

## pH and Temperature Dependent Gut Enzyme Niche in a Stomachless Herbivorous Freshwater Fish *Amblypharyngodon mola* (Hamilton, 1822)

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### Abstract

The activities of digestive  $\alpha$ -amylase (E. C. 3.2.1.1), total proteases, and bile salt-activated lipase (E. C. 3.1.1.-) along the digestive tract (lengthwise divided into five equal parts) of a stomachless freshwater fish (n = 10, weight =  $4.354 \pm 0.316$  g, standard length =  $21.641 \pm 2.271$  cm) were measured at different pH and temperature levels. Different optimum pH and temperature for the activity of  $\alpha$ -amylase (8-9, 35°C), proteases (7-8, 45°C), and lipase (8, 45°C) were observed. The first two regions of the digestive tract showed comparatively higher activity of all enzymes. The hierarchical clustering technique revealed three different enzymatically active regions, more inclined to pH in the digestive tract of the studied fish. The present study also supports that the stomachless gut of *A. mola* has substantial resemblances to the intestinal part of the digestive tract of fish.

**Keywords:**  $\alpha$ -amylase; *Amblypharyngodon mola*; Bile salt-activated lipase; Herbivorous fish; Proteases; Stomachless gut.

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### 1. Introduction

Digestion is a fundamental process in the metabolism of animals because it determines the availability of nutrients needed for all biological concerns where digestive enzymes play a key role [1]. In case of fish, Saikia [2] suggested enzyme assay from gut as a more convincing way to explain the qualitative selection of feeds in the environment. In all aquatic species including fish, digestive enzyme activity serves as a good indicator of their feeding ecology and trophic niche in natural conditions [3].

*Amblypharyngodon mola* (family Cichlidae) has recently received attention as a potential animal crop for alleviating malnutrition in human populations because of its capability to retain high amounts of micronutrients [4-6]. It is reported to be exceptionally rich in vitamin A, calcium and iron [7-9]. This species thrives in small freshwater bodies and is indigenous to south and south-east Asia, including India, Bangladesh, Pakistan, Myanmar, Nepal and Bhutan [10]. In IUCN databases, this fish is recorded as 'least

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concern', although unreported cases of vast loss of the habitat of this species have occurred. Several research works have been carried out on its growth, longevity [11-14], feeding [15,16] and reproductive biology [17-19]. However, the biochemical feature of the gut has not been considered while characterizing its nature of feeding. For culture practices, it is necessary to understand the behavior of the gut enzymes to biochemically classify its food and feeding environment. An attempt has, therefore, been made to understand the digestibility pattern to correlate the existing knowledge on the feeding ecology of this fish. Such knowledge of digestibility is also important for the formulation of different feeds to grow out culture of the fish. The study also aimed to examine how these enzymes are distributed along the digestive tract of the fish and whether their action follows any spatial dependent action regulated by pH or temperature or both.

## 2. Materials and Methods

### 2.1. Experimental animals

The fish were collected from a local fish pond, Hatserandi, Birbhum, West Bengal, India (23°37'N, 87°49'E) in July 2018 using gill net during early hours (08:00-09:00 am) and brought alive to the laboratory and kept unfed for 48 h in fiberglass aquaria (45×30×30 cm, 30 L). The temperature of the aquarium was maintained at  $24.67\pm 1.87$  °C; dissolved oxygen (D. O.)  $7.52\pm 0.21$  mg/L; pH  $7.47\pm 0.32$ ; conductivity  $616\pm 20.9$   $\mu$ S and photoperiod 12:12 (light hour: dark hour). The measurements of pH, temperature and conductivity were recorded with the help of a digital multi-parameter tester (PCSTestr 35, Oakton). D. O. was measured by an oxygen meter (DO-5510, Lutron).



Fig. 1. Gross morphology of adult Mola (*Amblypharyngodon mola*).

### 2.2. Chemicals

p-nitrophenyl palmitate was purchased from Sigma, UK. Other chemicals used in this study were purchased from Sisco Research Laboratories Pvt. Ltd. (SRL), India. All chemicals used were of analytical grade.

### **2.3. Measurement of gut index**

Several gut parameters were calculated, i.e. relative gut mass (RGM) = gut mass (g) × body mass (g) [20], relative gut length (RGL) = gut length (mm)/standard length (mm) [21], and Zihler's Index (ZI) = gut length (mm)/10 × [body mass (g)]<sup>1/3</sup> [22].

### **2.4. Preparation of extracts**

Before dissection of fish a small amount of food was introduced to stimulate the digestive system for a short time. The whole gut was removed and washed in chilled distilled water. The gut was divided into five segments of equal length and identified as Mola anterior (MA), middle 1 (MM1), middle 2 (MM2), middle 3 (MM3) and posterior (MP). Samples (n=10) were pooled and homogenized with a micro pestle (Tarsons) at 4 °C in 0.1 M Tris-HCl buffer, pH 7.4 then centrifuged (K2015R, UK) at 10,000 g at 4 °C for 10 min, and the supernatants were collected carefully.

### **2.5. Estimation of digestive enzymes**

The activity of  $\alpha$ -amylase (E. C. 3.2.1.1) was measured following Bernfeld [23] and the enzyme activity was expressed as the measure of U (1  $\mu$ g maltose liberated per hour) per  $\mu$ g protein. Casein was used [24] to measure the activity of total proteases according to Walter [25]. One unit of enzyme activity in each sample was expressed as the amount of enzyme required to liberate 1  $\mu$ g of tyrosine in one hour. Bile salt-activated lipase (E. C. 3.1.1.-) activity was assayed according to German et al. [3] using 4-nitrophenyl palmitate as substrate and results were expressed in U (1  $\mu$ g 4-nitrophenol liberated per hour) per  $\mu$ g protein. Determination of the protein was done following Lowry et al. [26].

### **2.6. Determination of optimal pH and temperature range**

At first, the optimal pH for each type of enzyme activity was determined by spectrophotometer (UV-1800, Shimadzu, Japan) using different buffer solutions ranging from pH 1 to 10 as an assay medium with appropriate substrate solution and the homogenized extract, which was prepared from each gut region containing the active enzymes into it. The buffers used were 0.2 M KCl-HCl buffer (pH 1 and 2), 0.2 M glycine-HCl buffer (pH 3), 0.1 M citrate buffer (pH 4, 5, and 6), 0.2 M tris-HCl buffer (pH 7, 8, and 9), 0.2 M glycine-NaOH (pH 10) respectively. Particularly for lipase activity, the range of pH was fixed from pH 7 to 10, as it can be active only in neutral to the alkaline environment [27-29]. Optimal temperatures for enzymes from each gastrointestinal tract region were determined by assaying their activity at different temperatures in a standard shaking water bath (Instrumentation India) starting from 25 to 65 °C (for amylase), 60 °C (for proteases) and 50 °C (for lipase) with 5 °C interval under optimal pH until a very low value of activity was observed. The spectrophotometric activity assays were performed in triplicate and the whole work has been done thrice.

## 2.7. Data analysis

One-way ANOVA was performed and followed by Tukey's post hoc test to elucidate the proper distribution of active digestive enzymes along the length of the gut. For two-way ANOVA, pH responses were divided as low to moderate (pH 1-6) and moderate to high (pH 5-10) for both amylase and proteases activity. Considering heavy tailed distribution of the data, Scheirer-Ray-Hare nonparametric test of two ways ANOVA was used to understand the strengths of impact of different pH, temperature and gut regions upon the activity of different enzymes. The alpha level was taken as 0.05 for all statistical analysis. Results were reported as mean values with SE.

Furthermore, the similarity patterns of the enzyme activity among the said gut regions with the change of temperature and pH were studied through clustering technique on scaled variables. Here, "single linkage agglomerative clustering" was performed which merges similar objects by 'nearest neighborhood' strategy based on the Euclidean distance matrix. Additionally, for double-checking, Ward's "minimum error variance within cluster" criterion of partitioning was adapted and to verify whether a similar type of partitioning was achieved or not, the corresponding dendrograms were showcased. Subsequently, silhouette analysis was performed to check the quality of clustering, hence scrutinizing the validity of consistency within the cluster of data. All the statistical analyses were done using R Studio (version 3.5.1) and some graphs were prepared in GraphPad PRISM (version 5.01).

## 3. Results and Discussion

### 3.1. Digestive tract index data

In fish, the digestive tract is a thin long straight continuous tube with or without a stomach. Earlier, guts from stomachless fish were divided into four equal regions as proximal, middle, distal and rectal [30, 31]. However, in the present case of *A. mola* it was divided into five equal regions for a precise understanding of the functional features of enzymatic activity. Calculated RGM, RGL and ZI were  $2.02 \pm 0.65$ ,  $3.59 \pm 0.17$  and  $14.10 \pm 1.87$  respectively. The high value of RGM, which is a relative indicator of herbivory [32] and is an attribute of the digestive tract in fish [21], suggested *A. mola* as an herbivore. The high RGL and ZI values also supported the similar nature of the fish [33,34].

### 3.2. Determination and distribution of digestive enzymes

The enzyme,  $\alpha$ -amylase is the primary glucosidase found in fish [35]. Distribution of amylase activity along the intestine of *A. mola* is summarized in Fig. 2. The higher activity of amylase was associated with the proximal parts (MA, MM1) over the middle (MM2, MM3) and distal (MP) parts of the gut. Higher activity of amylase may be due to the proximal association of the hepatopancreas, but the source of moderate amylase

activity in the distal part was unknown. A similar pattern was reported in herbivorous fish *Arrhamphus sclerolepi*, where higher amylase activity in the anterior part of the gut portion was followed by the subsequent parts of the intestine [30]. High amylolytic activity is coordinated with starch digestion and glucose absorption, which occur mainly in the anterior part of the intestine [36,37]. Being a herbivore fish, *A. mola* mainly feeds on phytoplankton [16], a good source of carbohydrate and this fact could be supplemented by the findings of this study when amylase activity was predominant over protease and lipase activity in the gut. Generally, herbivorous fish have high amylolytic activity than carnivorous fish, which have a low-carbohydrate diet [38].

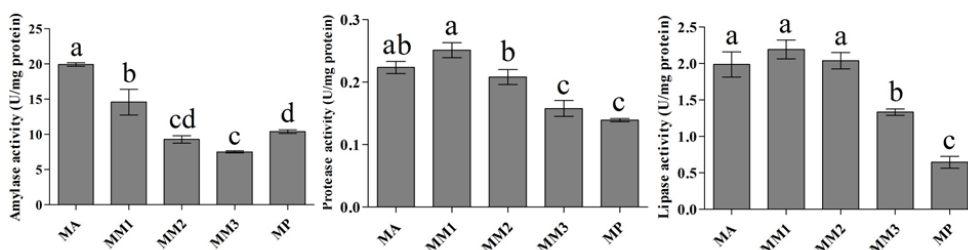


Fig. 2. Activity of amylase (left) along the digestive tract of *Amblypharyngodon mola* (n = 10). Values are expressed as mean±SE. Bars marked with differing letters are significantly different (one-way ANOVA, P< 0.05).

The activities of the amylase enzyme in MA and MM1 segments had an optimum pH of 9 (Fig. 3.) and the other segments shared a common optimum pH of 8. Optimum pH for the enzyme activity may vary along the digestive tract [29,39] and within different fish species [40,41] as several isoforms may be present for a particular enzyme.

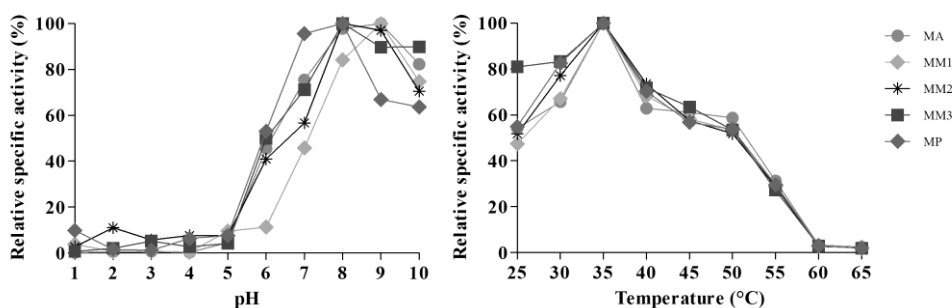


Fig. 3. Effect of pH (left) and temperature (right) on relative specific activity of  $\alpha$ -amylase along the digestive tract of *Amblypharyngodon mola* (n = 10). Enzyme activity is expressed as relative specific activity (RSA).  $RSA\% = (Z_i/Z_{max}) \times 100$  [ $Z_i$  = enzyme activity at specific pH;  $Z_{max}$  = maximal enzyme activity at optimum pH].

Generally, amylase activity peaks at natural or alkaline pH (with a wide range of 7 to 9) in the fish intestine [27,39,42]. Some authors also reported that in some fish the optimum pH

for amylase activity was slightly acidic from 4.5 to 6.7 [40,43,44]. The optimum temperature for the amylase activity was 35°C (Fig. 3). It was known that temperature ranging from 30° to around 55 °C favors amylase activity in wild fish [27,35,39,41].

The studies of proteases mean the study of pepsin, trypsin, chymotrypsin, aminopeptidase, carboxypeptidase that act as a battery of enzymes [45,46]. The first two regions of the gut of *A. mola* (i.e. MA and MM1) were found to be the maximum proteolytically active (Fig. 2) and it fell further beyond MM2. This pattern was more or less similar to an herbivore fish *Sarotherodon mossambicus* where the proteases activity was high at the anterior and decreased towards the remaining region of the gut [47]. Proteases activity was highest at pH 7 in the MM3 region of the intestine whereas in the other regions it was 8 (Fig. 4). The optimum pH for alkaline proteases was 8 in MA, MM1, MM2 except for MM3 where it was 7. So, the alkaline nature of gut favours protease digestion in *A. mola*. These findings were congruent with other studies where the optimum activity of proteases was reported close to pH 8 to 10 [48,49].

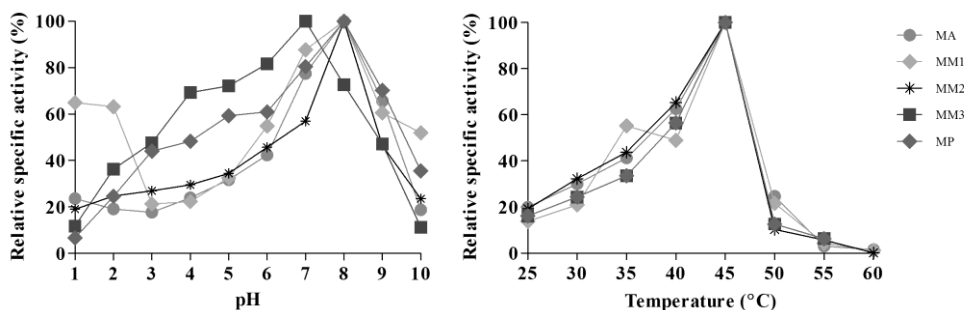


Fig. 4. Effect of pH (left) and temperature (right) on relative specific activity of proteases along the digestive tract of *Amblypharyngodon mola* (n = 10). Enzyme activity is expressed as relative specific activity (RSA).  $RSA\% = (Z_i/Z_{max}) \times 100$  [ $Z_i$  = enzyme activity at specific pH;  $Z_{max}$  = maximal enzyme activity at optimum pH].

The MM1 region showed some degree of acid proteases activity at pH 1-2, which was very unusual for a stomachless fish and may be possible due to some exogenous enzyme sources. Concerning temperature, in the current study, the maximum activity of proteases was found to be at 45 °C (Fig. 4). Earlier studies showed the activity of proteases was optimum in a range of 35-40 °C [43,48]. However, some other classical studies reported a slightly higher optimum temperature of proteases activity ranging from 50 to 55 °C [41,47,50].

Lipase is a very important digestive enzyme and responsible for the hydrolysis of ester bonds within the triacylglycerol at the hydrophilic-hydrophobic boundary [51,52]. The first three regions of the gut viz. MA, MM1, MM2 showed significantly higher activity of lipase compared to MM3 and MP (Fig. 2). The opening of the gall-bladder along with the hepatopancreas may be associated with the higher activity of lipase in the

anterior region. Such an outcome was consistent with another stomachless fish *Hyporhamphus regularisar delio* [30]. The optimum pH for lipase was 8 (Fig. 5).

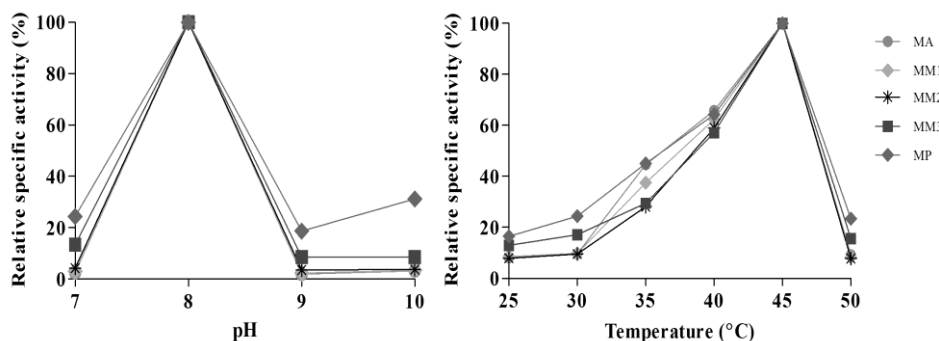


Fig. 5. Effect of pH (left) and temperature (right) on relative specific activity of lipase along the digestive tract of *Amblypharyngodon mola* (n = 10). Enzyme activity is expressed as relative specific activity (RSA).  $RSA\% = (Z_i/Z_{max}) \times 100$  [ $Z_i$  = enzyme activity at specific pH;  $Z_{max}$  = maximal enzyme activity at optimum pH].

Different studies reported the optimum pH for lipase within alkaline range i.e. 8 to 9 [23, 32,51]. On the other hand, Fu *et al.* [53] recorded neutral or very close to neutral pH as optimum for lipase activity. In the present case, the optimum pH for lipase was found to be 8.0 (Fig. 5). The optimum temperature for lipase activity was 45 °C in the studied fish (Fig. 5) similar to that of the Pacific bluefin tuna [27]. There are different optimum temperature reported for lipase activity in fish such as 35 °C [28], 40 °C and 50 °C [39].

The physiological processes in fish are correspondent with the environmental temperature since the seasonal variation of temperature is one of the main extrinsic factors, which can influence the metabolic rate of aquatic organisms [54]. Digestive enzymes reach maximum activity at higher temperatures than those at which they currently act to allow for thermal adaptations [27]. For surface or bottom water levels, the optimum activity of enzymes at different temperatures may be an adaptation.

### 3.3. Interaction of pH, temperature and gut regions

The influence of pH (Table 1) in the exposition of enzymes appeared as highly significant ( $p < 0.05$ ). On the contrary, for proteases (both acidic and alkaline) and amylase (alkaline), the influence of gut regions on the digestive system was insignificant. But the influence of gut regions on the activity of acidic amylase and lipase showed prominence ( $p < 0.05$ ).

Table. 1. Results of two-way ANOVA analyses testing the influence of different gut regions, pH and their interactions on the activity of five different types of digestive enzymes.

Dependent variable	Source	Degree of freedom	p-value
Acidic Proteases	Gut region	4	0.193
	pH	5	0
	Gut region x pH	20	0.999
Alkaline Proteases	Gut region	4	0.083
	pH	5	0
	Gut region x pH	20	0.999
Acidic Amylase	Gut region	4	0
	pH	5	0
	Gut region x pH	20	0.327
Alkaline Amylase	Gut region	4	0.052
	pH	5	0
	Gut region x pH	20	0.372
Lipase	Gut region	4	0.058
	pH	3	0
	Gut region x pH	12	0.993

Table. 2. Results of two-way ANOVA analyses testing the influence of different gut regions, temperatures and their interactions on the activity of three different types of digestive enzymes.

Dependent variable	Source	Degree of freedom	p-value
Proteases	Gut region	4	0
	Temperature	5	0.013
	Gut region x Temperature	20	0.997
Amylase	Gut region	4	0
	Temperature	5	0
	Gut region x Temperature	20	0.999
Lipase	Gut region	4	0.059
	Temperature	5	0
	Gut region x Temperature	20	0.999

In the second Table (Table 2) too, the influence of gut regions on amylase and proteases were significant ( $p < 0.05$ ) while it fails to be significant in lipase. In contrast to the influences of gut regions, the temperature had a strong significant effect on amylase and lipase but a very weak effect on proteases. The temperature has already been shown as an independent factor to influence amylase activity [54]. Gelman *et al.* [55] highlighted that enzyme adaptation to temperature is genetically determined and iso-enzyme may catalyze the same substrate with different maxima at different temperatures. A similar explanation may be forwarded for pH also. This is the reason why the interaction between two explanatory variables viz. gut region and pH or gut region and temperature for each type of enzymes portray as insignificant ( $p < 0.05$ ) which clarifies the nature of independence of effect of each explanatory variable with respect to other in the study of the response. Apart from pH and temperature, different regions of the gut can significantly affect the



activity of a particular digestive enzyme. Because, along the gut, the distribution of enzyme producing cells or the microenvironment for the digestive activity was not similar, and these attributes were strictly associated with the lumen of the gut only.

### 3.4. Clustering of gut regions

In case of temperature as a variable (Fig. 6), both Single linkage and Ward’s method direct towards partitioning the whole gut regions in terms of three clusters viz.  $C_1$ : (MM1, MA),  $C_2$ : (MM2),  $C_3$ : (MM3, MP). In three cluster-partitioning the region MM2 ascertains its borderline character which might be sorted out if it was merged with (MM1, MA), hence forming  $C_1$ : (MM1, MA, MM2) and  $C_2$ : (MP, MM3).

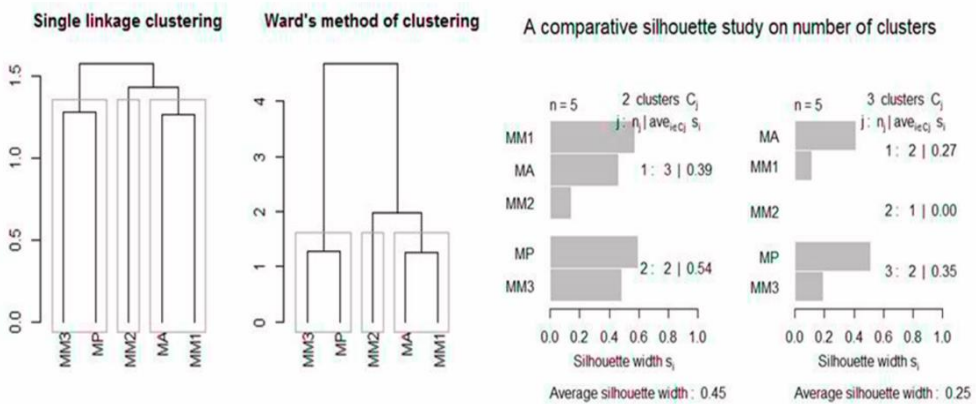


Fig. 6. Clustering of Gut regions in terms of enzyme secretion taking temperature as variable. Both Single linkage clustering and Ward’s methods are shown. The comparative silhouette plots were also shown to describe the best numbers of clusters.

This merging would enhance within cluster silhouette width strengthening within cluster bond. But two cluster partitions are too trivial to deal with, so it is better to stick to three cluster partitioning. Similarly, in case of pH as a variable (Fig. 7.), both Single linkage and Ward’s method uncover the same partitioning of gut regions viz.  $C_1$ :MM2,  $C_2$ : (MA, MM1) and  $C_3$ : (MM3, MP). Moreover, the following silhouette analysis projects the average silhouette width as 0.54 which strongly validates the separation distance between the three resulting clusters.

### 3.5. Probable enzyme niche

The five regions of the tract discussed in our study are more or less different in terms of acting as a zone for the studied digestive enzyme. But, there lies a strong possibility for two or more gut regions to exhibit optimum enzyme activity in a similar manner. The region-wise activity of enzymes based on pH and temperature showed gut regions with similar enzyme activity and such homogeneous regions were termed as ‘enzyme niche’.

To check such ‘enzyme niche’ across the different gut regions of *A. mola*, the clustering technique was performed on the selected scaled variables. In the case of pH based clustering, all gut regions revealed three clusters in terms of the activity of all enzymes studied. Subsequently, similarity patterns of these gut regions with respect to the activity of the same enzymes with the range of temperature also exhibited three clusters. In both cases, the first enzyme niche comprises of MM1 and MA, whereas the second comprises of MM3 and MP and the third one was MM2. On comparison of silhouette analysis, the pH based clustering gave more strong niche formation in terms of gut functioning. In an agastric situation and loss of pyloric caeca, having a long intestine is an adaptive feeding in fish [56]. The existence of ‘enzyme niche’ as in the case of *A. mola* may be another feature to compensate agastric situation or mobilization of digestive functions.

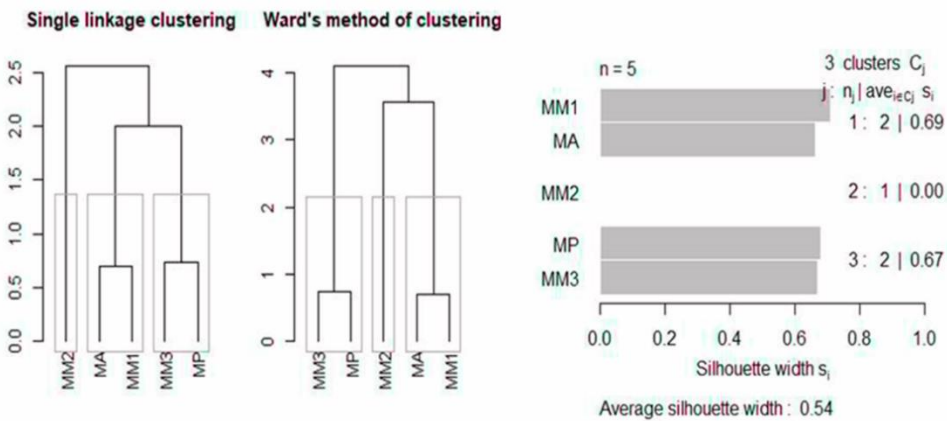


Fig. 7. Clustering of Gut regions in terms of enzyme secretion taking pH as variable. Both Single linkage clustering and Ward’s methods are shown. The comparative silhouette plots were also shown to describe the best numbers of clusters.

Therefore, in *A. mola*, clustering of gut regions in terms of enzyme activity from the perspective of pH delivers much explicit grouping than the same through the aid of temperature. The MM1 and MA, by their optimum proteolytic activity within a range of pH 8-9 may represent the anterior intestine of this fish. Similarly, MM3 and MP may represent the posterior intestine with low digestive activity. Interestingly, MM2 stands with moderate digestive nature between anterior and posterior intestine.

#### 4. Conclusion

Digestive physiology in fishes is still incomplete with several gaps, and as such, our knowledge of digestion in stomachless fishes may not be conclusive. The present study provides an unexplored area of the feeding ecology of *A. mola*. It was evident that the activity of amylase in the gut is highest than the other two enzymes. All three types of enzymes had optimum pH value ranging from neutral to alkaline and the optimum

temperatures were more or less within the range of 35-45 °C. The clusters further indicate an evolutionary direction where the agastric nature of progressively optimized digestive features of highly evolved fishes is reflected.

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