

Comparative Studies of Biodegradation of Wastes Using Cellulase from Garden Snails (*Limicolaria flammea*) and Giant African Snails (*Archachatina marginata*)

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ABSTRACT

The generation of solid waste is a massive and ever-growing problem that affects the entire world, particularly developing countries. In Africa, waste recycling is not fully embraced and performed; as a result, these waste products are viewed as physical contaminants that can harm the ecosystem, generate pollution, and cause a lot of environmental risks. The use of synthetic compounds in the decomposition of wastes could harm the ecosystem and lead to global warming, thus biocatalysts are a preferred option. Cellulase, a hydrolytic enzyme, could digest cellulose, a main component of solid waste. Garden snail (*Limicolaria flammea*) and Giant Africa snail (*Archachatina marginata*) are organisms that feed on dead, decaying and actively growing plants due to their possession of cellulolytic enzyme. Cellulase from the gut of *Archachatina marginata* haemolymph and *Limicolaria flammea* had specific activities of 1.15 U/mg and 1.14 U/mg respectively. It also showed high level of degradation. *Limicolaria flammea* haemolymph showed high activity of 504.11% on soluble starch, 529.39% on kick tree and 993.77% on carton paper. This study suggests that cellulase from both *Limicolaria flammea* and *Archachatina marginata* has biodegrading potentials on waste materials. While their effects and the rate of degrading wastes differ, they could both be relevant in waste management especially on kitchen waste.

Keywords: Cellulase, *Archachatina marginata*, *Limicolaria flammea*, waste, carboxyl methyl cellulose, haemolymph.

Introduction

Waste could be described as any material which has been used and is no longer wanted because the valuable or useful part of it has been removed (Awosusi, 2010). In 2016, major cities created 2.01 billion tonnes of solid trash; however, due to the high population rate and urbanization, annual waste creation is anticipated to rise by 70% from 2016 levels to 3.40 billion tonnes in 2050 (Ezima

et al., 2014). Cellulose is acquired either directly or indirectly from forest production or from waste materials. These might be in solid, gaseous or liquid form. Some are even hazardous, and are primarily produced by human activity (Vijayalakshmi, 2020). Solid wastes are divided into three categories: municipal wastes (trash created by households), industrial wastes

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(hazardous wastes), and infectious wastes (hospital generated waste). Market waste includes food waste, paper, cardboard, plastics, textiles, rubber, and a variety of other materials (Awosusi, 2010; Ebna *et al.*, 2013). Cellulose is consumed by a wide range of species, including fungi, bacteria, plants, and protists, as well as a wide range of invertebrate animals, such as insects, crustaceans, annelids, mollusks, and nematodes (Watanabe and Tokuda, 2001); cellulolytic microorganisms produce a variety of enzymes known as cellulases that are catalytic. Cellulase plays a vital part in natural biodegradation processes, where waste cellulosic materials are degraded or transformed into valuable products to meet the needs of an ever-increasing population (Lakhundi *et al.*, 2015). Cellulases account for a significant portion of the global industrial enzyme market, accounting for 20% of the total enzyme market worldwide. It has found application in a wide range of industries, such as in biofuels production, textiles, pulp and paper production, and food production for industrial purposes. It is also reported to be used as an additive in detergents and improving the digestibility of animal feeds (Zhang and Zhang 2013, Srivastava *et al.*, 2015). The invertebrate mollusk, African giant snail (*Archachatina marginata*), feeds on any edible plant or animal material (Ademolu *et al.*, 2005). These range from succulent fruits and vegetables e.g. paw-paw, banana and their leaves to ripe or overripe fruits, decaying organic material and grains. The nature of their food will require an effective cellulase system for degradation and digestion (Ojo and Zira, 2017). *Limicolaria* species are known to occur in abundance in forest edges and modified habitats such as plantations and farms. Hiding *Limicolaria* snails are extremely difficult to locate and it is known that they spend a considerable amount of time in the soil (Raut and Barker, 2002).

Materials and Methods

Dinitrosalicylic acid (DNSA) and Potassium sodium tartrate were obtained from Trust Chemical

laboratories. Carboxymethylcellulose (CMC), sodium hydroxide and bovine serum albumin (BSA) were obtained from Sigma-Aldrich Chemical Company, Limited, St. Louis, MO., U.S.A. Ethylenediaminetetraacetic acid (EDTA) was obtained from NAAFCO Scientific Supplies Ltd., New York. Sodium acetate and ammonium sulphate were purchased from BDH laboratory supplies Pooles, England. All other reagents were of analytical grades and were obtained from reputable sources. Glass distilled water was used for all preparations of solutions. Giant African Snail (*Archachatina marginata*) and Fresh Garden Snail (*Limicolaria flammea*) were acquired from Sagamu Local Market, Sagamu, Ogun State. They were taken to the laboratory in a well-ventilated container. These were identified and authenticated at the Department of Zoology, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria.

Homogenization and Enzyme Extraction

The gut and haemolymph of *Archachatina marginata* (African Giant Snail) and *Limicolaria flammea* (fresh garden snail) were washed and rinsed with distilled water. These were then homogenized in 10 mM acetate buffer pH 5.0. The homogenates were filtered with a superfine mesh with consistent stirring. The supernatant which served as the crude enzyme was collected and stored at 4 °C (Ezima *et al.*, 2014)

Enzyme Purification of Cellulase from Garden Snail and Giant African Snail

Ammonium Sulphate Precipitation and dialysis

The crude enzyme from homogenization was brought to 65% Ammonium Sulphate saturation by the continuous addition of solid ammonium sulphate with continuous agitation using a magnetic stirrer. The suspension was refrigerated overnight to improve the efficiency of the precipitation. The precipitated enzyme was collected by centrifugation at 2500 rpm for 15 minutes. It was

dissolved in 10 mM sodium acetate buffer pH 5.0 and dialyzed against the same buffer.

Enzyme Assay

Cellulase activity was assayed according to the method of Zhang *et al.* (2006) using Carboxymethyl-cellulose as the substrate. The reducing sugars were determined by estimating amount of sugars released from 1% solution of CM-cellulose. The reaction mixture consisted of 0.06 ml of crude enzyme, 0.6 ml of CMC-buffer and 0.6 ml of 3,5-Dinitrosalicylic acid (DNSA) reagent and boiled at 100 °C in a water bath for 2 minutes. The reaction mixture was allowed to cool to room temperature. After incubation at 37 °C, the reaction was stopped by adding 0.6 ml of DNSA reagent and boiled at 100 °C for 2 minutes. The activity was determined by the measurement of absorption at 540 nm. One unit of Cellulase activity was expressed as the amount of enzyme that released 1 μmol of reducing sugars (glucose equivalent) in 1 min under the above conditions. A standard calibration curve of glucose was constructed and used for the estimation.

Protein Determination

Protein concentration was determined by the method of Bradford (1976) using Bovine Serum Albumin (BSA) as the standard. The reaction mixture consists of 0.1 ml of the enzyme solution and 1.0 ml of Bradford reagent. The absorbance was read at 595 nm.

Effect of Temperature on the Cellulase Activity of Garden Snail and Giant African Snail

The activity of the cellulase was assayed at temperatures between 30° C and 100 °C to investigate the effect of temperature on the activity of the enzymes and to determine the optimum temperature of the enzyme. The assay mixture was first incubated at the initial temperature for 10 min before starting the reaction by the addition of an aliquot of the enzyme which had been equilibrated at the same temperature.

Effect of pH on the Enzymes Activity

The activity of cellulase in different buffers at different pH values ranging between pH 3.0-10.0 (10 mM citrate buffer pH 3-6; 10 mM phosphate buffer pH 7-10) was investigated. The substrate solution was prepared with different buffer concentrations and each enzyme assay was carried out accordingly as described.

Determination of Kinetic Parameters

The kinetic parameters of the enzyme were also estimated using the Lineweaver-Burk plot, by varying the concentrations of CMC solution between 0.1 ml and 1.0 ml.

Biodegradability Test

Biodegradability test of the samples were carried out in triplicate. The reaction mixtures contained 0.6 ml of the buffer-substrate solution (0.25g of wood waste in 25 ml of 10 mM Sodium Acetate buffer) and 5 g of leaves waste in 100 ml of 10 mM Sodium acetate buffer, pH 5.0, 0.06 ml of the enzyme, 0.6 ml of DNSA, boiled for 2 minutes after which 2.0 ml of distilled water was added. A control with CMC as substrate was run alongside each waste. The blank was prepared adding all components except buffer-substrate to the mixture while the activity of the enzyme remained as carried out. The samples were thoroughly mixed and the absorbance of the samples was read at 540 nm.

Results

Purification Table

The enzyme was partially purified using 65% ammonium sulphate precipitation. The summary of the Purification profile of cellulase from the gut and haemolymph of *Archachatina marginata* and *Limicolaria flammea* is shown in Tables 1-4.

Table 1: Summary of the purification of cellulase from *Archachatina marginata* gut

Purification step	Total Protein (mg)	Total Activity ($\mu\text{mol}/\text{min}$)	Specific Activity	% Yield	Purification fold
Crude Extract	1162.61	805.21	0.69	100	1
65% Ammonium sulphate	538.26	637.24	1.18	46.32	1.72

Table 2: Summary of the purification of cellulase from *Archachatina marginata* haemolymph

Purification step	Total Protein (mg)	Total Activity ($\mu\text{mol}/\text{min}$)	Specific Activity	% Yield	Purification fold
Crude Extract	652.33	812.32	1.25	100	1
65% Ammonium sulphate	241.12	629.11	2.61	37	2.09

Table 3: Summary of the purification of *Limicolaria flammea* (Garden Snail) Gut cellulase

Purification step	Total Protein (mg)	Total Activity ($\mu\text{mol}/\text{min}$)	Specific Activity	% Yield	Purification fold
Crude Extract	312.41	229.48	0.73	100	1
65% Ammonium sulphate	167.92	191.31	1.14	53.74	1.56

Table 4: Summary of the purification of *Limicolaria flammea* (Garden Snail) Haemolymph cellulase

Purification step	Total Protein (mg)	Total Activity ($\mu\text{mol}/\text{min}$)	Specific Activity	% Yield	Purification fold
Crude Extract	513.93	483.81	0.94	100	1
65% Ammonium sulphate	315.33	392.72	1.25	61.36	1.32

Effect of Temperature on Cellulase Activity

The effect of temperature on the rate of activity of cellulase is as presented in Figures 1-4. An optimum temperature of 60°C was obtained for cellulase from *Archachatina marginata* haemolymph. Cellulase from Garden snail gut and haemolymph had an optimum temperature of 50 °C,

while *Archachatina marginata* gut had an optimum temperature of 80 °C.

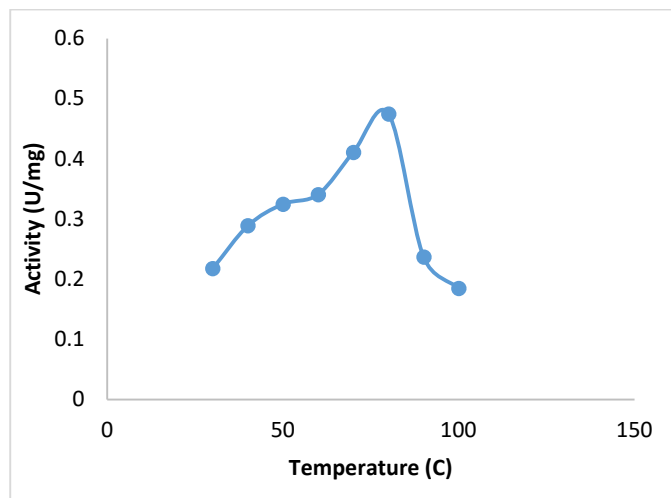


Figure 1: Effect of Temperature on Cellulase from *Archachatina marginata* gut

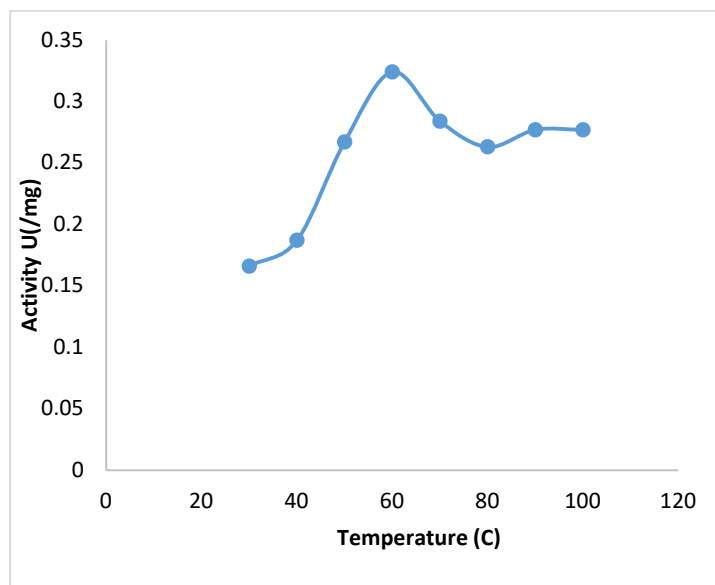


Figure 2: Effect of Temperature on Cellulase from *Archachatina marginata* haemolymph.

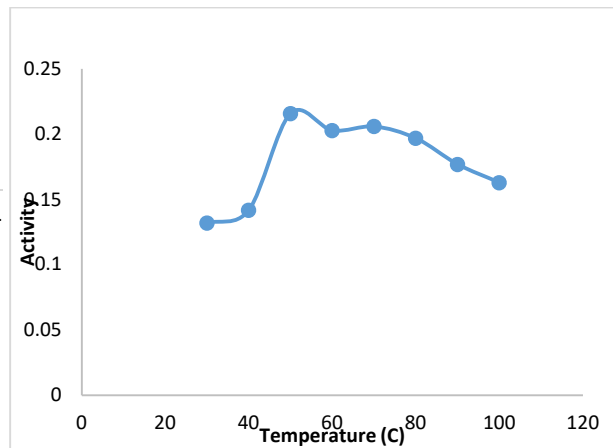


Figure 3: Effect of Temperature on Cellulase from *Limicolaria flammea* gut

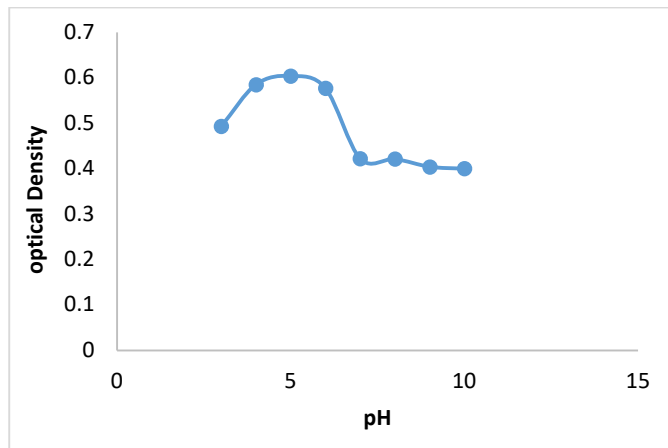


Figure 5: Effect of pH on Cellulase from *Archachatina marginata* gut

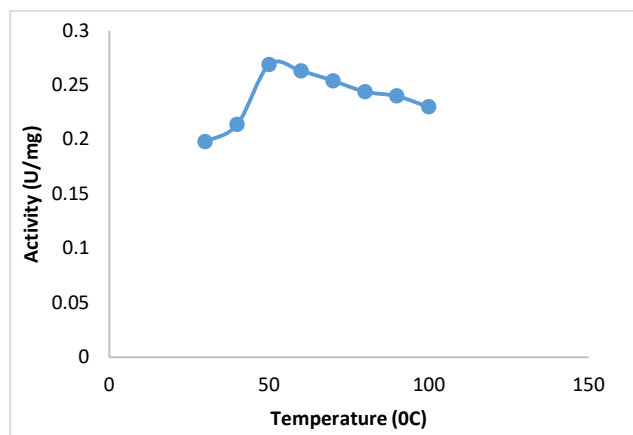


Figure 4: Effect of Temperature on Cellulase from *Limicolaria flammea* haemolymph

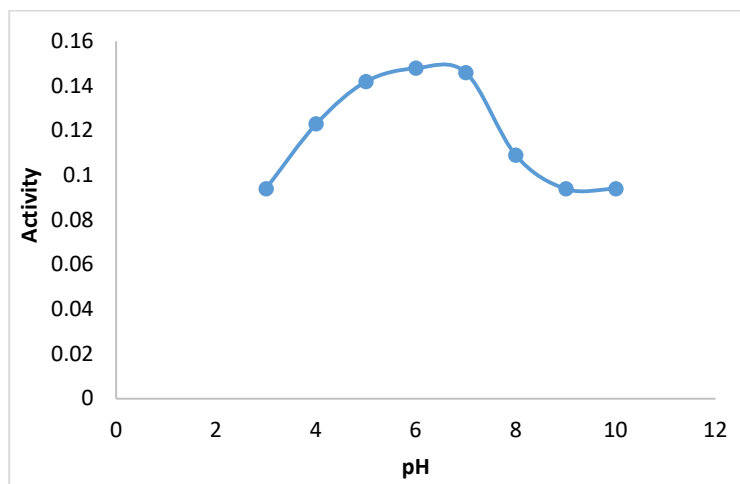


Figure 6: Effect of pH on Cellulase from *Archachatina marginata* haemolymph.

Effects of pH on the Enzyme Activity

The effects of pH on the activity of cellulase is as presented in Figures 5-8. An optimum pH of 5.0 was obtained for cellulase from *Archachatina marginata* gut and *Limicolaria flammea* gut, a pH of 6.0 was obtained for cellulase from *Archachatina marginata* haemolymph and an optimum pH of 7.0 from *Limicolaria flammea* haemolymph.

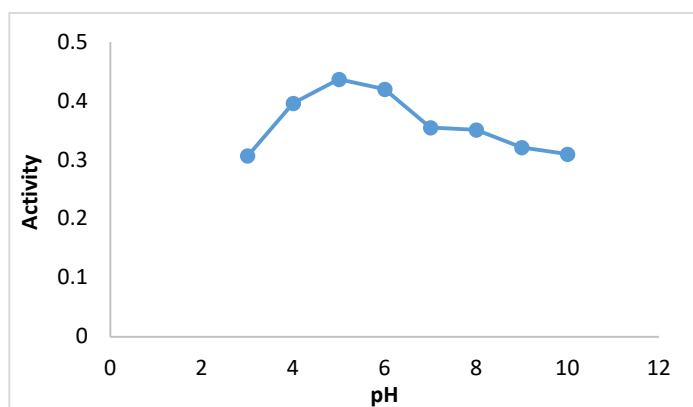


Figure 7: Effect of pH on Cellulase from Garden Snail (*Limicolaria flammea*) gut

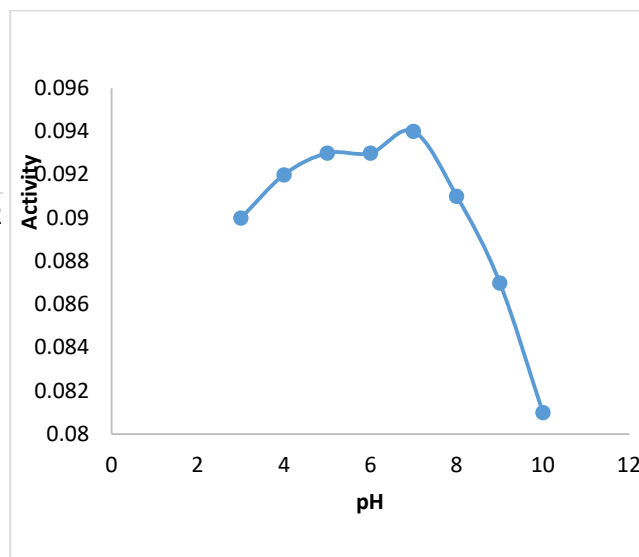


Figure 8: Effect of Temperature on Cellulase from *Limicolaria flammea* haemolymph

Determination of Kinetic Parameters

The summary of K_m and V_{max} of cellulase obtained from gut and haemolymph of *Archachatina marginata* and *Limicolaria flammea* is shown in Table 5.

Biodegradation

Action of cellulase from gut and haemolymph of African giant snail and Garden snail on kitchen waste, industrial waste, leaves, paper and wood was determined and compared in terms of their activity because carboxyl methyl cellulose is a natural substrate for the cellulase enzyme, the absorbance of 1% CMC to cellulase is used as a standard measure of samples to cellulase. The percentage inhibition activity of all the samples utilized in this study is presented in the tables.

Table 5: Summary of Kinetic Parameters

	African Giant Snail (<i>Archachatina marginata</i>) Haemolymph	African Giant Snail (<i>Archachatina marginata</i>) Gut	Garden Snail (<i>Limicolaria flammea</i>) Gut	Garden Snail (<i>Limicolaria flammea</i>) Haemolymph
Km (mg/ml)	2.20	4.80	2.40	5.2
Vmax (Umol/min)	0.05	0.15	0.80	0.062

Table 6: Action of Cellulase from gut and haemolymph of *Archachatina marginata* and *Limicolaria flammea* on industrial wastes

	African Giant Snail (<i>Archachatina marginata</i>) Haemolymph	African Giant Snail (<i>Archachatina marginata</i>) gut	Garden Snail (<i>Limicolaria flammea</i>) Gut	Garden Snail (<i>Limicolaria flammea</i>) Haemolymph
Groundnut shell	63.55	59.95	29.96	168.62
Sorghum	67.59	67.10	66.73	375.59
Millet	73.33	70.68	68.30	384.83
Rice Hulls	101.39	68.19	82.10	462.14
Wheat	111.93	77.51	90.73	384.43
Soluble starch	107.63	76.50	89.56	504.11

All results are presented as % activity of the enzyme on CM-cellulose.

Table 7: Action of Cellulase from gut and haemolymph of *Archachatina marginata* and *Limicolaria flammea* on wood

	African Giant Snail (<i>Archachatina marginata</i>) Haemolymph	African Giant Snail (<i>Archachatina marginata</i>) Gut	Garden Snail (<i>Limicolaria flammea</i>) Gut	Garden Snail (<i>Limicolaria flammea</i>) Haemolymph
Fig tree (<i>Ficus carica</i>)	18.22	15.02	27.35	153.92
Oak Tree (<i>Quercus</i>)	16.27	11.84	3.54	19.92
Palm plum Tree (<i>Hyphaene thebaica</i>)	17.62	13.36	30.39	171.10
Teak Tree (<i>Tectona grandis</i>)	173.09	25.35	94.05	529.39
African Balsam Tree (<i>Daniella oliver</i>)	28.65	16.87	30.91	173.97
Maple Tree (<i>Acer</i>)	16.32	28.31	14.79	83.19
Kapok Tree (<i>Ceiba pentandra</i>)	53.56	17.41	32.35	182.07
Mugwort Tree (<i>Artemisia vulgaris</i>)	14.37	19.88	9.16	51.56
Kola Tree (<i>Cola acuminata</i>)	14.17	15.81	9.03	50.81
Ohugbo Tree (<i>Triplochiton scleroxylon</i>)	1.17	17.86	8.67	48.85

All results are presented as % activity of the enzyme on CM-cellulose

Table 8: Action of Cellulase from gut and haemolymph of *Archachatina marginata* and *Limicolaria flammea* on paper

	African Giant Snail (<i>Archachatina marginata</i>) Haemolymph	African Giant Snail (<i>Archachatina marginata</i>) Gut	Garden Snail (<i>Limicolaria flammea</i>) Gut	Garden Snail (<i>Limicolaria flammea</i>) Haemolymph
Carton paper	353.96	177.43	176.55	993.77
Brown paper	133.10	109.84	115.13	648.07
Tissue paper	56.76	76.52	78.76	443.34
Art paper	259.36	142.30	144.29	812.20
Jotter paper	243.33	136.35	146.99	827.39
Newspaper	107.68	156.52	144.25	811.96
Office file	133.10	161.87	132.96	748.44
Notebook	108.58	196.08	136.66	769.24

All results are presented as % activity of the enzyme on CM-cellulose.

Table 9: Action of Cellulase from gut and haemolymph of *Archachatina marginata* and *Limicolaria flammea* on leaves

	African Giant Snail (<i>Archachatina marginata</i>) Haemolymph	African Giant Snail (<i>Archachatina marginata</i>) Gut	Garden Snail (<i>Limicolaria flammea</i>) Gut	Garden Snail (<i>Limicolaria flammea</i>) Haemolymph
Orange leaf (<i>Citrus sinensis</i>)	180.87	113.09	90.53	509.58
Mango leaf (<i>Magnifera indica</i>)	348.32	148.14	141.90	798.75
Kolanut leaf (<i>Cola acuminata</i>)	218.62	117.41	95.36	536.74
Cassava leaf (<i>Manihot esculenta</i>)	162.30	78.57	74.58	419.80
Lemon leaf (<i>Citrus lemon</i>)	148.02	84.79	73.78	415.32

All results are presented as % activity of the enzyme on CM-cellulose

Table 10: Action of Cellulase from gut and haemolymph of *Archachatina marginata* and *Limicolaria flammea* on kitchen wastes and some foodstuff

	African Giant Snail (<i>Archachatina marginata</i>) Haemolymph	African Giant Snail (<i>Archachatina marginata</i>) Gut	Garden Snail (<i>Limicolaria flammea</i>) Gut	Garden Snail (<i>Limicolaria flammea</i>) Haemolymph
Garri	137.89	57.16	35.94	202.24
Egg Shell	61.85	82.73	19.14	107.72
Yam Flour	49.97	74.76	40.20	226.28
Yam peel	91.16	65.42	53.74	302.49
Orange Peel	452.37	179.95	195.51	1100.49
Pineapple peel	94.90	129.10	115.71	647.69
Onion peel	428.0	175.41	185.73	1045.45
Beans peel	122.06	90.98	62.59	352.30

All results are presented as % activity of the enzyme on CM-cellulose.

Discussion

Cellulases are found in a wide range of plants and have been researched in both lower and higher plants (Ezima, 2014). In addition to a wide range of microbiological sources, including fungi such as *Trichoderma viride* (Ezima, 2014), bacteria in which they are known as 'cellulosomes,' e.g. thermophilic or thermostable bacteria and invertebrates and insects such as termite (Ezima *et al.*, 2014). Cellulase from the gut of *Archachatina marginata* and *Limicolaria flammea* had a specific activity and yield of 1.18 U/mg;46.32% and 1.14 U/mg;53.74% respectively. A specific activity of 2.61 U/mg was obtained from the cellulase of

Archachatina marginata haemolymph. The haemolymph of *Limicolaria flammea* had a specific activity of 1.25 U/mg with a percentage yield of 61.36. These values are higher than cellulase obtained from *Aspergillus niger* fungi as reported by Hurst *et al.* (1977) which had a specific activity of 0.0452 U/mg. Fagbohunka *et al.* (1997) reported a specific activity of 65.5 U/mg for cellulase from the haemolymph of *Archachatina marginata*. Higher values of specific activities have been reported for cellulase from termite sources *Nasutitermes exitosus* and *Copotermes lacteus* (O'Brein and Slaytor 1982).

Enzyme from the gut and haemolymph of *Archachatina marginata* had optimum temperatures of 80 °C and 60 °C respectively. An optimum temperature of 50 °C was obtained for cellulase from the gut and haemolymph of *Limicolaria flammea*. These optimum temperature values obtained was the same in comparison to that obtained with the cellulase from the *Reticulitermes speratus* (Kolbe), a Japanese Subterranean Termite (Watanabe *et al.*, 1997). A similarly high optimum temperature of 55 - 60 °C was observed for the cellulase produced by *Streptomyces malaysiensis* (Nascimento *et al.*, 2009). Fagbohunka *et al.* (2016) reported an optimum temperature of 50 °C for cellulase from the termite worker *Ametermes eveuncifer* (Silverstri).

The cellulase activity was affected by the pH of the reaction mixture. Cellulase from *Archachatina marginata* and *Limicolaria flammea* gut had an optimum pH of 5.0. Optimum enzyme activity was found at pH of 7.0 for *Limicolaria flammea* haemolymph, while *Archachatina marginata* haemolymph showed an optimum pH at 6.0. An optimum pH of 5.0 for enzyme activity with broad ranges of pH stability was observed in *Thermomonospora sp.* (George *et al.*, 2001). A pH of 8.0 was reported for cellulase from termite worker *Ametermes eveuncifer* (Silverstri) (Fagbohunka *et al.*, 2016).

Kinetic studies on the enzyme revealed the K_m and V_{max} of the enzyme from *Limicolaria flammea* haemolymph as 5.2 mg/ml and 0.062 μ mol/min respectively. The K_m and V_{max} of the enzyme were 2.20;0.05;4.80;0.15; and 2.40mg/ml;0.80 μ mol/min for *Archachatina marginata* haemolymph, *Archachatina marginata* gut and *Limicolaria flammea* gut respectively. A high K_m indicates weak binding, that is, low affinity of the enzyme for the substrate while a low K_m indicates strong binding that is high affinity of the enzyme for the substrate (Nelson and Cox, 2004).

Based on biodegradation of waste materials from the results above, it was observed that cellulase gotten from the haemolymph and guts of each snail have high or low activities on similar waste. On industrial waste, *Archachatina marginata* haemolymph had a high activity on wheat (111.73%) and low activity on groundnut shell (63.55%); *Archachatina marginata* gut had a high activity on wheat (77.51%) and low activity on groundnut shell (59.95%). *Limicolaria flammea* haemolymph cellulase had a high activity on soluble starch (504.11%) and low activity on groundnut shell (168.62%). Cellulase from *Limicolaria flammea* gut had the highest activity on wheat (90.73%) and lowest degradation activity on groundnut shell (29.96%). On wood waste, *Archachatina marginata* haemolymph cellulase had a high activity on kick tree (173.09%) and low activity on ohugbo tree (1.17%). *Archachatina marginata* gut had a high activity on maple tree (28.31%) and low activity on oak tree (11.84%); *Limicolaria flammea* haemolymph had a high activity on kick tree (529.39%) and low activity on oak tree (19.92%); *Limicolaria flammea* gut had a high activity on kick tree (94.05%) and low activity on oak tree (3.54%).

With paper waste, *Archachatina marginata* haemolymph cellulase had the high activity on carton paper (353.96%) and low activity on newspaper (107.68%); cellulase from *Archachatina marginata* gut had a high activity on note book (196.08%) and low activity on tissue paper (76.52%); *Limicolaria flammea* haemolymph has a high activity on carton paper (993.77%) and low activity on tissue paper (443.34%); *Limicolaria flammea* gut has a high activity on carton paper (176.55%) and low activity on tissue paper (78.76%). The different degrees of activities of the enzyme on the wood samples may be attributed to the different texture of the wood and the type of cellulose components the wood is made of. (Klyosov, 1990). On leaf

wastes, *Archachatina marginata* haemolymph cellulase had a high activity on mango leaf (348.32%) and low activity on lemon leaf (148.02%); *Archachatina marginata* gut cellulase had a high activity on mango leaf (196.08%) and low activity on cassava leaf (78.57%). Cellulase from *Limicolaria flammea* haemolymph had the highest activity on mango leaf (798.75%) and low activity on lemon leaf (415.32%). The enzyme from *Limicolaria flammea* gut reportedly had a high activity on mango leaf (141.90%) and low activity on lemon leaf (73.78%). This high degradation activity may be due to the fact that the leaves are readily susceptible to attack of the cellulase from the garden snail and giant African snail.

The highest enzymatic degradation of *Archachatina marginata* haemolymph cellulase was recorded on orange peel (452.37%) and lowest activity on yam flour (49.97%). *Archachatina marginata* gut cellulase was shown to have the highest degradation on orange peel (179.95%) and low activity on garri (57.16%). *Limicolaria flammea* haemolymph has a high activity on orange peel (1100.49%) and low activity on egg shell (107.72%). Cellulase from *Limicolaria flammea* gut had a high activity on orange peel (195.51%) and low activity on egg shell (19.14%).

Conclusion

Our findings showed that cellulase can be isolated from snails, and that the high activities of the enzyme, particularly the haemolymph of each snail on the wastes tested, makes it a better choice for large-scale cellulase production in the recycling, re-use, reduction, and management of cellulosic waste, particularly kitchen waste that is commonly disposed of improperly and contributes to environmental pollution and the ozone layer's depletion. It was also established that the cellulose gotten from the gut of *Archachatina marginata* with the temperature of

80 °C is thermally stable but it has a low enzymatic degradation activity.

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Competing interests

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

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