Extraction and characterization of endogenous cellulases in *Achatina fulica* for lignocellulose digestion

Mudasir Ahmad Dar[1,2], Jyoti M Chintalchere[1], Radhakrishna S Pandit[1*]

[1]Department of Zoology, Savitribai Phule Pune University, Pune, Maharashtra 411007, India
[2]School of the Environment and Safety Engineering, Jiangsu University, Zhenjiang, Jiangsu province 212013, China

ARTICLE INFORMATION	ABSTRACT
	The giant African land snail, *Achatina fulica* (Gastropoda: Pulmonata) acts as a prolific pest of over 500 crop species. Its major digestive enzymes responsible for digestion and assimilation of lignocellulose are not well studied. In this regard, we attempted to investigate the endogenous cellulases secreted by the snail which have been largely ignored due to more focus on its gut symbionts. The pH of the gut regions were found to be acidic ranging between pH. 3.0 to 6.2 in most of the regions which plays a pivotal role for the cellulose digestion. The study revealed that snail also secrets its own enzymes depicting some evolutionary significance. The crop, stomach and intestinal extracts showed maximum activity at 60 °C. The enzyme extracts except crop region exhibited susceptibility to temperature showing least stability. Similarly, the optimum pH of the gut extracts was depicted to be 3.0 showing a positive corroboration with digestive physiology of the snail. The undertaken gut extracts showed more preference towards cellulosic substrates such as carboxymethyl cellulose (CMC), avicel and xylan, signifying the herbivorous pest nature of the snail. The presented study provides vital baseline data to prospect for novel cellulase genes within snails by using functional-genomic analysis. **Keywords:** Pest, snail, *Achatina fulica*, cellulose, endogenous cellulases

1 Introduction

Snails are known to cause significant damage to agriculture causing tremendous economic loss across the world (GISD, 2013). The farmers in Philippines, Thailand and Western Australia are estimated to spend cumulatively about 7.2 billion dollars annually for snail management in crops (Joshi et al., 2017). The African land snails especially *Achatina fulica* Bowdich, 1822 are the most destructive pests of tropics and subtropical agriculture, causing devastating damage to farms, domestic gardens and commercial plantations such as citrus, papaya, rubber and cacao, etc (Raut and Barker, 2002). It dwells on trees, decaying matter and next to garbage disposing sites (Mead, 1995; de Vasconcellos and Pile, 2001). The snail feeds on almost everything from stones to partially degraded bones for its shell development during different stages of the life. These animals are known to digest the plant matter chiefly composed of cellulose with the aid of gut symbionts like protozoa and bacteria. These gut symbionts enable the host to develop a complex system of enzymes called cellulases which actively hydrolyze cellulose fibrils (Tomme et al., 1995) into monosaccharide residues or short chain oligosaccharides. This theory was put forth by Cleveland, while working on cellulose digestion in termites. Where he demonstrated the disappearance of the viability of *Reticulitermes flavipes* after defaunation of protozoa from the hindgut (Cleveland, 1924), thus concluding that a hindgut protozoan fauna was responsible for the cel-

lulose digestion in the host gut. Since his demonstration, symbiotic cellulase production became the most favored explanation for cellulose digestion in higher animals (Chapman, 1998; Martin, 1991). However, the dogma stating that digestion of lignocellulosic matter is confined to gut symbionts only was nullified first in 1978 by Martin and his coworker. The authors (Martin, 1991) demonstrated that cellulolytic activities in animals are also attributed by endogenous secretions of the gut- organs (e.g., salivary glands of termites or defaunated guts of *Macrotermes natalensis*). Which was followed by purification of several endogenous cellulase components in many herbivorous invertebrates (Watanabe and Tokuda, 2001; Yokoe and Yasumasu, 1964). However, initially this research impacted very little to change the general view concerning animal cellulose digestion. Due to the advances in molecular biology methods, the origins of animal cellulases have been firmly established (Watanabe et al., 1998). Consequently a lot of research has been reported that explored the endogenous cellulases of different animals (Brune, 2014). To date, cellulase genes have been reported from insects (Tokuda and Watanabe, 2007), cray fishes, nematodes and molluscs (Teng et al., 2010). Recently the cellulase that can hydrolyze filter paper was extracted from the visceral organs of garden snail, *Cornu aspersum* (Ndlovu and Wyk, 2019). Similarly Ozioko et al. (2013) extracted and partially purified three cellulases from the gut of *Achatina fulica*. However, they used whole guts for extraction whereas the present study highlights distribution of these cellulases in different regions of the snail gut.

Besides arthropods, several cellulases have been reported in molluscs like snails (Marshall, 1973; Maeda et al., 1996), a periwinkle (Elyakova et al., 1968), slug (Anzai et al., 1984) and some bivalves (Xu et al., 2000; Imam et al., 1993). In these animals many organs like hepatopancreas, gastric teeth, and crystalline styles (Siddall, 2003) are known to secrete the cellulases. Most of these studies have demonstrated quantitative analyses of cellulolytic systems. The purification of these proteins by using different chromatography methods and subsequent analysis on molecular levels has revealed functional differences among cellulases. Consequently, two different action systems have been recognized wherein first method depicts a synergistic action of three free enzymes such as endoglucanase (EG), exoglucanase (CBH) and β-glucosidase. These cellulase enzyme systems act in a coordinated manner to hydrolyze the cellulose efficiently. The endoglucanase (EC3.2.1.4) hydrolyzes the long chains of cellulose molecule into short stretches by cleaving internal β-1,4 glycosidic bonds. These short chains are further degraded by the exoglucanase (EC3.2.1.91) also known as cellobiohydrolase (CBH) releasing terminal cellobiose units. Cellobiohydrolase is usually more active against the crystalline

cellulose chains as compared to endoglucanase (Breznak and Brune, 1994). Although cellobiohydrolases are of two types based on functionality (reducing end E.C.3.2.1.176 and non-reducing end E.C.3.2.1.91), but they are known to act uni-directionally on the long chain polysaccharide cellulose (Wahlström et al., 2013). Lastly, the β-glucosidase (EC3.2.1.21) that converts cellobiose units into monomer sugar residues are also of 2 sub families, A and B while the later being abundantly observed in rumen bacteria (Park et al., 2011). The other system includes cellulosome comprised of multiple binding components assembled together into a larger structure (Bégum and Lemaire, 1996; Bayer et al., 1998). In animals, some EG show similar action like CBHs which can break cellobiose units while attacking cello-oligomers (Watanabe et al., 1997; Tokuda et al., 1997). This quality of animal EG components may help them to digest native-form cellulose in food whose surface area is limited compared to that of carboxymethyl cellulose (CMC, a chemical constituent) in which frequent substitution of carboxymethyl groups on glucose residues protects the chains from enzyme attack (Klyosov, 1990). Additionally, some herbivorous invertebrates possess grinding or crunching assets, such as mandibles of insects, crystalline styles and sacs of bivalves, and the radula teeth of herbivorous molluscs, which can break down food material into fine particles (Snodgrass, 2018). The function increases the surface areas of the food debris into substrates which the enzymes can then easily hydrolyze.

The cellulases of animal origin were first studied by Biedermann and Moritz (1898), in *Helix* spp. at the end of 19th century. This was subsequently contradicted by the findings of many researchers around the world. Therefore, in view of the incompleteness of the evidence and less understood process of cellulose digestion by *Achatina fulica*, we considered it worthwhile to elucidate the possibility of endogenous nature of cellulase in this animal. Herein reported are the results of extraction of gut enzymes that are capable to convert cellulosic biomass into value added metabolites like sugars thereby contributing energy to the host. Further there are very few reports that reveal the existence of endogenous cellulases in animals signifying the importance of this work.

2 Materials and Methods

2.1 Snail rearing and sample preparation

The small, young snails ($n = 5$) collected from the urban area around Govt. polytechnic college, Pune, Maharashtra, India were maintained *in vitro* at a temperature of $25 \pm 2\,°C$, relative humidity $65 \pm 5\%$, and a 12:12 h (L:D) period. The shell height and weight of the collected snails were 7.0 ± 1.5 cm and 24 ± 7 g, respectively. The animals were maintained on a natural

diet of cabbage leaves soaked in 10 mM streptomycin in order to clear the gut microbes of the snails. The snails were fed ad libitum continuously for a period of 25-30 d after which the animals were sacrificed for further studies. In order to have uniformity in results, the snails that stopped feeding or underwent aestivation during rearing were eliminated from the study. Generally the physiological studies in molluscs have been carried out over week's period only (Gomot, 1990). But due to large body size of *A. fulica*, the period of study was extended to analyze the possible alterations occurring in the gut microflora.

2.2 Determination of pH of the gut

To determine the pH in the gastro-intestinal tract of *A. fulica*, methods described by (Anderson and Bignell, 1980) were followed. The snails were dissected under aseptic conditions as discussed previously (Dar et al., 2015). Each region of gut from dissected snails ($n = 5$) was cut and mounted on a microscope slide, then 5µL of pH indicator solutions were added to each gut region. Indicator dyes used for the purpose included 0.1% each of Bromophenol blue (pH: 3.0-4.6), 0.1% Methyl red (pH: 4.4-6.2), 0.1% Congo red (pH: 3.0-5.2), 0.1% Phenol red (pH: 6.4-7.4), and Phenolphthalein (pH: 8.3-10) with varying pH range.

2.3 Cellulase extraction

For enzyme assay, the snails were sacrificed after surface sterilization by 70 % ethanol. The gut regions were transferred to pre-weighed micro centrifuge tubes containing 0.85% NaCl solution. The gut contents were thoroughly homogenized on ice in sodium citrate buffer (SCB, pH 5.6) containing 1% proteinase inhibitor solution (Complete Mini EDTA-free; Roche, USA) in distilled water with a sterile glass homogenizer. The homogenized mixture was vortexed and sonicated at 68Hz (35s × 5 times) at 4 °C. The samples were centrifuged at 20000 rpm for 20 min at 4 °C and the supernatants were collected in separate tubes. The pellets obtained were again suspended in SCB and centrifuged at 20000 rpm for 20 min, 4 °C. The supernatants obtained were pooled and the pellets were finally suspended in cell-lysis buffer, vortexed for 20 s, followed by incubation on ice for 10 min. Lastly the suspension was centrifuged at 20,000 rpm, for 20 min. The respective supernatants collected from each region were now pooled and treated as enzyme extracts. The enzyme extracts were used immediately or occasionally stored temporarily at 4 °C.

2.4 Cellulolytic hydrolysis

For qualitative tests 20 µl of the enzyme extracts were spread on the agar plates containing 0.5% CMC (w/v) dissolved in SCB (pH 5.4). The plates were incubated at 37 °C for 1 h to check the activity. After incubation the plates were stained with grams iodine staining solution in order to visualize the zone of cellulose clearance by the enzyme extracts. The gram's iodine staining solution was preferred over congo red staining due to its higher sensitivity, inexpensive and fast action (Kasana et al., 2008).

2.5 Temperature and cellulase activity

The enzyme assays were carried out as per the method of Tokuda and Watanabe (2007) with few modifications, where 250 µL of CMC substrate (1% w/v) dissolved in SCB (pH 5.6) was mixed with 50 µL of enzyme extract and incubated at a range of temperatures (20 to 80 °C) for 60 min. To determine thermal stability, gut extracts were pre-incubated at various temperatures (20-80 °C) for 1 h at pH 7.0 (optimum pH) without substrate thereafter the enzyme assays were carried out by following standardized protocol. The residual cellulase activity after pre-incubation for one hr at 60 °C was measured as mentioned below after mixing the above contents of the reaction. The reaction was terminated by adding 500 µL of DNSA solution and heating in a boiling water bath for 5 min. Subsequently, the reaction mixtures were cooled and absorbances read at 540 nm. All the reactions were carried out in three or more independent replicates. The absorbances read at 540 nm were used to calculate specific activity (IU mL^{-1} Extract) using glucose as standard. For determination of protein content in gut extracts, we used Bradford method (Bradford, 1976). The enzyme activities were determined in international units (IU mL^{-1} of gut extract) where 1 IU is the amount of enzyme that liberated 1 µMol of glucose equivalents per min under assay conditions.

2.6 pH and cellulase activity

To determine the optimum pH required for the maximum cellulase activity of the gut extracts, 50 µL of gut extracts were mixed with 250 µL of (1% w/v) CMC substrate in 50 mM buffers of different pHs (SCB, 2.0–5.0; PB, 5.6–7.0; Tris buffer TB, 7.0–9.0) and incubated at 37 °C for 60 min. Like thermal stability, to test for pH tolerance, the gut extracts were pre-incubated in 50 mM solutions of above-mentioned buffers for 1 h at 4 °C and then the residual activities were measured as per the above mentioned protocol. The reactions were terminated by adding 500 µL of DNSA reagent (Miller, 1959) and heated in a boiling water bath for 5 min. The tubes were cooled and absorbances were recorded at 540 nm. All the reactions were carried out in at least three replicates.

2.7 Substrate specificity of gut extracts

The substrate specificity of gut extracts was carried out by using various substrates, such as CMC, Xylan, Avicel, Cellobiose, Starch and Glycogen. All the substrates were prepared in 50 mM TB (pH, 7.0). The substrate specificity of each gut extract was estimated according to the standard method as described above. To check the substrate preference of the enzyme extracts for maximum production of sugars, 250 µL of 1% (*w/v*) respective substrates dissolved in TB (pH, 7.0) were added to 50 µL of gut enzyme extracts and incubated at 37 °C for 1 h. Then enzyme assays were carried out under assay conditions using glucose as standard. All the readings were recorded in multi-plate reader (Multiskan Ex spectrophotometer, Thermo Scientific, Finland) using Ascent multiskan software using a filter for 540 nm.

2.8 Statistical analysis

Results obtained are reported as mean ± standard deviations of three or more independent replicates. Analyses of data were carried out by using Microsoft office suite and SPSS software version 22 (IBM SPSS, NY) wherever applicable. One-way analysis of variance (ANOVA) was carried out by using Dunnett multiple comparison test (p<0.01) maintaining a parametric model in SPSS software. Graphs were prepared in Origin Lab. software version 8.1 as well as in Microsoft excel.

3 Results

3.1 pH of the gut

In all regions except intestine, the pH was found to be acidic ranging from 3.0 to 6.2, whereas pH of the intestine was slightly neutral as shown in Table 1. Therefore, it can be stated that overall pH of the gut in *A. fulica* is acidic.

3.2 Cellulolytic hydrolysis

Preliminary screening of cellulase activities, oesophagus and rectum regions did not show any activity due to which they were eliminated from the study and correspondingly the crop, stomach and intestine extracts which depicted positive for cellulose digestion were taken into consideration (Fig. 1). Among these three regions the maximum zone of carboxymethyl cellulose clearance was displayed by intestinal extract. While the crop and stomach extracts exhibited comparatively lower activities of 10 and 12 mm respectively. Hereafter only these 3 regions *viz.*, crop, stomach and intestine were taken for further analysis.

3.3 Temperature and thermal stability

The optimum temperature was found to be 60 °C where crop, stomach and intestinal extracts exhibited maximum relative activity (Fig. 2a). The lowest activities were obtained at 20 °C showing the inactive nature at low temperatures which could be correlated with the metabolic adaptation of the animal. For determination of thermal stability, the extracts were pre-incubated at different temperatures for 1 h. At all the tested temperatures a drastic reduction in the cellulolytic activity was observed showing the susceptibility of the endogenous enzymes towards temperature variations. Among the gut regions, the enzymes extracted from stomach and intestine regions, showed maximum alteration in the activity with a 15-80% decrease in activity. In contrast the residual activity of the crop extract was least affected at tested temperatures with a notable reduction of just 50 to 57% in activity as observed at 20-60 °C (Fig. 2b).

3.4 Optimal pH and pH tolerance

Since pH plays a vital role in maintaining the apt metabolism within the cell. To identify the optimum pH required by the cellulases extracted from different regions were incubated in various buffers containing pH between 3 and 9 and enzyme activities were measured at the respective pHs. The optimum pH of the crop extract was found to be highly acidic (pH 3.0) showing a positive correspondence with the digestive physiology of the mollusc. However, in case of stomach and intestinal extracts the highest cellulase activities were observed at neutral or higher pH values (Fig. 3a). The present study demonstrates that the endogenous cellulase enzymes of anterior region of gut prefer acidic pH while the posterior sections like intestine works better under alkaline conditions. These results show the paradigm shifts in the pH milieu along the gut of giant African land snail probably showing correlation with digestive functioning of the animal where different regions or sections of the gut perform hydrolysis and assimilation of different chemical constituents in food. The methodology employed here was very effective in estimating enzymatic behavior under different pH and temperature conditions.

In case of pH stability however, the results were quite astonishing showing that the stomach and intestinal extracts confer least/no activity at lower pH, i.e., below pH 5.0. However, as mentioned earlier the activity was drastically reduced in all the gut extracts after pre-incubation in different buffers. It was observed that the gut extracts showed very less tolerance to the pH alterations. There was 75% reduction in the cellulase activity of crop extract after preincubation (Fig. 3b). Similarly in case of stomach and intestine a marked reduction of 94% and 47% was observed respectively at their optimum pH values.

Table 1. The pH range of different regions of the gastro-intestinal tract of giant African land snail, *A. fulica*

Indicator dye (pH)	Colour	Gut region				
		Oesophagus	Crop	Stomach	Intestine	Rectum
Bromophenol blue (3.0-4.6)	Yellow to purple	−	−	−	−	−
Congo red (3.0-5.2)	Violet to orange	−	+	+	−	−
Methyl red (4.4-6.2)	Red to orange	+	+	+	−	−
Phenol red (6.4-7.4)	Yellow to violet	+	−	−	+	+
Phenolphthalein (8.3-10.0)	Colourless	−	−	−	−	−
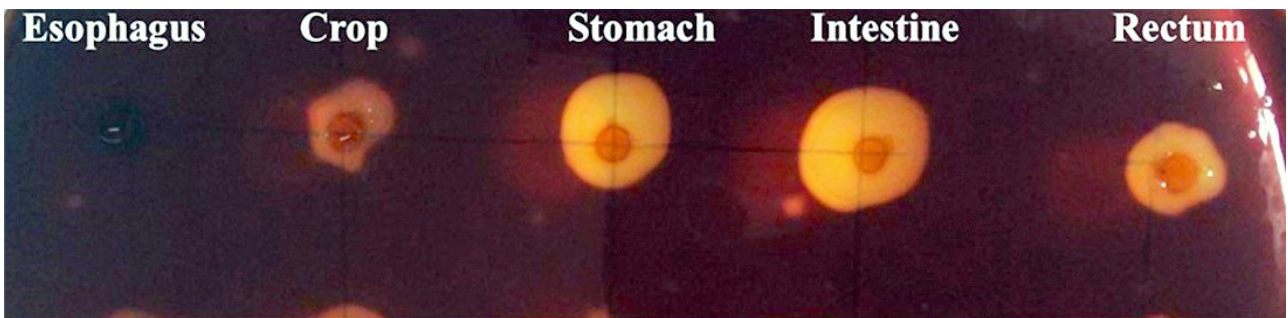
Figure 1. Plate based assay depicting the clearance of CMC substrate (yellow zones) by the gut extracts after 1 hr of incubation at 37 °C. The CMC-agar plat after incubation was stained with Gram's iodine solution to display the zone of clearance. Sometimes the staining was followed by a brief wash of 1% acetic acid glacial in water (*v/v*) to enhance the resolution of the clearance zone.
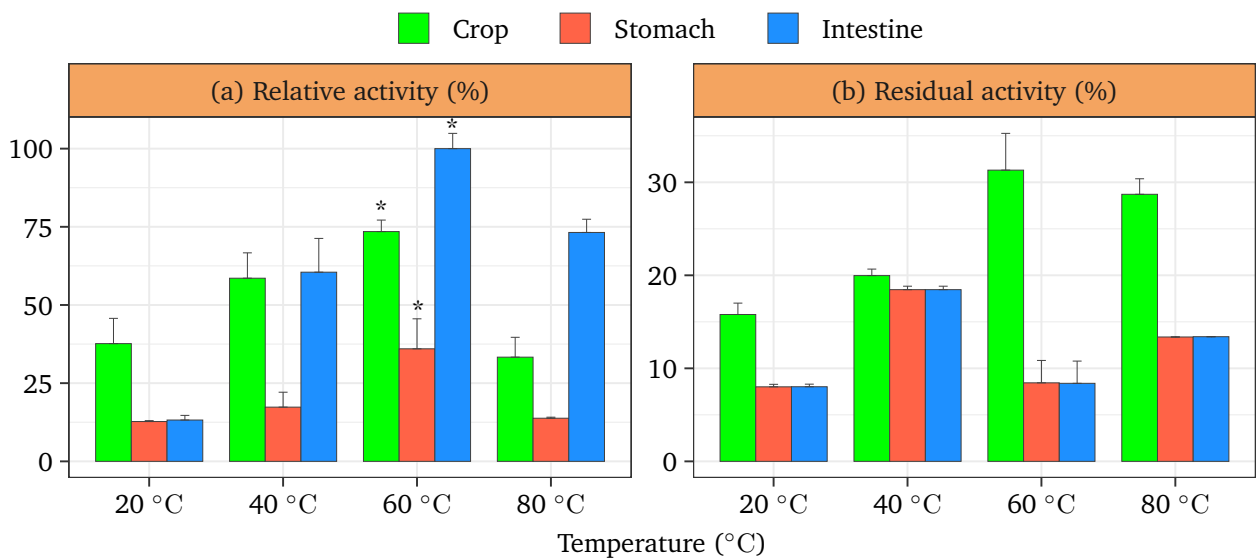
Figure 2. Effect of temperature on the activity of extracted cellulases from different gut regions. (a) Temperature optimization to examine the best suited temperature for higher cellulolytic activity; (b) Thermal stability of the gut enzyme extracts after a preincubation of 60 min at varying temperatures. Data analyzed by One-way ANOVA, followed by Dunnett test and significance at p<0.01 between the activities at different temperatures of individual gut extracts.
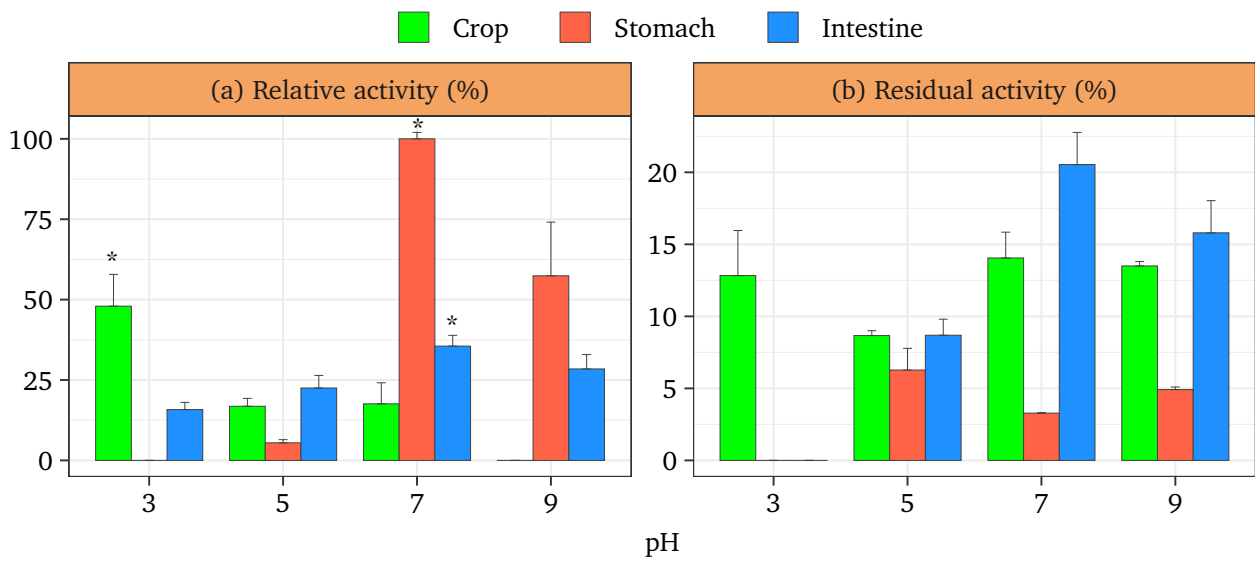
Figure 3. Effect of pH on the activity of extracted cellulases from different gut regions of the snail. (a) Optimum pH where extracts show highest activity; (b) pH stability of the gut extracts after preincubation in different buffers. Data analyzed by One-way ANOVA, followed by Dunnett test and significance at $p<0.01$ between the activities in different buffers of individual gut extracts.
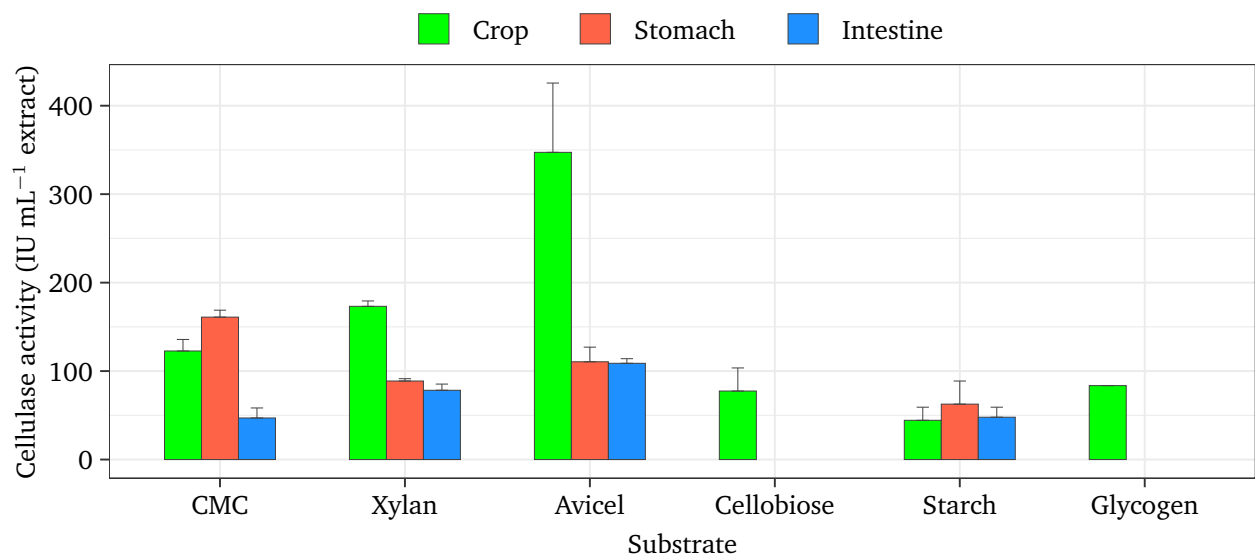

Figure 4. Substrate specificity of the gut extracts showing affinity towards different carbon sources.

3.5   Substrate specificity

The gut extracts showed typical preference towards cellulosic substrates. The more affinity was observed towards microcrystalline cellulose, i.e., Avicel among the tested substrates followed by Xylan and CMC. The crop and intestinal extracts preferred Avicel as substrate over the other tested compounds showing a 347.21 IU mL^{-1} extract and 108.11 IU mL^{-1} extract activities respectively, which was preceded by Xylan and CMC in both cases as shown in Fig. 4.

With stomach extract, it was observed that it showed more preference towards CMC (160.3 $\pm$ 8.5 IU mL^{-1} extract) then followed by Avicel and Xylan. However, among the tested substrates it was observed that stomach and intestinal extracts did not use Cellobiose and Glycogen showing endoglucanase nature which makes random attacks on the inner chains of cellulose. Therefore, these inferences suggest that these enzymes could be used for hydrolysis of many substrates for the production of reducing sugars which can be readily used for different purposes by the snail.

4 Discussion

The alimentary tract of the snail is very simple probably due to its terrestrial life style comprised of buccal mass, oesophagus, crop, stomach, intestine and rectum. The crop being the largest part of the foregut acts for temporary storage and primary digestion of ingested food material (Ghose, 1963). The intestine is usually narrow and long coiled tube representing a typical herbivorous gut (Ley et al., 2008). The physiology of land snails is determined by surrounding environmental conditions (Nowakowska et al., 2010) where pH of the gut is vital that determines the characteristic nature of the enzymes involved in digestion (Terra and Ferreira, 1994). Though pH of animal guts is generally correlated with feeding habits it also regulates dissociation or coagulation of ingested proteins, controlling the solubility or toxicity of stomach toxins. Our results show that oesophagus, crop and stomach regions of *A. fulica* are highly acidic (pH 3.0) while in intestine and rectum regions the pH was in the range of pH 6.4 to 7.4 depicting a slightly neutral nature. Similar findings have been reported for many invertebrates like lepidopteran species such as *Trichoplusia ni*, *Ephestia cautella* (Srivastava and Mathur, 1966), *Corcyra cephalonica* (Srivastava AK, 1976) which share similar feeding habits.

Hitherto, cellulases have been isolated from fungi, bacteria and many invertebrate animals like arthropods, molluscs, etc (Dar et al., 2018a; Maeda et al., 1996). A relative diversity of cellulases have been reported in insects like termites (Tokuda et al., 1997) and beetles, stick insects, lepidopterans (Dar et al., 2018b), etc. Similarly, some molluscs like bivalves have been reported to secrete endogenous cellulases. Among molluscs, snails like *H. pomatia* (Marshall, 1973) and *Achatina fulica* are one of the earliest animals reported as being cellulolytic (Maeda et al., 1996). Some snails are known to secrete endogenous cellulases (Calow and Calow, 1975) which correlates with their evolutionary adaptations. Although endosymbiont owning herbivores secret some endogenous enzymes for digestion of plant matter, the efficiency of these enzymes is relatively low when compared to their exoenzymes (enzymes contributed by the endosymbionts). Assuming the low efficiency of endogenous enzymes in snail gut, the extracts were incubated for 60 min to hydrolyze the substrate in order to have a profound conclusion related to cellulase activity. Apart from snails, the secretion of endogenous cellulases has been reported in many other animal species such as blue mussel *Mytilus edulis* (Xu et al., 2000), sea slug *Dolabella auricularia* (Anzai et al., 1984), termites (Tokuda and Watanabe, 2007), etc. The cellulase enzyme isolated from the gastric teeth of the *Dolabella auricularia* by Anzai et al. (1984) had a molecular weight of 44 kDa. Not only that many other cellulases have been isolated and purified from several molluscs ranging in molecular weight from 19.702 kDa in *Mytilus edulis* (Xu et al., 2000) to 51 kDa in *Dolabella auricularia* (Anzai et al., 1988). These inferences suggest that *A. fulica* might also serve as a potential source for industrially important enzymes. The cellulase activity observed in this study is far greater than the activity reported by Dini et al. (2019) which is only 0.123 IU mL^{-1}. The observed activity could be higher than the previous reports due to the short reaction time used by the researchers previously, such as 30 min by Dini et al. (2019). The longer the incubation time offered to enzyme to react with substrate, the higher is the cellulase activity as the enzyme gets sufficient time to hydrolyze the complex polysaccharide, cellulose (Dini et al., 2019).

The optimum temperature for the endogenous cellulases extracted from crop, stomach and intestinal regions were found to be 60 °C showing resemblance with AfEG22 endoglucanase which exhibited maximum activity at 50 °C (Teng et al., 2010). While in case of thermal stability the enzymes were affected to varying extents but affected least below 40 °C. The optimum temperature of the enzyme extracts was higher than the crude extracts of same animal which showed higher activity at 37 °C. However, the thermal stability of extracted cellulases contradicted with earlier report, where AfEG22 endoglucanase showed high thermal stability by retaining more than 90% activity after incubation at 60 °C for 1 h. This change in optimum temperature could be due to the dilution of the enzyme extracts before mixing the reactants (Saryono, 1991). Nevertheless, in another case authors stated that low temperatures and short period of incubation causes less collision and reduced binding of enzymes with substrate resulting lower activities. Our results are in congruence with previous observations where authors stated that the cellulases extracted from snails are thermophilic in nature as they can withstand temperatures above 45 °C (Dini et al., 2019).

The pH stability was checked in terms of residual activity of the extracted gut cellulase after incubation in buffers with different pH at room temperature (RT). However, the optimum pH also varied for the 3 gut extracts. The crop extract revealed maximum activity at a pH of 3.0 showing physiological correlation with the gut environment as the crop region is acidic in nature. With stomach and intestinal extracts, the neutral pH i.e., 7.0 was found to be optimum. Our observations were contrasting with results stated by Ozioko et al. (2013) where the gut extract from the same animal showed optimum activity at a pH of 5.5. Possible reasons for this variation in pH optima could be the mixing of the extracts from whole gut. Our results were also in line with the activity reported by Teng et al. (2010) in case of endoglucanase from gut fluid of *A. fulica*. The authors stated that the snail endoglucanase works better at a pH of 4.0 and re-

tains more than 90% activity at pH 3.0-5.0 (Teng et al., 2010) while exhibiting very less activity (20% activity) under alkaline conditions. The pH stabilities of the extracted cellulases were very less as compared to the AfEG22 endoglucanase isolated from the same animal. The activity of all the gut extracts were far higher than the activity of many animal cellulases which also function under low pH (acidic) conditions (Ndlovu and Wyk, 2019; ichi Suzuki et al., 2003; Xu et al., 2000; Li et al., 2004, 2009). The acidophilic property shown by the gut extracts would play an important role in many biotechnological applications, such as acidic cellulases could be readily used as animal feed supplements to improve the animal health and hygiene. Moreover, these enzymes might find some role in bioethanol production from lignocellulosic biomass due to their pH stability suitable for industrial scale processes and thus help to reduce production costs (Huang et al., 2005). Being different in origin the gut extracts also showed different affinities towards the carbon sources. The crop and intestinal extracts depicted higher preferences towards Avicel substrate, which is also called as microcrystalline cellulose being very difficult to degrade. Since Avicel is a complex substrate with a minute particle size of ∼50 μM and its degradation indicates the true cellulolytic nature of the gut extracts. The most favored substrate of the stomach extract was observed to be CMC followed by Avicel and Xylan carbon sources. The higher preference for CMC by stomach extract could possibly be due to physiological role of organ in digestion process where many enzymes proteases and amylases are contributed to support digestion of the plant matter. These results were similar to the finding of Guo et al. (2007) and Guo et al. (2008) who observed more specificity of His6-tagged β-endoglucanases isolated from mollusc, *Ampullaria crossean* towards CMC substrate.

Since giant African land snail, *A. fulica* is a complex holobiont system, it is imperative from the results achieved in this study and our previous observations (Dar et al., 2015, 2018b) that digestion of lignocellulose within the snail gut is a combined effort from the host and its gut bacterial symbionts. However, in order to elucidate the exact origin and structure of these enzymes, more investigation is needed to purify and sequence them. The further research will not only explicate their molecular structure but might help to distinguish them from the cellulases contributed by snail's gut symbionts.

5 Conclusion

Cellulases extracted from different gut regions of *A. fulica* show acidic nature. The overall observations provide evidences for the existence of endogenous cellulases in the snail which are responsible for the cellulose digestion in different parts of the gut. This report provides much needed insight into the cellulase enzyme system of snails in general and *A. fulica* in particular which could be exploited for designing species specific molluscicides for its strategic control. However, whether these enzymes have been solely contributed by the gut-symbionts to this animal during the course of evolution or the snail has evolved its own digestive strategies to combat the different feeding habits over the period of time is worth further investigation. The future perspectives of this study would be captive rearing of the snails for generations and design more systematic tactics to clear the maximum load of gut flora in order to confirm the molecular origin of these enzymes in the animal. The molecular biology of these proteins will provide more insights into their evolution and mechanism involved in lignocellulose digestion within the snails.

Acknowledgments

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Ethical Approval

The animal experimentation was performed following ethical guidelines established for animal usage by Savitribai Phule Pune University, Pune, India.

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