Nigerian Journal of Biochemistry and Molecular Biology
Volume 26, Number 2, 2011, pp. 108-115 (Printed in Nigeria)
0189-4757/96 $3.0 + 0.00

NJBMB-020-11

Purification and Some Kinetic Parameters of Endo-1,4-β-glucanase From Digestive Tracts of Giant African Land Snail (*Achatina achatina)*

Ozioko, P. C., *Eze, S. O. O. and Chilaka, F. C.
Department of Biochemistry, University of Nigeria, Nsukka. Enugu State, Nigeria

ABSTRACT
Crude endo-1,4-β-glucanase was extracted from the digestive tracts of matured *A. achatina* and subjected to a 3-step purification process of 40% ammonium sulphate saturation, dialysis and gel filtration. This yielded 60.52 purification fold with three prominent peaks in the chromatogram, designated A, B, and C with Peak A having relatively high endoglucanase activity and optimum pH and temperature of 7.5 and 45^{o}C respectively. The kinetic studies showed a V_{max} and Km of 955.11 µmole/min and 2.39 mg of Sodium Carboxymethyl Cellulose. This study showed that endoglucanase component of cellulase from digestive tracts of *A. achatina* could be used to degrade cellulose-containing materials and may find applications in laundry, detergent, pulp and paper industries.

Keywords: *Achatina achatina*, Endoglucanase, Purification, Kinetic parmaeters, Sodium Carboxymethyl Cellulose

INTRODUCTION

The cellulase enzyme complex consists of enzymes (endoglucanase, exoglucanase and β-glucosidase) acting synergistically on cellulose for complete degradation (Bhat and Bhat, 1997). Endoglucanases (1,4-D-glucan-4-glucanohydrolase; EC 3.2.1.4) attack cellulose chains in a random manner and liberate cellodextrins in the process. The potential applications of cellulases are in food, textile, detergent and the pulp and paper industry (Cavaco-Paulo, 1998; Ikeda *et al*., 2006). Another exceptional use area of cellulases are in the bioconversion of cellulosic material for bio-ethanol production while enormous amount of agricultural and industrial cellulosic wastes have been produced annually (Cherry and Fidantsef, 2003; Kim *et al*., 2003). Thus, this enzyme plays a key role for achieving huge benefits for effective resource utilization.

Cellulases are produced chiefly by fungi, bacteria, protozoans, and termites (Lee *et al*., 2000; Watanabe *et al*., 1998). However, there are also cellulases produced by other types of organisms such as plants, molluscs, and animals (Watanabe and Tokuda, 2001). In animals, cellulase is produced mainly by symbiotic bacteria in the ruminating chambers of herbivores. The abundance of snails in tropical rain forest, which have been shown to contain a large amount of cellulolytic material, makes snail a good choice in the estimation of endoglucanase. Thus, this work focuses on purification and characterization of endoglucanase from molluscs (*Achatina achatina*).

***Corresponding Author**
Tel.: +2347066090552;
E-mail: sabinus.eze@unn.edu.ng; ezeaug22@yahoo.com

MATERIALS AND METHODS

Materials

A number of matured Giant African Land Snails, (*Achatina achatina)*, were bought from Ogige Main-Market, at 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 time-intervals.

Reagents and Chemicals

3, 5-dinitrosalicyclic acid (DNS) ($C_7H_4N_2O_7$) was a product of Lab. Tech. Chemicals, Avighkar, India, while Sodium Carboxymethyl Cellulose (Na-CMC) and Sephadex G-200 were products of BDH Chemicals Ltd. Poole, England) and Superfine, India, respectively. Other chemicals and reagents used were of analytical grade and were freshly prepared unless otherwise stated.

Methods

Isolation of cellulase from the digestive tracts of Achatina achatina

Dissection of the snails was carried out as described by Umezurike (1976). The snails were deshelled and their digestive glands (digestive tracts) removed. This was frozen in 50 mM of sodium acetate buffer, pH 5.5, at about -15^{o}C. When needed, the digestive tracts were homogenized using pestle-and-mortar. The homogenate was centrifuged at 1790 x *g* for 15 minutes at 4^{o}C. The supernatant was collected as crude enzyme.

Purification of enzyme

The crude enzyme was brought to 40% ammonium sulphate saturation. This was then centrifuged at 1790 x *g* for 15 minutes after which the precipitate was dissolved in 50 mM of Na-acetate buffer, pH 5.5. This was then dialyzed overnight in the same buffer as previously mentioned with change of buffer at every 6 hours. The dialyzed enzyme was introduced onto Sephadex G-200 packed column (2.6 x 61.50cm) pre-equilibrated with 50 mM of Na-acetate buffer, pH 5.5. The protein was eluted with 50 mM of Na-acetate buffer, pH 5.5 at a flow rate of 1.8 ml/hour. The fractions with high cellulase activity were pooled together and rechromatographed on Sephadex G-200 at elution flow rate of 1.5ml/hr. Protein content and cellulase activity from each of the eluted fraction were monitored by absorbance at 280nm and 540nm respectively.

Assay of endo-β-1, 4-glucanase activity

The endoglucanase activity assay was carried out according to the method of Mandels *et al* (1976) with slight modification. A known volume (0.1 ml) of 2% Sodium Carboxymethyl Cellulose (Na-CMC) (high viscosity) in 50 mM of Na-acetate buffer, pH 5.5 was pipetted into a test tube, followed by addition of 0.1ml of the enzyme. This was incubated in a temperature controlled water bath at 50^{o}C for 30 minutes. The reaction mixture was then made up to 2.5 ml with 50 mM of Na-acetate buffer, pH 5.5. 0.5 ml of DNS reagent was then added and the reaction mixture was boiled in a temperature controlled water bath for 10 minutes. This was cooled on ice and the absorbance was read at 540 nm.

Determination of protein and glucose concentration

The protein and glucose concentrations were determined according to the procedures described by Lowry *et al* (1951) and Miller (1959) respectively.

RESULTS AND DISCUSSION

Gel filtration on sephadex G-200 column chromatography resulted in elution profile of multiple peaks whose protein bands did not coincide with cellulase activity bands (Figure 1). Similarly, the three prominent cellulase activity peaks which coincided with protein peaks are shown in Figure 2. Assay of the different fractions revealed that peak A had relatively high endoglucanase activity. Figure 3 showed the activity of endo-1, 4-β-glucanase on the three peaks (A, B and C). The endoglucanase (peak A) gave respectively a purification fold and activity yield of 60.52 and 1.76% (Table 1). This result suggests that the enzyme solution is composed of three isozymes of which one is endoglucanase. This agrees with previously published works of Henrissat (1994), Boisset *et al* (2000) and Zverlov *et al* (2002) who reported independently that cellulase is composed of three components as evidenced by the three prominent peaks (A, B, C) of the elution profile in the present study (Figure 2).

Assay of enzyme activity at different pH and temperature revealed that the optimum pH and temperature were 7.5 and 45^{o}C respectively (Figures 4 and 5). The optimum pH of 7.5 obtained in the present study contrast those of previously published works of Howard *et al* (2003) and Onyike *et al* (2008) that reported acidic pH values. However, Aygan *et al* (2011)

reported a highest activity at an alkaline pH of 10.0 from *Bacillus licheniformis* C108. Optimum pH within the alkaline region suggests that endoglucanases from *A. achatina* in the present study could be explored in the detergent, textile, laundry, paper and pulp industries, as well as in the production of ethanol. Furthermore, Ito (1997) and Bajpai (1999) reported that neutral cellulases acting at pH 6-8 can be used for abrasion of denim in textile and laundry as well as pulp and paper industries.

Temperature is also an important factor in enzymology. An increase in temperature above this optimu temperature (45^{o}C) showed a sharp decrease in activity. This could be as a result of denaturation of the secondary and tertiary structures of endoglucanase which might result from dissociation of catalytic domain and cellulose binding domain (CBD) of this enzyme. Cellulase binding domain has been reported to bind cellulose, thus bringing the later in close proximity to the catalytic domain (Bhikhabhai *et al*., 1984; Ong *et al*., 1989). Most of the reports by previous researchers indicated an optimum temperature range of 45-60^{o}C from different sources (Li *et al*., 1998; Onyike *et al*., 2008; Bajaj *et al*. 2009; Quay *et al*., 2011). However, Howard *et al* (2003); Karnchanatat *et al* (2008) and Aygan *et al* (2011) reported temperatures of 70^{o}C, 70^{o}C and 30^{o}C respectively.

The highest endoglucanase activity was at a substrate (Sodium Carboxymethyl Cellulose) concentration of 1.5% (Figure 6). Studies on the substrate effect on the enzyme activity revealed a decline in activity at high substrate concentrations without assuming the hyperbolic shape of Michaelis-Menten plot. The non-Michaelis-Menten behaviour of this enzyme could be attributed to product inhibition by cellobiose, glucose, and cello-oligosaccharides (cellodextrins). Both glucose and cellobiose have been reported to reduce the solubilization of cellulose substrates and thus inhibit the activity of the enzyme. The inhibitory effect caused by the reaction products could be due to the binding of sugars to endoglucanases. However, cellobiose shows a stronger inhibitory effect on endoglucanase activity than glucose (Xiao *et al*. 2004; Karnchanatat *et al*., 2008).

The endoglucanase showed a maximum velocity, Vmax, of 955.11μmole/min and Michaelis-Menten constant, Km, of 2.39 mg/ml Na-CMC (Figure 7). Onyike *et al* (2008) reported a Km value of 14 mg/ml from micro-organisms. This indicates a significantly low substrate affinity in comparison to the one in the present study. Watanabe *et al* (1997) and Tokuda *et al* (1997) reported in separate studies that endoglucanases from different species of termites had Km values of 1.83 mg/ml and 8.7 mg/ml Na-CMC.

In conclusion, as most work on endoglucanases has been from microbial sources especially fungi, this work contributes to new and alternative sources of endoglucanases. This study has also shown that purified enzyme solution from *A. achatina* is a complex enzyme which can find applications in laundry, detergent, pulp and paper industries Therefore, endoglucanase from the digestive tracts of *A. achatina*, could be a better and cheaper source of cellulase for hydrolyzing the lignocellulosic biomass to utilizable products (especially glucose) for production of ethanol.

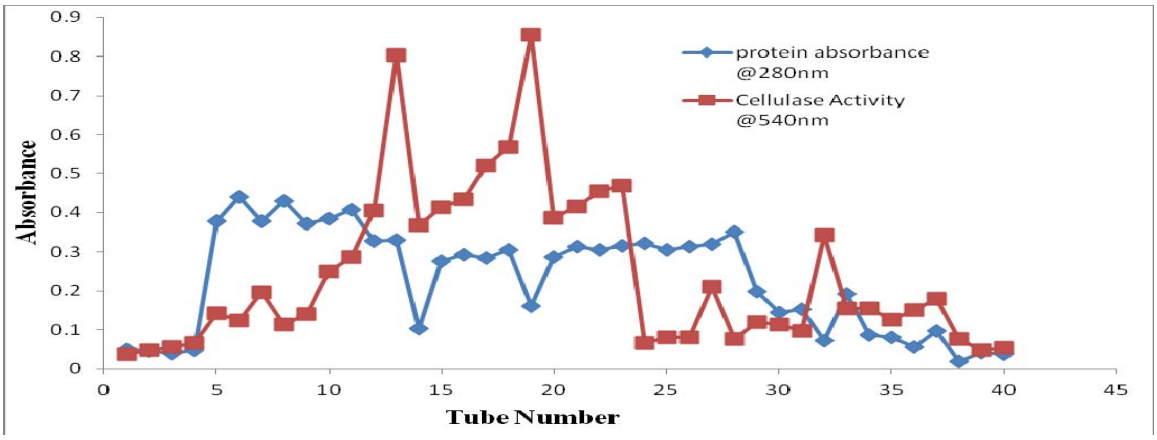

Fig. 1: Elution profile of the first gel filtration

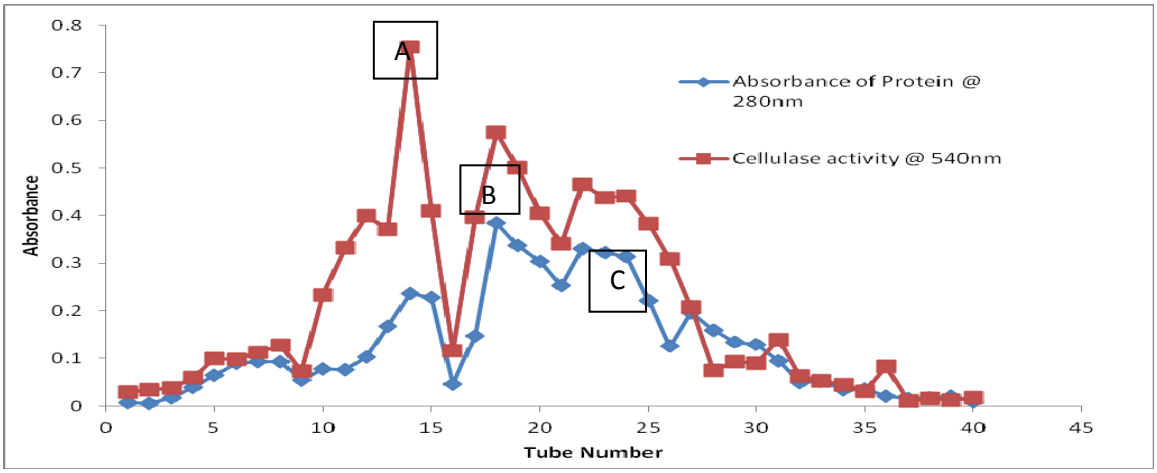

Fig. 2: Elution profile of second (rechromatographed) gel filtration

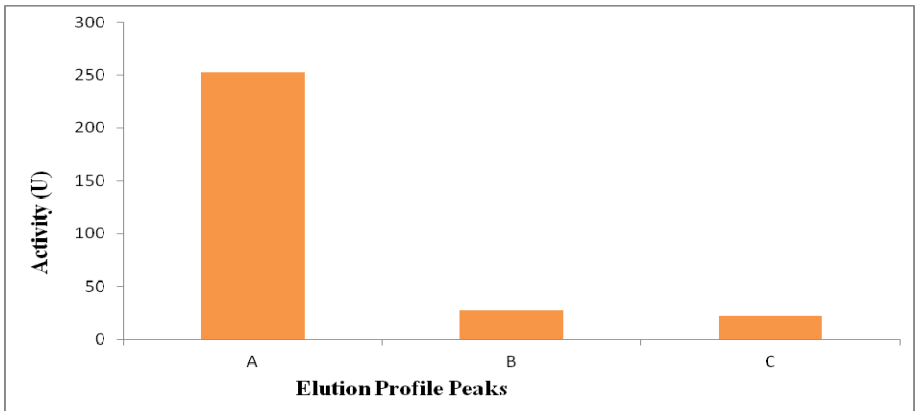

Fig. 3: Endo-1,4-β-glucanase activity on different elution peaks

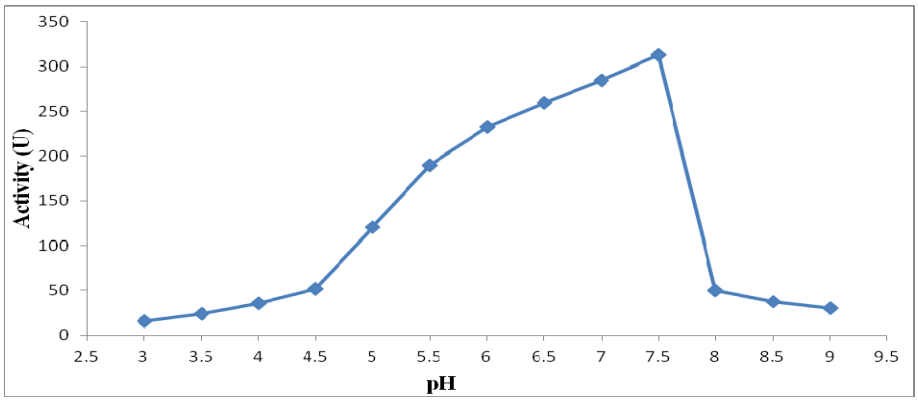

Fig. 4: pH profile of Endo-1,4-β-glucanase

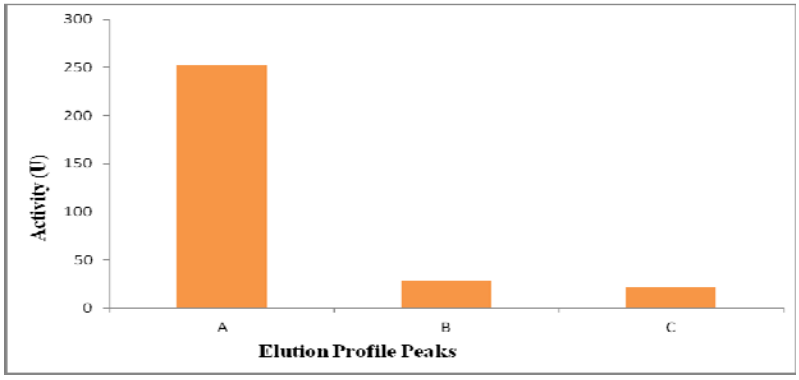

Fig. 5: Temperature profile of Endo-1,4-β-glucanase

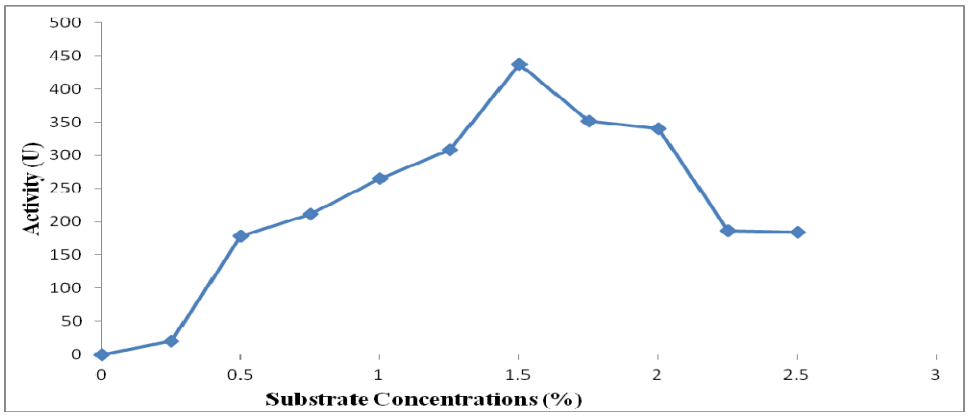

Fig. 6: Effect of substrate concentrations on Endo-1,4-β-glucanase activity

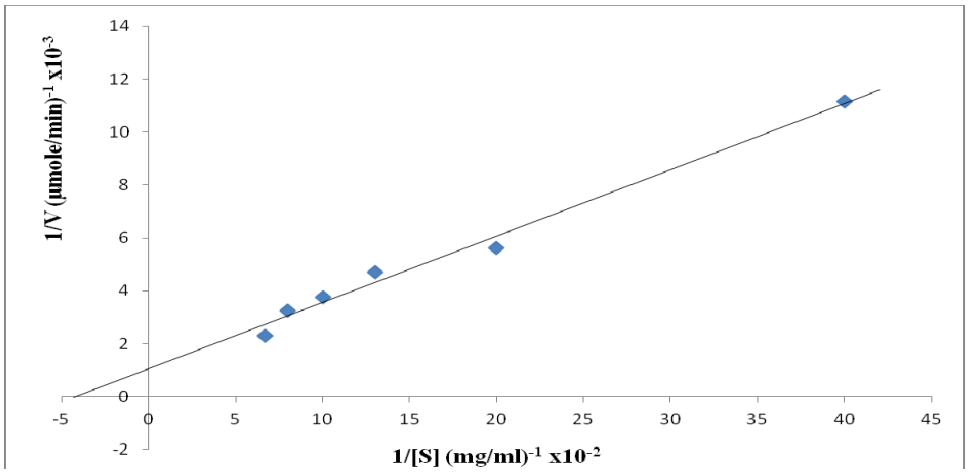

Fig. 7: Lineweaver-Burk plot of Endo-1,4-β-glucanase

Table 1: Purification profile of Endo-1,4-β-Glucanase

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	51836.00	5.32	100.00	1.00
40% $(NH_4)_2SO_4$ Saturation	20.00	1522.72	26096.00	17.14	50.34	3.22
Dialysis	22.00	1420.32	24750.46	17.43	47.75	3.28
Gel Filtration Chromatography	10.00	2.84	914.41	321.98	1.76	60.52

A unit activity (U) is defined as the amount of enzyme required to produce 1 μmole of reducing sugars per minute under the experimental condition.

REFERENCES

Aygan, A., Karcioglu, L. and Arikan, B. (2011). Alkaline thermostable and halophilic endoglucanase from *Bacillus licheniformis* C108. African Journal of Biotechnology 10(5): 789-796.

Bajaj, B. K., Pangotra, H., Wani, M. A., Sharma, P. and Sharma, A. (2009). Partial purification and characterization of a highly thermostable and pH stable endoglucanase from a newly isolated *Bacillus* strain M-9. Indian Journal of Chemical Technology 16: 382-387.

Bajpai, P. (1999). Application of enzymes in the pulp and paper industry. Biotechnological Progress 15(2): 147-157.

Bhat, M. and Bhat, S. (1997). Cellulose degrading enzymes and their potential industrial applications. Biotechnology Advance 15: 583-620.

Bhikhabhai, R., Johasson, G. and Pettersson, G. (1984). Cellobiohydrolase from *Trichoderma reesei*: internal homology and prediction of secondary structure. International Journal of Pepetide Protein Research 25: 368-374.

Boisset, C., Fraschini, C., Schulein, M., Henrissat, B. and Chanzy, H. (2000). Imaging the enzymatic digestion of bacterial cellulose ribbons reveals the endo character of the cellobohydrolase *Cel*6A from *Humicola insolens* and its mode of synergy with cellobiohydrolase *Cel*7A. Applied Environmental Microbiology 66, 1444-1452.

Cavaco-Paulo, A. (1998). Mechanism of cellulase action in textile processes. Carbohydrate Polymerization 37: 273-277.

Cherry, J. R. and Fidantsef, A. I. (2003). Directed evolution of industrial enzymes: An update. Current Opinion in Biotechnology 14: 438-443.

Henrissat, B. (1994). Cellulases and their interaction with cellulose. Cellulose 1: 169-196.

Howard, R.L., Abotsi, E., Jansen VanRensburg, E.L. and Howard, S. (2003). Lignocellulose biotechnology: issues of bioconversion and enzyme production. African Journal of Biotechnology 2: 602-619.

Ikeda, Y., Park, E.Y. and Okida, N. (2006). Bioconversion of waste office paper to gluconic acid in a turbine blade reactor by the filamentous fungus, *Aspergillus niger*. Bioresource Technology 17: 1030-1035.

Ito, S. (1997) Alkaline cellulases from alkaliphilic Bacillus: enzymatic properties, genetics and application to detergents. Exremophiles 1(2): 61-66.

Karnchanatat, A., Petsom, A., Sangvanich, P., Piapukiew, J., Whalley, A.J.S., Reynolds, C. D., Gadd, G. M. and Sihanonth, P. (2008). A novel theermostable endoglucanase from the wood-

decaying fungus, *Daldinia eschscholzii*. Enzyme and Microbial Technology 42: 404-413.

Kim, K. C., Yoo, S. S., Oh Young, A. and Seong-Jun, K. (2003). Isolation and characterization of Trichodeema harzianum FJ1 producing cellulase axylanase. Journal of Microbiology and Biotechnology 13: 1-8.

Lee, I., Evans, B. R. and Woodward, J. (2000). The mechanism of cellulase action on cotton fibers: evidence from atomic force microscopy. Ultramicroscopy 82: 213-221.

Li, X., Lin, W., Gao, P. and Chen, E. (1998) Endoglucanase S, a novel endoglucanase exhibiting exoglucanase activity from a newly isolated *Streptomyces* Sp. LX. Journal of Applied Microbiology 85: 347-356.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with Folin-Phenol reagent. Journal of Biological Chemistry 193: 265-275.

Mandels, M., Andreotti, R. and Roche, C. (1976). Measurement of saccharfying cellulase. Biotechnology and Bioengineering Symposium 6, 21-34.

Miller, G.L. (1959). Use of dinitrosalicyclic reagent in determination of reducing sugars. Analytical Chemistry 31: 426-442.

Ong, E., Greenwood, J., Gilkes, N., Kilburn, D. G., Miller, R. C. and Warren, R. A. J. (1989) The cellulose-binding domains of cellulases: tools for biotechnology. Trends of Biotechnololgy 7: 239-243.

Onyike, E., Auta, R. and Nok, A. J. (2008). Isolation, partial purification, and characterization of endoglucanase from *Aspargillus niger* SLI using corn cobs as carbon source. Nigerian Journal of Biochemistry and Molecular Biology 23(2): 1-11.

Quay, D. H. X., Bakar, F. D. A., Rabu, A., Said, M., Illias, R. M., Mahadi, N. M., Hassan, O. and Murad, A. M. A. (2011). Over-expression, purification, and characterization of the *Aspergillus niger* endoglucanase, EglA, in *Pichia pastoris*. African Journal of Biotechnology 10(11): 2101-2111.

Tokuda, G., Watanabe, H., Matsumoto, T. and Noda, H. (1997). Cellulose digestion in the wood-eating higher termite, *Nasutitermes takasagiensis*. Zoological Science 14: 83-93.

Umezurike, G. M. (1976). The β-glucosidase in the gut contents of snail, *Achatina achatina*: energy-dependent modification of structure and activity. Biochemistry Journal 157: 381-387.

Watanabe, H. and Tokuda, G. (2001). Animal cellulases. Cellular and Molecular Life Science 58:1167-1178.

Watanabe, H., Nakamura, M., Tokuda, G., Yamaoka, I., Scrivener, A. M. and Noda, H. (1997). Site of secretion and properties of endogenous endo-β-1,4-glucanase components from *Reticulitermes speratus* (Kolbe), a Japanese subterranean termite. Insect Biochemistry and Molecular Biology 27: 305-313.

Watanabe, H., Noda, H., Toguda, G. and Lo, N. (1998) A cellulase gene of termite origin. Nature 394: 330-331.

Xiao, Z. Z., Zhang, X., Gregg, D. J. and Saddler, J. N. (2004). Effects of sugar inhibition on cellulases and β-glucosidase during enzymatic hydrolysis of softwood substrates. Applied Biochemistry and Biotechnology 133: 1115-1126.

Zverlov, V. V., Velikodvorskaya, G. A. and Schwarz, W. H. (2002). A newly described cellulosomal cellobiohydrolase, CelO, from *Clostridium thermocellum*: Investigation of the exo-mode of hydrolysis, and binding capacity of crystalline cellulose. Microbiology 148: 247-255.