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Partial purification and characterization of cellulases from digestive tracts of the African giant snail (*Achatina achatina*)

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Abstract: Crude cellulase was extracted from the digestive tracts of 30 mature *Achatina achatina* and subjected to a 3-step purification process of ammonium sulfate precipitation, dialysis, and gel filtration. This purification procedure gave 3 prominent enzyme activity peaks that coincided with protein peaks and were designated A, B, and C, corresponding to high endoglucanase, β-glucosidase, and total cellulase activities, respectively. Temperature optima of 50 °C were recorded for β-glucosidase and total cellulase while 45 °C was recorded for endoglucanase. Total cellulase, glucosidase, and endoglucanase showed maximum activities at pH values of 5.5, 4.5, and 7.5, respectively. Kinetic studies show that total cellulase has a V_{max} and K_m of 2427.18 μmol/min and 15.12 mg cellulose and endoglucanase has values of 955.11 μmol/min and 2.39 mg Na-CMC, while the values of β-glucosidase are 946.97 μmol/min and 4.3 mM cellobiose, respectively. This study shows that cellulases from digestive tracts of *A. achatina* could be utilized for degradation of cellulose-containing materials because of their high thermostability and acid/alkali stability, which reflect the potential commercial significance of the enzyme.

Key words: *Achatina achatina*, cellulases, purifications, characterization, sodium carboxymethyl cellulose (Na-CMC), cellobiose

1. Introduction

Cellulase (EC 3.2.1.4) refers to a class of hydrolase enzymes produced by fungi, bacteria, protozoans, and termites that catalyzes the hydrolysis of cellulose (1,2). Microorganisms constitute the most important source for the production of industrial enzymes that have great economic potential in many processes (3), but some animals, particularly ruminants and mollusks, degrade cellulose with the help of cellulases found in their guts (4). Cellulose and lignocelluloses are the most abundant renewable biomass available on our planet (5), and their biodegradation by cellulases produced by numerous microorganisms represent a major carbon flow from fixed carbon sinks to atmospheric carbon(IV) oxide (6), and is very important in several agricultural and waste treatment processes. Cellulose-based waste materials could also be widely used to produce sustainable bio-based products and bio-energy to replace depleted fossil fuels (7). The effective conversion of recalcitrant lignocellulolytic materials to fermentable sugars requires 3 sequential steps: size reduction, pretreatment/fractionation, and enzymatic hydrolysis (8). In general, cellulose is degraded to cellodextrin or cellobiose or glucose by the synergistic actions of the 3 cellulase systems: endo-1,4-β-glucanase, exo-1,4-β-glucanase, and β-D-glucosidase (9). Endo-glucanases cleave randomly intramolecular β-1,4-glucosidic linkages within the cellulose chain. Exo-glucanases (cellobiohydrolases), on the other hand, cleave the accessible ends of cellulose molecules to liberate glucose and cellobiose (10), while β-D-glucosidase hydrolyzes soluble cellobiose and other cellodextrins with a degree of polymerization (DP) up to 6, to produce glucose in the aqueous phase. Cellulases are relatively costly enzymes, and a significant reduction in cost will be important for their commercial use in bio-refineries. Hence, the need for natural and cheaper sources of cellulases is the driving force of this investigation.

2. Materials and methods

2.1. Materials

Mature giant African land snails (*Achatina achatina*) were bought from Ogige Main-Market, in Nsukka, Enugu State, and maintained in the laboratory at room temperature in a perforated metal box. The animals were fed mainly on fresh pawpaw (*Carica papaya*) leaves. The box was moistened with tap water at regular intervals.

2.2. Reagents/chemicals

3,5-Dinitrosalicyclic acid (DNS) ($C_7H_4N_2O_7$) was obtained from Lab. Tech. Chemicals, Avighkar, India; cellobiose ($C_{12}H_{22}O_{11}$) from Sigma Aldrich Chemicals Ltd, England;

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sodium carboxymethyl cellulose (Na-CMC) from BDA Chemicals Ltd., Poole, England; and sephadex G-200 from Superfine, India. Other chemicals/reagents were of analytical grade and were freshly prepared except where otherwise stated.

2.3. Methods

2.3.1. Isolation of cellulase from the digestive tracts of *Achatina achatina*

Dissection of the snails was carried out as described by Umezurike (11). The snails were deshelled and their digestive glands (digestive tracts) were removed. This was frozen in 50 mM sodium acetate buffer, pH 5.5, at about –15 °C. When necessary, the digestive tracts were homogenized using a pestle and mortar. The homogenate was centrifuged at 5000 rpm for 15 min at 4 °C. The supernatant was collected as crude enzyme (cellulases).

2.3.2. Enzyme purification

The crude enzyme was brought to 40% ammonium sulfate saturation. This was centrifuged at 5000 rpm for 15 min. The precipitate was dissolved in 50 mM Na-acetate buffer, pH 5.5, and dialyzed overnight in the same buffer with a change of buffer every 6 h. The dialyzed enzyme was introduced onto a Sephadex G-200 packed column (2.6 × 61.50 cm) pre-equilibrated with 50 mM Na-acetate buffer, pH 5.5. The protein was eluted with 50 mM Na-acetate buffer, pH5.5, at a flow rate of 1.8 mL/h. The fractions with high cellulase activity were pooled together and rechromatographed on Sephadex G-200 at an elution flow rate of 1.5 mL/h. Protein content and cellulase activity from each of the eluted fractions were monitored by absorbance at 280 nm and 540 nm, respectively.

2.3.3. Enzyme assays

2.3.3.1. Total cellulase activity assay

Total cellulase activity encompasses the activities of cellulase components (i.e. endoglucanase, exoglucanase, and β-glucosidase) sequentially working in synergy. The total cellulase activity was assayed according to the method of Mendels et al. (12), with slight modification. The assay mixture was made of 2 mL of 50 mM Na-acetate buffer, pH 5.5, and 0.5 mL of the enzyme. A strip of filter paper [Whatman No.1 filter paper strips measuring 1.0 × 6.0 cm (≈50 mg)] was immersed into the assay mixture and incubated in a water bath at 50 °C for 30 min, after which 0.5 mL of DNS reagent was added. The assay mixture was then boiled in a temperature-controlled water bath for 10 min. This was cooled on ice and the absorbance was read at 540 nm.

2.3.3.2. Endo-β-1,4-glucanase activity assay (carboxymethyl cellulase assay)

The endoglucanase activity assay was carried out according to the method of Mendels et al. (12) with slight modification. First, 0.1 mL of 2% sodium carboxymethyl celluose (Na-CMC) (high viscosity) in 50 mM Na-acetate buffer, pH 5.5, was pipetted into a test tube, followed by the addition of 0.1 mL of the enzyme. This was incubated in a temperature-controlled water bath at 50 °C for 30 min. The reaction mixture was then made up to 2.5 mL with 50 mM Na-acetate buffer, pH 5.5. Then 0.5 mL of DNS reagent was added and the reaction mixture was boiled in a temperature-controlled water bath for 10 min. This was cooled on ice and the absorbance was read at 540 nm.

2.3.3.3. β-1,4-Glucosidase activity assay

β-Glucosidase was assayed as described by Sternberg et al. (13) with slight modification. The reaction mixture consisted of 0.1 mL of 15.0 mM cellobiose in 0.05 M Na-acetate buffer, pH 5.5, and 0.1 mL of the enzyme solution. The assay mixture was incubated in a water bath at 50 °C for 30 min, after which the volume was made up to 2.5 mL with 0.05 M Na-acetate buffer. Next 0.5 mL of DNS reagent was added, and the mixture was boiled in a water bath for 10 min. After cooling on ice, the absorbance was read at 540 nm. All the experiments were in duplicate.

2.3.4. Protein estimation

Protein concentration was determined by the method of Lowry et al. (14).

2.3.5. Glucose estimation and enzyme characterization

Glucose concentration was estimated using the dinitrosalicylic acid (DNS) method as described by Miller (15). The effects of pH and temperature were determined according to the method of Uzyol et al. (3), and the kinetic parameters were calculated based on the Lineweaver–Burk transformation according to Dobrev and Zhekova (5).

3. Results and discussion

3.1. Enzyme purification

The giant land snails, *Achatina achatina*, are found more abundantly in areas with low vegetation, littered with decaying vegetable matter. Since cellulose forms a major source of carbohydrate for this animal, it is not surprising that cellulase (including β-glucosidase and endoglucanase) is found in large amounts in their digestive tracts. The total cellulase activity, endoglucanase activity, and β-glucosidase activity are 1822.87 units, 437.41 units, and 399.81 units, respectively (Table). These values are relatively high compared to the findings of Linton and Greenaway (16) from the gecarcinid land crabs *Gecarcoidea natalis* and *Discoplax hirtipes.*

The results show that cellulase from *A. achatina* is composed of endoglucanase, β-glucosidase, and probably exoglucanase, since peak C equally showed total cellulase activity. This is in agreement with work on cellulases from different sources (17–20).

Gel filtration on a Sephadex G-200 column results in an elution profile of multiple peaks whose protein peaks

Table. Purification profile of endoglucanase (A), β-glucosidase (B), and total cellulase (C).

Purification step		Volume (mL)	Total protein (mg)	Total cellulase activity (U)	Specific activity (U/mg protein)	Activity yield (%)	Purification fold
Crude enzyme		200.00	9735.20	60,960.00	6.26	100.00	1.00
40% $(NH_4)_2SO_4$ Saturation		20.00	1522.72	47,260.00	31.04	77.50	4.96
Dialysis		22.00	1420.32	45,450.00	32.00	74.56	5.11
Gel filtration chromatography	A	10.00	2.84	437.41	321.98	1.76	60.52
	B	10.00	2.13	399.81	624.43	2.22	100.71
	C	10.00	4.74	1822.87	347.34	2.70	55.49

A = Endoglucanase, B = β-glucosidase, C = Total cellulose. A unit enzyme activity (U) is defined as the amount of enzyme required to produce 1 µmol of reducing sugars per minute under experimental conditions.

do not coincide with cellulase activity peaks (Figure 1). Consequently, the different peaks indicating fractions with high cellulase activity were pooled together and rerun on a Sephadex G-200 column with a flow rate of 1.5 mL/h (Figure 2), which resulted in 3 prominent cellulase activity peaks that coincided with protein peaks. Assay of the different fractions reveals that peak A contains endoglucanase, peak B contains β-glucosidase, and peak C contains total cellulase. Endo-β-glucanase, β-glucosidase, and total cellulase activities from the 3 different fractions are shown in Figures 3, 4, and 5, respectively. Total cellulase activity represents a combination of endoglucanase, exuglucanase, and β-glucosidase activities. Double purification on a Sephadex G-200 results in 3 fractions with different purification folds. Total cellulase results in a 55.49-fold purification with a 2.70% activity yield, endoglucanase results in a 60.52-fold purification with an activity yield of 1.76%, and β-glucosidase fraction results in a 100.71-fold purification with an activity yield of 2.22% (Table).

3.2. Effect of pH

The pH optima for total cellulase activity, endoglucanase activity, and β-glucosidase activity are 5.5, 7.5, and 4.5 (Figures 6, 7, and 8, respectively). This result is in agreement with the work of Linton and Greenaway (16), which showed a similar pH optimum of 5.5 for total cellulase from the gecarcinid land crabs *Gecarcoidea natalis* and *Discoplax hirtipes*. Sørensen et al. (21) reported a pH optimum of 4.5 for β-glucosidase from *Aspergillus niger*. Kaur et al. (22) reported a pH of 6.0 from *Thermomyces lanuginosus*, and Gueguen et al. (23) a pH of 5.5 from *Leuconostoc mesenteroides* for β-glucosidases. Umezurike (11) has observed that β-glucosidases exhibit maximum activity at an acidic pH range of 4.0–6.0 from most of the organisms studied. An alkaline pH optimum of 7.5 was obtained in this study for endoglucanase, but optimum pH values in the acidic region have been observed from *Aspergillus niger* (24) and *Streptomyces omiyaensis* (25). However, Aygan et al. (26) reported an optimum activity

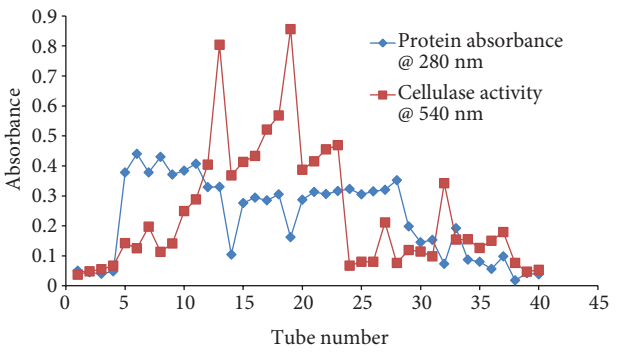

Figure 1. Elution profile of the first gel filtration fractions.

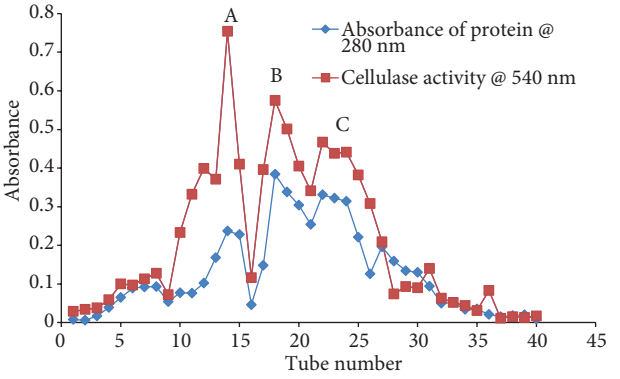

Figure 2. Elution profile of the second (rechromatographed) gel filtration fractions.

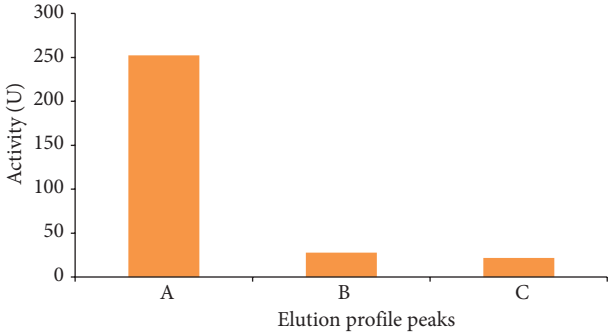

Figure 3. Endo-1,4-β-glucanase activity on different elution profile peaks.

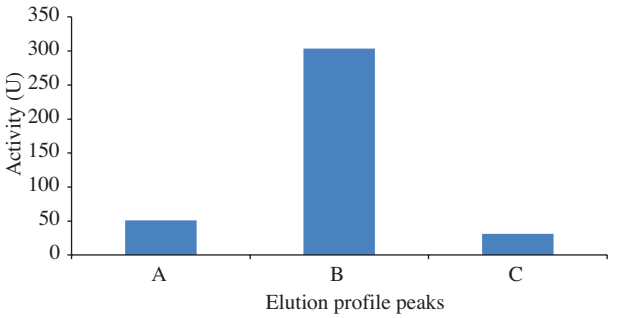

Figure 4. β-Glucosidase activity on different elution profile peaks.

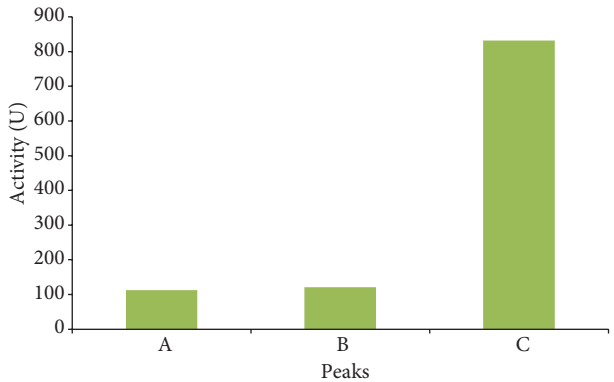

Figure 5. Total cellulase activity on different elution profile peaks.

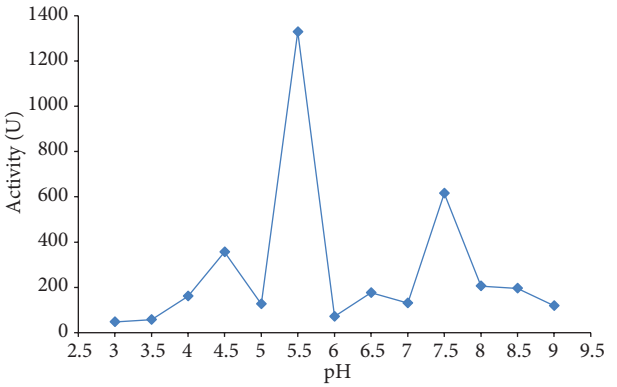

Figure 6. pH profile for total cellulase activity.

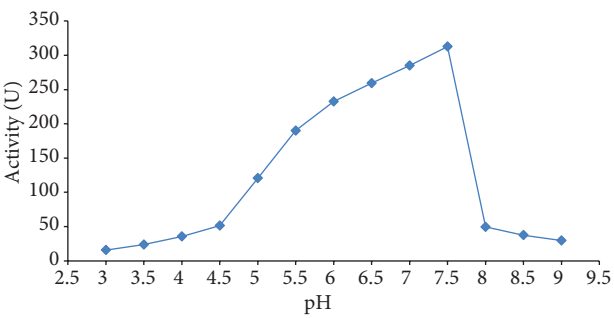

Figure 7. pH profile for endo-1,4-β-glucanase activity.

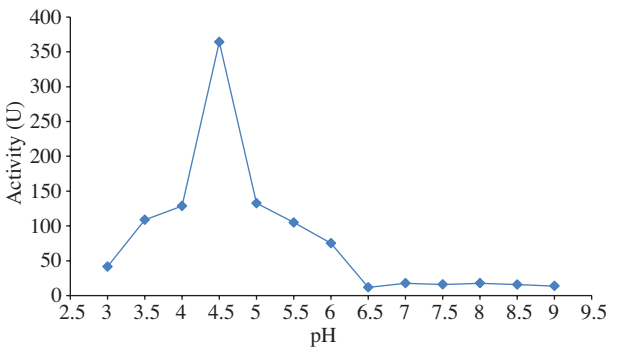

Figure 8. pH profile for β-glucosidase activity.

at an alkaline pH of 10.0 from *Bacillus licheniformis* C108. Optimum pH within the alkaline region suggests that endoglucanases from *A. achatina* could be applied in the paper and pulp, detergent, textile, and laundry industries (27,28), as well as in bio-ethanol production.

3.3. Effect of temperature

The temperature optima for total cellulase, endoglucanase, and β-glucosidase activity are 50 °C (Figure 9), 45 °C (Figure 10), and 50 °C (Figure 11), respectively. Above these temperatures there were sharp

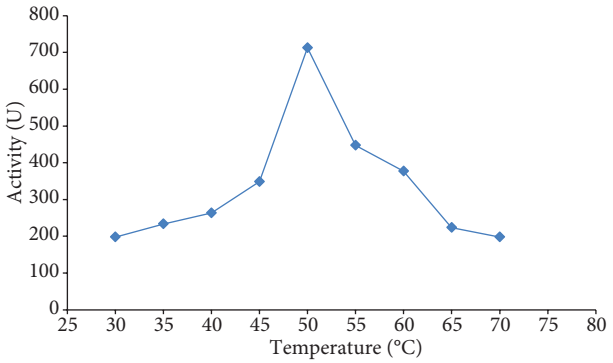

Figure 9. Temperature profile for total cellulase activity.

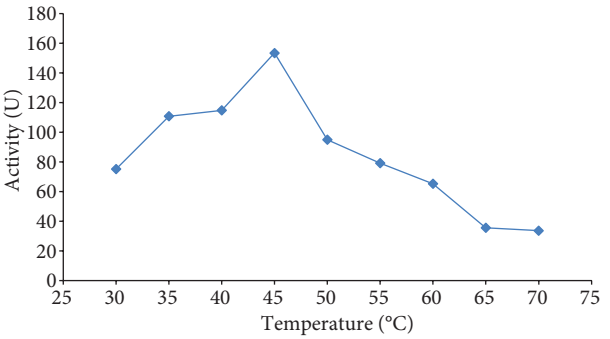

Figure 10. Temperature profile for endo-1,4-β-glucanase activity.

decreases in cellulase activity. Optimum temperatures within the range of 45–60 °C for both endoglucanases and β-glucosidases have been recorded by many researchers for these 2 enzymes from different sources (22–24,28–31). However, Howard et al. (25) and Aygan et al. (26) reported a temperature optimum of 70 °C and 30 °C, respectively, for endoglucanases. In the production of bio-ethanol from *Washingtonia robusta* fruits, Mazmanci (32) reported pH and temperature optima of 6.8–7.2 and 50 °C, respectively, using yeast. Similarly, both Mohanty et al. (33) and Ward and Ray (34) reported bio-ethanol production at pH and temperature optima of 6.0 and 30 °C from the mahula (*Madhuca latifolia* L.) flower and from cassava chips/flour using yeasts. These pH and temperature optima are close to the optimum conditions for cellulases activity in the present work. Thus, in a consolidated bioprocessing (CBP) system, cellulosic biomass can be utilized for bio-ethanol production since both β-glucosidase and yeast have a close range of operational conditions.

3.4. Kinetic studies

The total cellulase showed a maximum velocity, V_{max}, of 2427.18 μmol/min and a Michaelis-Menten constant, K_m, of 15.12 mg cellulose (Figure 12). Endoglucanase, on the other hand, showed a V_{max} of 955.11 μmol/min and a K_m of 2.39 mg Na-CMC (Figure 13), while β-glucosidase showed a V_{max} of 946.97 μmol/min and a K_m of 4.3 mM cellobiose (Figure 14). These results obviously indicate that the V_{max} for total cellulase activity is higher than the sum of the individual activity of the cellulases, suggesting a synergistic effect of cellulases. This could also be attributed to effective conversion of the cellobiose product of endoglucanase to glucose by the β-glucosidase activity of cellulose, hence reducing the inhibitory effect of cellobiose on endoglucanase (35,36). Linton and Greenaway (16) reported a lower V_{max} of 0.01 μmol/min and 0.03 μmol/min for total cellulase activity from the foregut of *Gecarcoidea natalis* and *Discoplax hirtipes*, respectively. Onyike et al. (24) and Wittman et al. (37) reported a K_m value of 14 mg and 13 mg Na-CMC for endoglucanases from microorganisms, respectively. These show low substrate affinity in comparison to the result of 2.39 mg Na-CMC obtained in this work. Watanabe et al. (38) and Tokuda et al. (39) also reported that endoglucanases from different species of termites had a K_m of 1.83 mg and 8.7 mg Na-CMC, respectively.

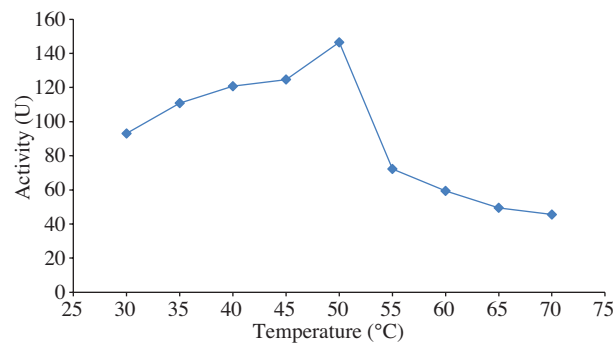

Figure 11. Temperature profile for β-glucosidase activity.

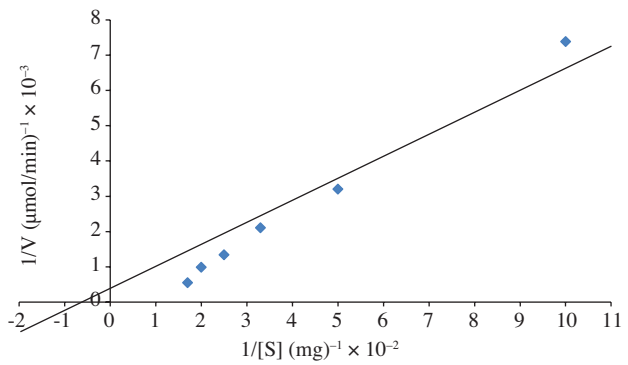

Figure 12. Lineweaver–Burk plot of total cellulase.

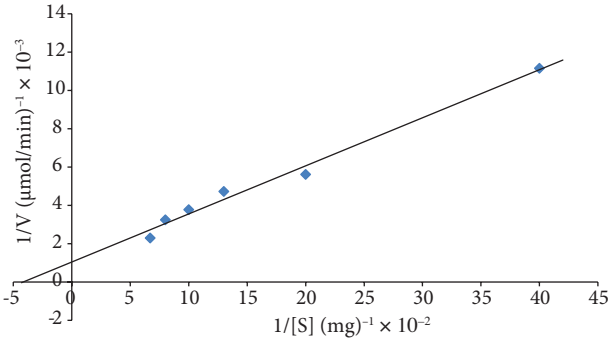

Figure 13. Lineweaver–Burk plot of endo-1,4-β-glucanase.

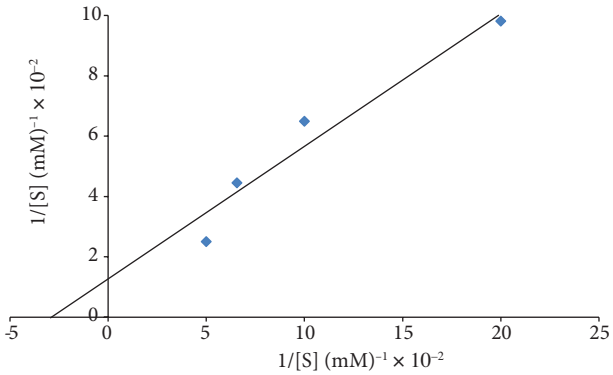

Figure 14. Lineweaver–Burk plot of β-glucosidase.

Although using p-nitrophenyl-β-glucoside, pNPG, as substrate instead of cellobiose for β-glucosidases, Kaur et al. (22) and Sørensen et al. (21) reported a K_m of 3.3 mM and 1.09 mM, respectively, for β-glucosidase from microorganisms. It could be that β-glucosidase has a higher affinity for pNPG than cellobiose. However, Linton and Greenaway (16) reported a K_m of 5.84 mM and 4.23 mM cellobiose for β-glucosidase from *Gecarcoidea natalis* and *Discoplax hirtipes*, respectively, when cellobiose was used as substrate. This is in agreement with our results.

In conclusion, as most of the studies on cellulases have been from microbial sources, especially fungi, this work contributes to new and alternative sources of cellulases. Cellulases from the digestive tracts of *A. achatina*, if purified to homogeneity and harnessed properly, could be a better and cheaper source of cellulase enzymes for the hydrolysis of cellulosic biomass to utilizable products (especially glucose) for the production of bio-products (such as bio-ethanol) and for other industrial purposes such as in the pulp and paper industries, textile and laundry industries, and detergent industries.

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