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Exogenous enzymes activities in the fore- and mid-gut of the African snail (*Archarchatina marginata*)

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Additional keywords
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SUMMARY

This study investigated the enzyme activities of two (2) microbial organisms in the fore- and mid- gut regions in the gastrointestinal tract, in Archarchatina marginata, a giant Land snail from the region of West Africa. Microbial analysis was conducted to verify the microbial organisms present in different parts of the snail gut while enzyme assay was performed to determine the type and level of enzyme activities taking place in the mouth and stomach regions. Results revealed the different bacteria inhabiting the regions of the alimentary tract in *Archarchatina marginata*. *Azobacter chroococcum* and *Pseudomonas syringae*, the two bacteria isolate that were further investigated, effectively digested starch, cellulose and casein. However, P. syringae exhibited the highest enzymatic activities for cellulase (8.72µmol/min/ml at 18h) and amylase (9.05 µmol/min/ml at 24h) while *Azobacter chroococcum* exhibited the highest enzymatic activity for protease (17.18 µmol/min/ml at 24h) during incubation. Consequently, the study validated amylolytic, cellulolytic and proteolytic bacterial activities within the digestive tract of *A. marginata*. This knowledge is informative for research and an aid to snail farmers in the choice of feeding materials for these land snails.

Actividades de enzimas exógenas en el intestino anterior y medio del caracol africano *(Archarchatina marginata)*

Palabras clave
Amilasa.
Celulasa.
Incubación.
Microorganismos.
Proteasa.

RESUMEN

Este estudio investigó las actividades enzimáticas de dos (2) organismos microbianos en las regiones del intestino anterior y medio del tracto gastrointestinal, en Archarchatina marginata, un caracol terrestre gigante de la región de África Occidental. Se realizó un análisis microbiano para verificar los organismos microbianos presentes en diferentes partes del intestino del caracol, mientras que se realizó un ensayo enzimático para determinar el tipo y nivel de actividades enzimáticas que tienen lugar en las regiones de la boca y el estómago. Los resultados revelaron las diferentes bacterias que habitan las regiones del tracto alimentario en *Archarchatina marginata*. *Azobacter chroococcum* y *Pseudomonas syringae*, las dos bacterias aisladas que se investigaron más a fondo, digirieron eficazmente almidón, celulosa y caseína. Sin embargo, *P. syringae* exhibió las actividades enzimáticas más altas para celulasa (8,72 µmol / min / ml a las 18 h) y amilasa (9,05 µmol / min / ml a las 24 h) mientras que Azobacter chroococcum exhibió la mayor actividad enzimática para la proteasa (17,18 µmol / min / ml a las 24 h) durante la incubación. En consecuencia, el estudio validó las actividades bacterianas amilolíticas, celulolíticas y proteolíticas dentro del tracto digestivo de *A. marginata*. Este conocimiento es informativo para la investigación y una ayuda para los criadores de caracoles en la elección de materiales de alimentación para estos caracoles terrestres.

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INTRODUCTION

The giant African land snail is a common delicacy in the menu of Nigerians and it is a cheap source of quality protein (Paiko *et al.*, 2016, p. 149-152; Sando *et al.*, 2012, p. 55). In recent time, interest in snail farming has gain momentum due to the many benefits of the snail as a whole. Snail meat contains iron, calcium, magnesium, potassium, phosphorus, zinc and essential amino acids such as isoleucine, leucine, lysine, and

phenylalanine (Ebenebe, 2000, p. 19; Ademolu *et al.*, 2004, p. 412). The protein-rich meat is low in sodium, fat and cholesterol levels thereby making this meat a good diet for persons suseptible to cardiac arrest, stroke, hypertension and heart attack (Nyaogba *et al.*, 2016, p. 94). Snail products has also found use in cosmetics and pharmaceuticals (Olagbende-Dada, 2015, p. 298). Although there is a high demand for snail products (Nyaogba *et al*., 2016, p.94) yet, deforestation and indiscriminate hunting of giant land snails (Jimoh *et al.*, 2020, p. 2) is causing a decrease in the population of snails in the wild. The captive rearing of snails requires lower inputs and less labour, resulting in more income for farmers. Furthermore, snail farming is a lucrative enterprise providing income for farmers, international traders, snail meat processor and resturant owners (Ndah et al., 2017, p. 2). Snail farmers would harness much more the benefits from captive rearing of the giant African land snails, when snail feeding for growth and reproduction is standardized.

Achievement of standardized snail feeding requires production of snail feed with acceptable nutritional requirements just like in the case of conventional livestock. To achieve this, a comprehensive understanding of the physiology of digestion in the snail is imperative and would therefore require the detailed study of the presence and biochemical activities of resident microorganisms within the snail alimentary tract. Earlier reports (Chevalier *et al*., 2003, p.127) affirmed that land snails feed on fresh vegetations with high calcium and protein contents, they join with other invertebrate animals living on the soil, to breakdown leaf litter (Bardgett, 2005, p. 42) as a result, they require suitable enzymes for the digestion of these vegetative materials. The land snail harbors active bacterial microflora in their gastrointestinal tract for their metabolism (Pawar *et al*., 2012, p.416). Studies (Charrier *et al*., 2006, p. 147; Van Horn *et al.,* 2011, p. 74; Oyeleke *et al.,* 2012, p. 15; Pawar *et al*., 2012, p. 416 & Ademolu *et al*., 2013, p.73) have revealed that microbial communities inhabit the snails' intestinal tissue. The dependence of gastropods on intestinal microbial activity is evident by their very efficient ability to digest plant tissues (Dar *et al*., 2017, p. 200). Generally, in animals the intestinal microflora is essential for maintenance of the animal through resistance to pathogens, metabolism of energy and detoxification process (Dong *et al.*, 2009, p.2). The microorganisms present in the stomach as normal flora are a reflection of the oral flora (Evaldson *et a*l, 1982, p. 9). Furthermore, the presence and activity of bacteria in different parts of the gut is dependent on factors, such as diet type, nutrient availability, bacterial adhesion, bacterial cooperation, and bacterial antagonism among others (Hao and Lee, 2004, p. 491). As a contribution to the understanding of the physiology of digestion in land snails and to assist snail farmers in making an effective choice of feeding stuff, this study was carried out therefore, to ascertain the microflora type and compare enzymatic activities of *Azobacter chroococcum and Pseudomonas syringae* in mouth and stomach regions in *Archachatina marginata* during digestion.

MATERIAL AND METHODS

Experimental animals and sample collection

The identification of snail gut regions in *Archachatina marginata* was conducted in the Animal science laboratory. Enzyme assays and microbial analysis were carried out at the Biochemistry and Microbiology laboratories respectively. Adult snails of 150 – 250 g liveweight, bought from snail markets, were sacrificed for this experiment. Snail tissues were excised as described by Segun (1975, p. 11). After dissection, the different organs of the digestive tract were exposed in order to obtain swabs of the surface epithelia of different portion of the gut. Each portion of the snail gut was wiped with sterile moist cotton swabs separately. The different sterile moist swabs were soaked in a preparation of nutrient broth using 13g of broth powder in deionized water (1 litre).

Preparations of micro-flora cultures from snail gut content

Nutrient agar media was used to isolate bacteria organisms. The agar media was prepared using the manufacturer's manual as a guide; it was sterilized in autoclave for 15 minutes at 121°C. An inoculating loop was used to smear a portion of the different section of the snail intestinal tissue onto nutrient agar plates which were then incubated for 18-24 h, for bacterial growth, at 37°C. Individual colonies observed were sub-cultured for 18-24 h, on nutrient agar plates and finally, pure cultures were preserved by growing them on agar slants. Biochemical tests, cultural characteristics, and colonial morphology were used to characterize the bacterial isolates (Oyeleke & Manga, 2008, p. 18). Using Bergey's manual, the bacterial isolates were identified by comparing the characteristics with those of known taxa. (Holt *et al*., 1994, p. 527-588).

Microbial cellulase and amylase enzyme assay and production

Bacteria isolates were grown in a salt medium to produce amylase and cellulase. The salt medium (500 ml) consist of 1g KH_2PO_4, 0.5g $(NH_4)_2SO_4$, 3.5g K_2HPO_4, 0.25 g sodium citrate, 0.05g $MgSO_4$, supplemented with starch (for amylase) and a carbon source, CMC (carboxymethyl cellulose) for cellulose production, using cooled distilled water (sterilized for 15 min at 121°C). Culture broth were sampled every 6 h during the growth period (which was at 37°C for 30 h), to determine activities of enzymes in relation to the yield of biomass obtained, by taking measurements with a spectrophotometer at 540 nm absorbance. Culture filtrate (Singh, 2003, p. 153) which served as the enzyme solution, was obtained by filtration through filter paper (Whatman No. 1), For amylase, culture supernatant fluid (0.5 ml) was incubated with 0.5 ml of starch (1%) in 0.05 M acetate buffer, at 40°C and pH 5.0 for 30 min while for cellulase, the culture supernatant fluid (0.5 ml) was incubated with 0.5 ml CMC (1%) in 0.05 M citrate buffer, at 40°C and pH 4.8 for 30 min. The DNSA (dinitrosalicylic acid) assay method for measurement of reducing sugars (Bertrand et al., 2004, p. 53-55) was carried out, using glucose as standard. Cellulase and amylase activities were analyzed by the measurement of reducing sugar liberated by CMC and starch respectively.

Microbial protease enzyme assay and production

Culture medium used for growing bacteria isolates contained 3.75g peptone, 3g casein, 0.05g $FeSO_4$, 2.5g each of glucose, KH_2PO_4, and $MgSO_4$ in 500 ml of cooled distilled water (sterilized at 121℃ for 15 min,) and incubated for 24 h. During growth, the culture broth was sampled every 6 h. The enzyme activity for protease was analyzed by the method of Lovrien *et al*, (1985, p. 261). The reaction mixture contains, the enzyme (0.1 ml) and casein (0.5%) in 0.1 M Tris-HCl buffer (2.95 ml), and 3 ml of this mixture was incubated at 40℃ and pH 8.0 for 30 min, the reaction was ended after 30 min. by addition of 10% cold trichloroacetic acid (3 ml). Filtrate of each culture was centrifuged after 1 h, for 5 min at 6,000 rpm, which resulted in removal of the precipitate. The spectrophotometer was then used to read absorbance of the supernatants at 540 nm. Calculations of the amount of Amino acid released were done using a standard curve plotted against a data of known tyrosine concentrations. Bertrand **et al,** (2004, p. 53-55) gave a unit of enzyme (u/ml/min) as the volume of enzyme that could liberate 1.5 g tyrosine per min. per ml during the analysis.

Data analysis

Data were subjected to descriptive analytical methods using line graphs.

RESULTS AND DISCUSSION

The list of bacteria isolated from the different gut regions of the alimentary tract of *A. marginata* is shown in **Table I**. The presence of different species of bacterial organisms in the gut of *A. marginata*, corroborates earlier reports on snails and other invertebrates (Warnecke *et al*., 2007, p. 561; Huang *et al.,* 2012, p. 2567; Dar *et al*., 2017, p. 200).

The characteristics shown by the bacteria found in the different gut regions are presented in **Tables II & III**.

The characteristics reveal the fermentative ability of some of the isolates, thereby showing the microbes' capacity to aid the host snail in digestion of carbohydrate and cellulosic feed materials. This observation agrees with the report of Mahejabin & Taranum (2015, p. 577-584) that bacteria isolates from the snail gut plays important role in the host digestion of pectin, lignin, and cellulose, found in plant tissues. The presence of bacterial organisms throughout the length of the gastrointestinal tract implies an endosymbiotic relationship between the snail and bacteria organisms. Bacterial endosymbionts are important in the evolution, ecology and physiology of host animals and thus affects adaptation (Sazama *et al.,* 2019, p. 130; Zilber-Rosenberg and Rosenberg, 2008, p. 725). This relationship between the snail and bacteria is mutualistic since bacteria organisms produce digestive enzymes useful for the snail host. Dar *et al*. (2017, p.198) suggested that snails developed symbiotic relationship with diverse bacteria during evolution. Earlier studies indicated that gut bacteria furnish host organisms with advantages such as improved defense against certain diseases, synthesis of vitamins and essential amino acids, conversion of sugars into fatty acids for energy generation, complex polysaccharide digestion, and preventing the growth of harmful organisms (Gonzalez et al., 2011, p. 780; Guarner & Malagelada, 2003, p. 512 & Hooper et al.,2002, p. 285). The need for in-depth study of the mechanism of microbial metabolism in the gut of giant Africa land snails is crucial in order to harness more resource for research, industry and enhance the productivity of these species. Other reports, from studying the hind gut microflora of termites, revealed interesting microbial metabolism which could facilitate biotechnological production of biofuel (Wamecke et al., 2007, p. 561).

The survivability and adaptable nature of the giant African snails is probably due to ability to host and alter its gut microflora to fit the environment.

This creates an exceptional capacity for lignocellulosic plant material hydrolysis and fermentation (Charrier & Daguzan, 1980, p. 147-166).The cellulose degrading activities of microbial isolates from the mouth and stomach region in *A. marginata* are shown in **Figure 1**. *Pseudomonas syringae* had the higher activity with a peak cellulase activity of 8.72µmol/min/ml at 18 h of incubation period and thereafter declined while *Azotobacter chroococcum* had a lower cellulase activity with a peak of 3.59µmol/min/ml at 18 h of incubation.

Cellulose is an important component of the plant cell wall materials consisting of complex β-1,4-linked glucan chains, and are often embedded within the cavity of other structural biopolymers like lignin and hemicellulose (Marchessault & Sundararajan 1993, p.

Table I. List of bacterial isolates from the fore- and mid-gut regions of the gastrointestinal tract of *A. marginata* (Lista de aislados bacterianos de las regiones del intestino anterior y medio del tracto gastrointestinal de *A. marginata*).

Mouth	Buccal mass	Salivary gland	Anterior oesophagus	Crop	Posterior oesophagus	Stomach	Intestine	Rectum	Digestive gland
Azotobacter chroococcum	Shigella sp	*Xanthomonas fragaride*	*Aerococcus viridians*	*Streptococcus feacium*	*Proteus morganii*	*Pseudomonas syringae*	*Erwinia amylovora*	*Aeromonas hydrophila*	*Enterobacter cloacae*
Kurthia zoopfii		*Micrococcus lutus*	*Klebsiella rhinosderomalis*		*Clostridium sporogenes*		*Corynebacterium zerosis*	*Erwinia amylovora*	*Staphylococcus epidermidis*

11-95; Sommerville, 2006, p. 54). These complex plant materials are however, the main source of nutrients utilized by giant African snails. Ruminants and invertebrate species like the land snails benefit tremendously from the enzyme producing activities of cellulose degrading microbes residing within the gastrointestinal tract (Koleva et al., 2015, p. 267; Wang & McAllister, 2002, p. 1660). The ability of *A. marginata* to derive nutrients from its cellulose degrading gut bacteria is of importance in the human dietary nutrition, since land snails are excellent source of protein in human diet. In addition, processes such as composting and anaerobic digestion also make use of microbial cellulose utilization activities (Lynd *et al.,* 2002, p. 506-577). The result of the assay of enzyme activity of bacterial isolates in this study, showed that *P. syringe* from the stomach

Table II. Characteristics of microbial isolates from the gastrointestinal tract of *A. marginata* (Características de los aislados microbianos del tracto gastrointestinal de *A. marginata*).

Organism	GR	Spore	GL	NR	IDL	MTL	CTL	MR	VP	OXL	URS
Azotobacter chroococcum	-			-	-		+	-	-		
Kurthia zoopfii	+	-	-	-	-	+	+	-	-	-	
Shigella sp	-		-	+	-	+	-	+	-		
Xanthomonas fragaride	-	-	+		+	-	-	-	-	-	-
Micrococcus lutus	+		-	-	-	-	+			-	
Aerococcus viridians	+	-			-		+	-	-		
Klebsiella rhinosderomalis	-	-	-	-	-	+	-			-	
Streptococcus feacium	+	-	-	-	-	+	-			-	
Proteus morganii	-	-	-	+	+	+	-	-	+		
Clostridium sporogenes	+	+	+	-	+	+					
Pseudomonas syringae	+	-	-	-	-	-	+	-	-		
Erwinia amylovora	-		-		+		+	+	-	-	-
Corynebacterium zerosis	+	-	-	+	-	-	+	-	-	-	-
Enterobacter cloacae	-	-			-	+		-	+		
Staphylococcus epidermidis	+		+ -				+				

Legend: GR= Gram reaction, GL=Gelatin liquefaction, MTL=Motility, MR= Methyl red, VP= Voges-Proskauer , NR= Nitrate reduction, IDL= Indole, CTL= Catalase, URS= Urease, OXL= Oxalate.

Table III. Characteristics of micro organisms isolated from the gastrointestinal tract of *A. marginata* **continued** (Características de los microorganismos aislados del tracto gastrointestinal de *A. marginata* continuaron).

Organism	Cell shape	GLC	SCR	LAT	MAL	MAN	HL test
Azotobacter chroococcum	Ovoid	UC	AG	UC		A	
Kurthia zoopfii	Rod		A	UC	UC	UC	
Shigella sp	MLR	A	UC	A	A	A	OX
Xanthomonas fragaride	Rod	UC	UC	UC	UC		OX
Micrococcus lutus	Sphere	A	A	UC	A	A	
Aerococcus viridians	Cocci	A	A	AG	A	AG	
Klebsiella rhinosderomalis	Rod	UC	UC	UC	UC	UC	
Streptococcus feacium	Cocci	UC	UC	UC	UC	AG	
Proteus morganii	LR	AG	UC	UC	A	UC	
Clostridium sporogenes	LR	NC	UC	UC	UC	UC	
Pseudomonas syringae	Cocci	UC	A	A	A	A	
Erwinia amylovora	Rod	AG	UC	UC	UC	UC	None
Corynebacterium zerosis	Rod	AG	A	UC	A	UC	
Enterobacter cloacae	SR	A	UC	UC	A	A	
Staphylococcus epidermidis	Rod						F

Legend: AG=Gas and acid production, UC=no change, A=acid production, F=fermentation, HL=Hough and Liefson, OX=oxidation, SR=Short Rod, MLR=Medium Long Rod, LR=Long Rod, GLC=Glucose, SCR=Sucrose, LAT=Lactose, MAL=Maltose, MAN=Mannitol.

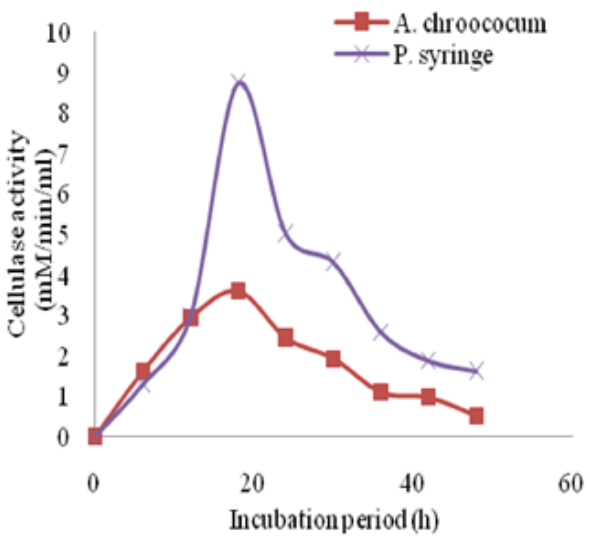

Figure 1. Cellulase activities of bacterial isolates from the mouth and stomach region of *A. marginata* (Actividades de la celulasa de aislados bacterianos de la región de la boca y el estómago of *A. marginata*).

had the highest cellulase activity. This observation disagrees with the findings of Mohamed *et al.* (2010, p. 35-42) & Oyeleke *et al*. (2012, p. 15-20) that reported maximum cellulase productivity after 24 h by *Bacillus sp*. The observed disparity in period of cellulase activities by the bacterial isolates may be due to the differences in the bacteria species. The structural component of plant cell wall can be degraded to glucose through enzymatic activities of cellulolytic complexes using endo-β-1,4-glucanases, exo-β-1,4-cellobiohydrolases, and β-glucosidases (Lynd *et al.*, 2002, p. 506-577). A true cellulolytic bacterium is one which produces multiple enzymes which act together for effective hydrolysis of cellulose (Leschine, 1995, p. 399-426). Ademolu *et al.* (2013, p. 73-77) reported the presence of cellulase enzymes in aestivating, *A. marginata* and suggested that the enzyme was produced by gut microbes.

The activity of protease from bacterial isolates from the mouth and stomach region in *A. marginata* is shown in **Figure 2**. *Azotobacter chroococcum* had a higher protease activity with a peak protease activity of 17.18µmol/min/ml at 24 h of incubation while *Pseudomonas syringae* had a lower protease activity with a peak of 16.67µmol/min/ml at 12 h of incubation. However, there is a decrease in protease activity by the bacterial isolates as the period of incubation increases. Godoy *et al*. (2013, p. 7) reported differences in protease activity in the different gut regions of the apple snail *P. canaliculata*. Some earlier reports revealed proteolytic enzyme activities in the gut of vetigastropods and stylommatophoran pulmonates while none was reported in ampullarids (Guionie *et al.*, 2003, p. 503-510; Martin *et.al.* 2011, p. 365-373).

Protease activity was higher for A. *chroococcum* than for *P. syringae* in the present study, however, Ariole & ilega, (2013, p. 128) reported that Pseudomonas spp had a higher protease activity in fresh water snail *Pila ovate* while Wellingta & Meire (2004, p.93) & Oyeleke *et al*. (2012, p. 18) reported highest protease activity by *Bacillus spp.* at 9 h and 18 h of incubation respectively. Endogenous proteases from the salivary gland, exogenous sources from microbes residing in the midgut and protease from uncertain sources were implicated in protein digestion in the apple snail, *P. canaliculata* (Godoy *et al*., 2013, p. 8). Amusan & Omidiji (1999, p. 1-16) revealed that snail in the wild and captive rearing effectively utilized mushroom, ants and earthworms. Protein digestion was effective due to the availability of proteases in the gut. Bacterial proteolytic activity was also reported in the gut of actively feeding *C. aspersum* (Koleva *et al*., 2015, p. 267). Proteolytic activity of bacterial organisms, apart from aiding the digestion of protein, is essential for decomposition or plant and animal protein matter for nutrient recycling and pollution-free environment (Dar *et al*., 2017, p.203)

Figure 3 shows the production of amylase by bacterial isolates from the gut of *A. marginata*.

The peak amylase activities were 9.05 and 8.02µmol/min/ml for *P. syringae* and *A. chroococcum*, at 24 and 18h of incubation respectively. Amylase hydrolyses starch which features in most animal feed materials. The heightened interest in heliciculture has necessitated the inclusion of grains in the diet of captive reared snails. The endosperm of cereal grains consists of about 60-90% starch granules depending on cereal type (Kent & Ever, 1994, p. 44). Prassanna *et al* (2014, p. 42) when studying the gut microbiota of *Bombyx mori*, reported that gut bacterial organisms produces a cocktail of enzymes which effectively digested starch compound in the silkworm. *Pseudomonas syringae* isolated from the stomach region of *A. marginata* which exhibited highest amylase activity after 24 hours is consistent with the report of Okukubo *et al*. (1964, p. 155-158), he revealed that amylase activity reached a

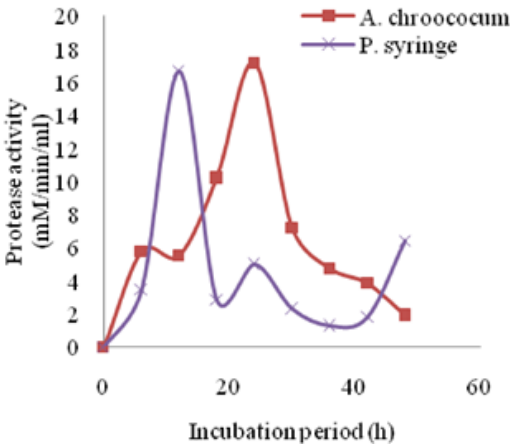

Figure 2. Protease activities of bacterial isolates from the motuj and stomach region of *A. marginata* (Actividades proteasas de aislados bacterianos de la región motuj y estomacal de *A. marginata*).

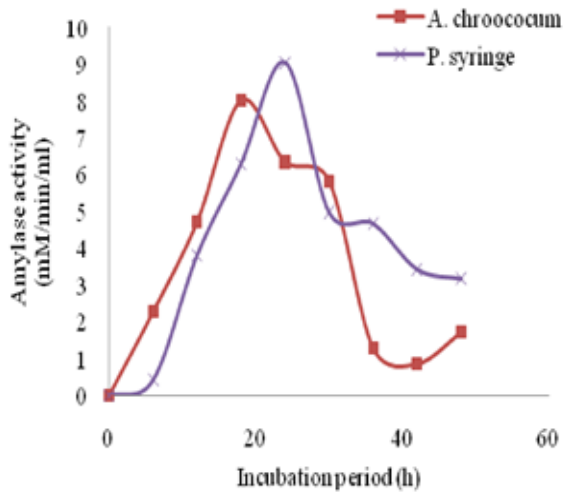

Figure 3. Amylase activities of bacterial isolates from the motuh and stomach of *A. marginata* (Actividades de la amilasa de aislados bacterianos del motuh y el estómago de *A. marginata*).

peak after 24 h, although for a different species of bacteria. The activities of enzyme producing bacteria in the gut of snails is an indication of the snail's intestinal tract being a good source of nutrients for microbes and consequently a symbiotic relationship exists between the microbes and the snail (Chow *et al*., 2010, p. 6). The significance of bacteria in the process of digestion still require further investigation, however, host phylogeny and diet type has been reported (Ley *et al*., 2008, p. 1650) to influence bacterial diversity in the host tissue, hence a bacterial community codiversifies with its host. Some studies revealed that bacteria from the gut of gastropods have the capacity to degrade substrates such as mannan, carboxymethyl cellulose, liminaran, and agarose (Charrier *et al*., 2006, p. 678; Kim *et al*., 2007, p. 2929). Reports by Brendelberger, (1997, p. 1636) & Erasmus *et al*., (1997, p. 381), revealed that snails that had antibiotics administered to them to remove bacteria in the gut were able to degrade polysaccharides, after being treated, this implies that the bacteria help but may not be indispensable in the process of digestion. Bacteria have been reported as food sources for some snail species (Martin *et al*., 2011, p.371) and this may be true for many other snail species, since most of the bacteria species reported in the present study had also been reported as being present in snail gut by different authors (Odiete & Akpata, 1983, p. 124; Oyeleke *et al*., 2012, p.18 & Cicero *et al*., 2015, p. 4196), implying that snails benefit from the presence of gut bacterial flora.

CONCLUSIONS

It can be concluded from this study that in the gastrointestinal tract of *A. marginata*, *A. chroococcum* and *P. syringae* from the mouth and stomach region, showed varied levels of cellulolytic, proteolytic, and amylolitic activities. The activity level of the digestive enzyme depends on the bacteria species within the gut region. Also, the bacterium existing within the snail gut seems to aid the snail's ability to adapt to different diet types and could be a guide in making a choice of the suitable feeding materials for *A. marginata* when it is reared in captivity.

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