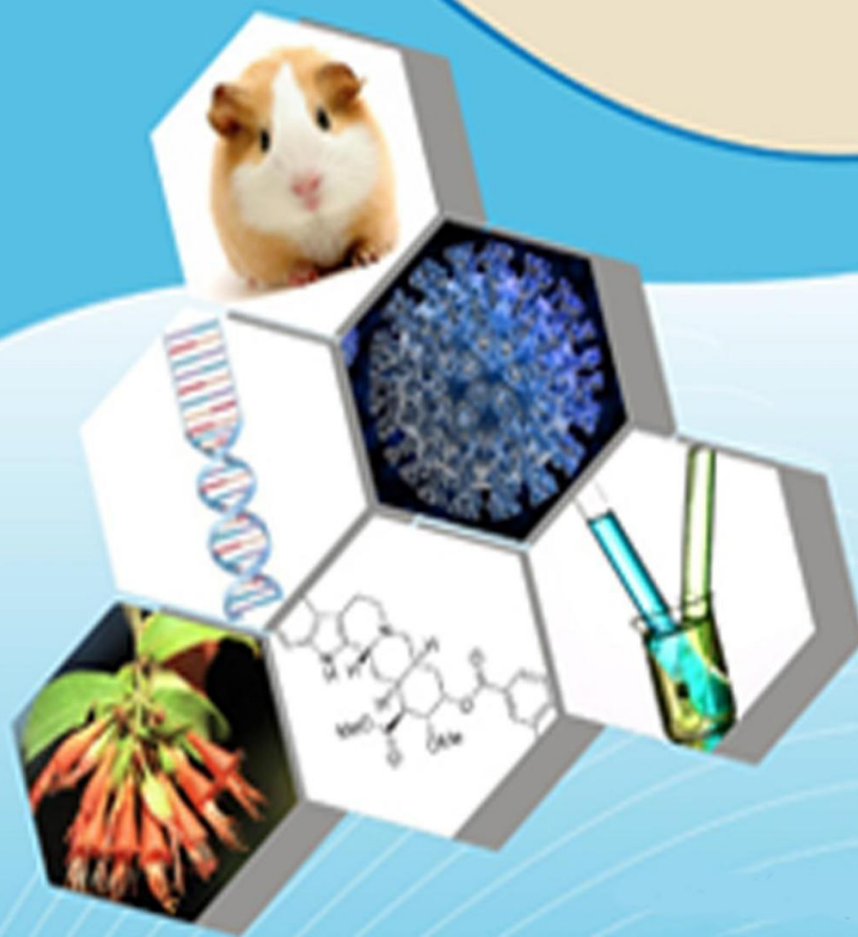




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“Analysis of Gut Microbial Communities in *Telescopium telescopium* from Coastal Mangrove Regions”

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Abstract

Telescopium telescopium is a common mangrove gastropod that plays an important role in organic matter decomposition. This study aims to analyze the gut microflora of *Telescopium telescopium* and its functional significance. Specimens were collected from mangrove habitats, and gut contents were examined using standard microbiological techniques. The results revealed the presence of diverse microbial populations, including bacteria involved in the digestion of complex organic materials. These microorganisms help in nutrient absorption and support the host's survival in the mangrove environment.

Key words: *Telescopium telescopium*, Gut microflora, Decomposition.

1 Introduction

Aquatic organisms have recently emerged as rich source for the isolation of enzymes. The enzymes are defined as “simple or combined proteins acting as specific catalysts”. The gut consists of a mouth, buccal cavity, oesophagus, stomach, intestine, rectum and anus. Digestion is always at least partly extracellular with few exceptions, the enzymes for extracellular digestion are produced by the salivary gland, oesophageal pouches, digestive caeca or a



combination of the structures. It is presumed that the alimentary tract is a major site of interaction between an animal ecosystem and its physiology (Subavathy *et al.*, 2017).

The digestive enzymes and extracellular enzymes of the associated microbes inside the tract of alimentary canal play an important role in the digestion of food in *T. telescopium*. The gastrointestinal tract of animals can serve multiple functions including digestion, osmoregulation and protection. There are several reports available on digestive enzymes of the different regions of the gut microflora of marine gastropods (Jeyaprabha, 2012).

The molluscs have surprising enzyme equipment. Indeed there seems to be no other group in the animal kingdom with such an array of digestive enzymes. Studies on the digestive enzymes of lamelli branches were first initiated by Younge (1926). In oyster Mansour and Zaki (1946) reported the presence of protease, peptidases and lipases from the stomach juice as well as digestive diverticula of *Unio prasedens*.

Digestive enzymes play an essential role in food degradation and are determinants of the digestibility and assimilation efficiency (Fernandez *et al.*, 2001; Picos Garcia, 2000). In fact the ability of an animal to digest and absorb nutrients depends on the presence and the quality of their digestive enzymes. (Kumar *et al.*, 2007; Alarcon *et al.*, 1998).

Bacterial flora of the digestive system of aquatic animals primarily integrates into the protective function of the organism and decomposition of natural and extraneous, useful and harmful substances as well as the synthesis of the missing ones (Janina Syvokiene *et al.*, 2008).

Knowledge of the digestive tract microbiome of an organism can provide insight into its physiological functions (Rup, 2012). The digestive tract is the primary site of microbe – host interactions (Vorn Horn *et al.*, 2012), Host diet, as well as the anatomy and physical and chemical conditions of the digestive tract, shape the microbial community structure. Digestive



microbial flora play a vital role in food biodegradation, digestion, nutrient absorption resistance to infectious pathogens and the health of host aquatic animals (Iehata *et al.*, 2015). For instance, gut microbes function in gut epithelial cell proliferation and differentiation and are known to modulate the innate immune response and nutrient metabolism in zebrafish (John Rawls *et al.*, 2004; Bates *et al.*, 2006). In the giant African snail *Achantia fulica*, several strains of cellulose producing bacteria from symbiotic gut micro – organisms contributed to cellulose degradation (Pawer *et al.*, 2012).

Two groups of digestive enzymes, carbohydrases and lipases have been identified in the crystalline style of bivalves (Owen, 1966; 1974; Morton, 1983). The presence of amylase in the style of bivalves was reported by Morton (1983). Other carbohydrases like glucosidases and galactosidases and structural polysaccharases like cellulase carboxy methyl cellulase and chitinase were also shown to be present in the style of many bivalves (Owen, 1966, 1974; Morton, 1983; Shahul Hameed and Paul Pandian, 1987; Tachibana *et al.*, 2005).

From a view point of enzymology the hepatopancreas of marine animals expected to be rich in various hydrolyzing enzymes. Since Yokoe and Yasumasu (1964) discovered cellulolytic activity in marine invertebrates, the presence of several glucan degrading enzymes including cellulase Anzai *et al.*, 1984, amylase (Tsao *et al.*, 2003); Onishi *et al.* (1985) extensively surveyed the distribution of glycan hydrolases in gastropods and bivalves and reported that a crude extract of 50 species of shellfishes possessed diverse enzymatic activities.

Amylases are known to be more active in herbivores than in carnivores due to a diet composed almost exclusively of carbohydrates (German *et al.*, 2010). In molluscs amylase has been described in herbivorous species, such as *Perna viridis* (Sababathy and Teo, 1992), *Hyriopsis bialata* (Areekijserree *et al.*, 2004), *Mytilus galloprovincialis* (Lombrana *et al.*, 2005).



and Several *Haliotis* species *Picos Garcia et al.*, 2000; *Tsao et al.*, 2003; *Viana et al.*, 2007; *Hsieh et al.*, 2008; *Nika pitiya et al.*, 2009 and *Conchoiepas concholepas* (*Alejandro et al.*, 2017).

Potamidid snails are mesogastropods which are unique in resembling bivalves in having crystalline style, a proteinaceous structure that produces digestive enzymes (Sreenivasan, 1995). Proteolytic enzymes in the digestive organs of invertebrates have been well documented and characterized eg: spiny lobster *Pannulirus interruptus* (*Celis-Guerrero et al.*, 2004), shrimps like *Artemesia longinaris* (*Fernandez Gimenez et al.*, 2002), the pacific oyster *Crossostrea gigas* (*Luna-Ganzolez et al.*, 2004) and various species of abalone *Haliotis rufescens* (*Garcia-Esquivel and Felbeck*, 2006), black abalone *Haliotis rubra* (*Edward and Condon*, 2001). However, studies of the digestive enzymes in mangrove gastropods are scarce. Hence the present study throws light on gut microflora and enzymes in the alimentary tract of *T. telescopium*.

2 Materials and methods

1 Enumeration of total viable heterotrophic bacterial counts

The fresh snails were sampled from the habitat and brought to the laboratory in a bucket containing the same water. 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 mollusc.

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.

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 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, 1 g of different regions of digestive tract of *T. telescopium* 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 75% sterile seawater. Further serial dilutions were made using 9 ml of 75% sterile seawater blanks.

4 Plating and enumeration of viable bacterial counts

The viable number of bacterial counts of foregut, midgut and hindgut of *T. telescopium* was enumerated using pour plate method. 1 ml of aliquots from the appropriate dilution was pipetted out into a sterile Petri dish. 20 ml of sterile molten nutrient agar were aseptically poured into the petriplates and were rotated in clockwise and anti clockwise 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.

5 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 *T. telescopium* 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.

5.2.6 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 out growth was recorded as positive or negative amylolytic reaction respectively.

7 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.

8 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 isolate. The formation of oleic acid around the edge of the halozone was recorded.

9 Qualitative assessment of digestive enzymes from the digestive tract of *T. telescopium*

The gut of *T. telescopium* was dissected and divided into three regions viz.

The foregut comprising buccal complex and esophagus.

The midgut comprising the stomach and digestive gland, crystalline style sac.

The hindgut comprising the intestine and rectum.

For qualitative analysis of the digestive enzymes of *T. telescopium* the digestive enzyme extracts were prepared by homogenizing weighed quantity of the various digestive region 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).

5.2.10 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 starch has been hydrolysed in the experimental tube.



11 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 (maltose) and there is no change in control.

12 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°C. After an hour, one drop of Fehling's 'A' and one drop of Fehling's '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.

5.2.13 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°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.

14 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°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.

3 Results

The occurrence and activity of various digestive enzyme present in the digestive tract of *T. telescopium* are given in Table (1) Plate (.1, 2).



The results of total heterotrophic bacterial counts (THB) and various physiological groups were enumerated from different parts of the alimentary tract of *T. telescopium* and are shown in (Table 2) (Plate 1). The THB recorded in the foregut, midgut and hindgut were 6.8×10^4 (cfu/g), 4.8×10^5 (cfu/g) and 8.9×10^6 (cfu/g) respectively.

In the foregut of *T. telescopium* the carbohydrase amylase was prominent and this region showed very weak maltase activity and no invertase activity was observed in the foregut region of *T. telescopium*. It showed a moderate concentration of the enzymes protease and lipase (Table 1). The physiological bacterial populations like amylolytic, caseinolytic and lipolytic groups were also identified from different regions of the alimentary tract. Foregut harboured 32.93% of amylolytic, 45.12% of caseinolytic and 21.95% of lipolytic bacterial groups (Table 5.3).

The enzyme activity in the midgut, protease and lipase were found to be more robust whereas carbohydrases were weak. Protease and lipase activities were found to be more significant in the midgut, whereas carbohydrase activities were found to be moderate (Plate 5.3). In the midgut 40.63% of amylolytic, 34.38% caseinolytic and 25% of lipolytic bacterial populations were recorded (Table 3).

The highest bacterial counts were recorded in the hindgut region than the other two locales. The hindgut showed 30.01% of amylolytic, 32.00% of caseinolytic and 38.00% of lipolytic bacterial populations. Lipolytic activity was high in the hindgut followed by caseinolytic and amylolytic activities.

Plate 1 : THB in different locales of gut of *T. telescopium*



Foregut



Midgut



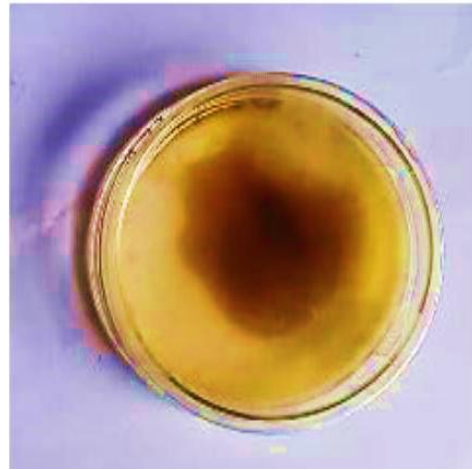
Hindgut

Plate 2 : Physiological groupings in *T. telescopium*

Amylolytic activity



Positive
Caseinolytic activity



Negative



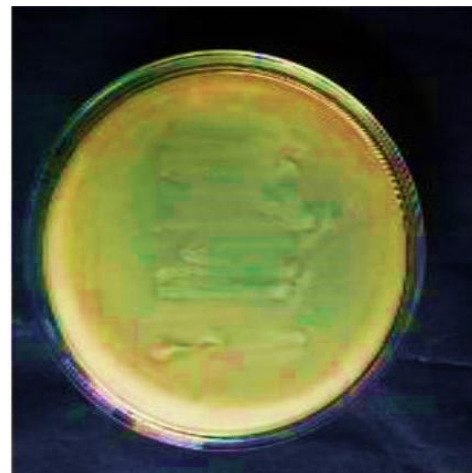
Positive
Lipolytic activity



Negative

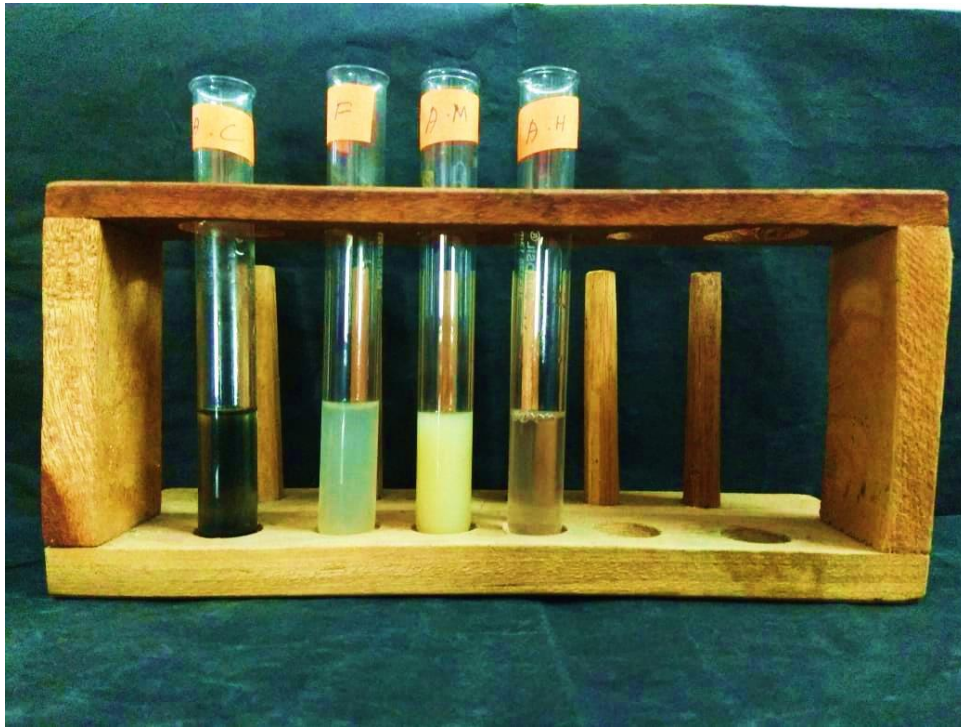


Positive
Plate 3 : Enzyme Activities



Negative

1. Amylase activity



2. Maltase activity



3. Invertase activity



4.

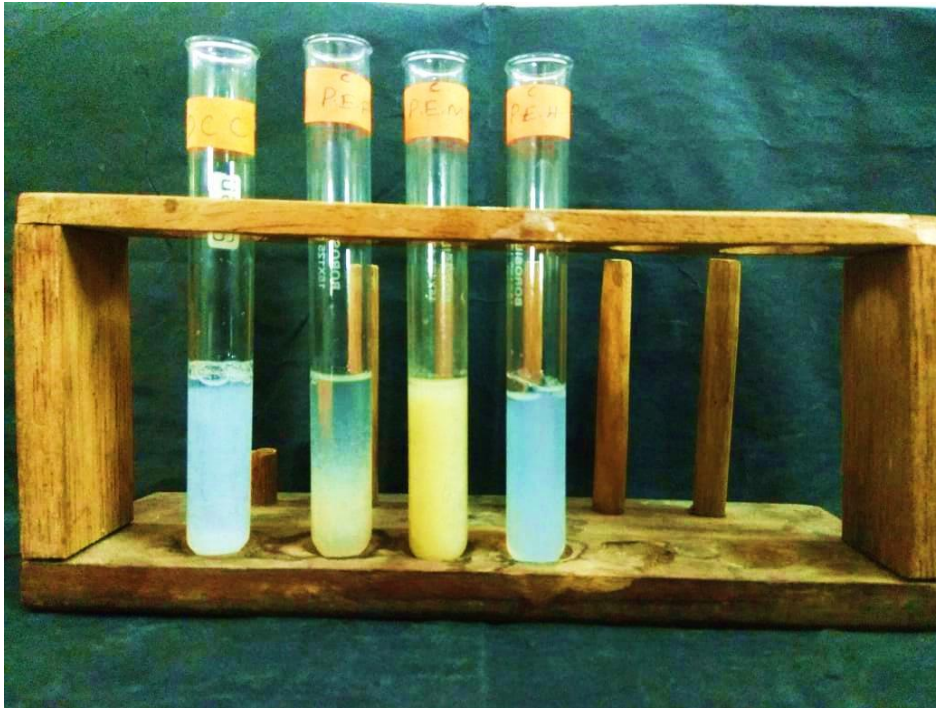
a. Biuret test

Proteaseactivity





b. Conjugation test



5. Lipaseactivity



**Table 1 : Digestive enzymes activity of the extract of different regions of the alimentary tract of *T. telescopium***

Enzymes	Regions of the Alimentary Canal		
	Foregut	Midgut	Hindgut
I			
Carbohydrate			
Amylase	+++	++	+
Invertase	-	-	-
Maltase	+	+++	+
II			
Protease			
Conjugation test	+	+++	+
Biuret test	++	+++	+
III			
Lipase	++	+++	+

+ = Weak activity

++ = Moderate activity

+++ = Very high activity of enzymes

- = No activity

**Table 2 : Incidence of total heterotrophic bacterial counts (THB) (cfu/g) of the various parts of the alimentary tract of *T. telescopium***

Source	Bacterial Counts (CFU/g)
Foregut	6.8×10^4
Midgut	4.8×10^5
Hindgut	8.9×10^6

Table 3 : Enumeration of various physiological, bacterial counts associated with different regions of alimentary canal of *T. telescopium*

Bacterial Type	Bacterial Counts (CFU $\times 10^5$) Different regions of alimentary canal of <i>T. telescopium</i>		
	Foregut	Midgut	Hindgut
Amylolytic	27 (32.93%)	26 (40.63%)	15 (30.01%)
Caseinolytic	37 (45.12%)	22 (34.38%)	16 (32.00%)
Lipolytic	18 (21.95%)	16 (25.00%)	19 (38.00%)

Values in parenthesis are percentage of bacterial colonies showing the activity

4 Discussion

The total heterotrophic bacterial population was higher in the hindgut followed by foregut and midgut in the experimental organism. The largest population of bacteria was discovered in the hindgut (Plante *et al.*, 1990; Harris *et al.*, 1993). Due to the fact that invertebrate food is absorbed in the hindgut, and since the production and activity of digestive enzymes are weak or absent in the hindgut, Plante *et al.* (1990) hypothesized that the invertebrate hindgut would be a prime bacterial habitat. The enzyme activity of *T. telescopium* are likewise lower in the hind intestine compared to the other two regions, as shown in the current research.



In *T. telescopium*, the digestive process began in the mouth and foregut. According to reports, *Tegula Funerbralis* foregut has potent amylolytic action (Galli and Giese, 1959). Carbohydrates were found to be predominate in the foregut of the experimental animal, suggesting that this area is likely where carbohydrates are digested (Subavathy *et al.*, 2017).

T. telescopium midgut, which is equipped with digestive glands, is where the majority of the digestive enzymes are produced. According to (Jeyaprabha, 2012), the hindgut is the place of reduced digestive action and absorption, which may explain why there is less enzyme activity there. Similar trends were seen in the current investigation, with less bacteria in the midgut and foregut as compared to the hindgut. The highest concentration of bacteria was found in the hindgut, whereas the lowest concentration was found in the midgut. The presence of low bacterial counts in the midgut was also reported in *Bullia vittata* (Vellammal, 1987), *Bursa spinosa* (Petchimuthu, 1985), *Rapana rapiformis* (Rajakumar, 1995); Shenbaga valli (2009) also reported higher bacterial load in the hindgut followed by foregut and midgut. The new study findings corroborate those previous ones quite well.

In addition to their involvement in feeding, gut bacteria have significant ecological importance in detritus habitats due to their ability to fill the ecology with nutrients. Bacterial flora in the intestines degrades waste stuff for nutritional use, relieving pressure on the animal own digestive enzymes (Jayaprabha, 2012).

In addition, Payne *et al.* (1972) found that the bacterial enzymes produced by the gut microflora would be of significant aid in digesting when the production of enzymes is minimal or nonexistent. Herbivores, whose diet consists nearly entirely of carbohydrates, have more active amylases than carnivores (German *et al.*, 2010). Herbivorous molluscs including the *Hyriopsis bialata* (Areekijsee *et al.*, 2004), *Mytilus galloprovincialis* (Lombrana *et al.*, 2005), and a number of *Haliothis* species have been characterized as possessing amylase (Viana



et al., 2007; Hsieh *et al.*, 2008; Nikapitiya *et al.*, 2009). Carnivorous fishes of high aquaculture value, such as salmonids, have also been reported, however their amylase activity is lower than their proteolytic activity (Hidalgo *et al.*, 1999). *T. telescopium* in this investigation was found to eat debris from mangrove forests (deteriorated and semi decayed leaves of mangrove plants). Amylase activity was highest in the foregut, lowest in the midgut, and highest in the hindgut.

Muricid gastropods have a highly developed gland of leiblen, which is responsible for producing digestive proteases such as trypsin, chymotrypsin, carboxypeptidases, and amino peptidases (Andrew and Thorogood, 2005). Proteolytic enzymes are essential for breaking down proteins in diet and storing chemical energy (Sainz *et al.*, 2004). Since they are responsible for the breakdown of dietary proteins, protease activity in the current investigation was highest in the midgut and lowest in the foregut.

It is to be stated that in detritivorous mesogastropod. *T. telescopium*, the bacteria seem to play an active role indigestion. To a very great extent, they complement the activities of enzymes from the host. In general, the number of bacteria and the rate of enzyme activity of the various parts of the gut seem to be inversely proportional to each other, if bacteria become complementary in function and help in digestion of food (Thilaga *et al.*, 2010). Thus the gut microflora of *T. telescopium* has a significant role to play in digestion.

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