ansinet
Asian Network for Scientific Information

RESEARCH ARTICLE

OPEN ACCESS

Partial Purification and Characterization of Cellulase from the Haemolymph of the African Giant Land Snail (*Archachatina marginata*)

[1]Omolola E. Omotosho, [1]A.C. Omonhinmin, [1]S.O. Rotimi, [1]E.E.J. Iweala, [1]O.A. Rotimi and [2]Ayoade Femi
[1]Department of Biological Sciences, College of Science and Technology, Covenant University, PMB 1023, Ota, Ogun State, Nigeria
[2]Department of Biological Sciences, Redeemer's University of Nigeria

ABSTRACT

The aim of this study is to partially purify and characterize the cellulase extracted from the snail haemolymph of the African giant land snail of the species, *Archachatina marginata*. The protein concentration of the haemolymph was found to be 168 mg mL^{-1} and the specific activity of the crude cellulase was found to be 0.007 U mg^{-1} protein using Carboxymethyl-cellulose (CMC) as the substrate. The partial purification on Sephadex G-200 produced two activity peaks. The molecular weights of the two cellulase fractions were estimated to be 112,202 and 67,000. The Michealis Menten constant (km) for cellulase was 4.3 and 2.8 mg mL^{-1} for the higher and lower molecular weight fractions, respectively. The multiplicity of cellulase complexes indicated that the haemolymph of *A. marginata* may convert derived cellulose in foodstuffs such as garlic, mushrooms and domestic wastes into sources of fuel. It is suggested that the enzyme can be used to produce value-added products, such as ethanol, citric acid, amino acids and vitamins including primary metabolites from cellulosic wastes.

Key words: Hydrolyzing enzyme, cellulosic wastes, haemolymph, gel electrophoresis, cellulase

INTRODUCTION

The plant cell wall represents the most abundant reservoir of organic carbon in the biosphere and also represents an important industrial substrate (Topakas and Christakopoulos, 2007). As a major component of the plant cell wall, cellulose is the most abundant renewable polysaccharide in nature. cellulases (1,4-β-D-glucanglucanohydrolase, EC 3.2.1.4) are multi enzyme complexes, comprising three main components; endo-β-glucanase (EC 3.2.1.4), exo-β-glucanase (EC 3.2.1.9.1) and β-glucosidase (EC 3.2.1.21), which have been shown to act synergistically in the hydrolysis of cellulose (Lambert *et al.*, 2013).

Cellulases are being studied increasingly due to their application in the hydrolysis of cellulose, the most abundant biopolymer and potential source of utilizable sugars, which serve as raw materials in the microbial production for a wide variety of chemicals, food and fuel. Cellulose is obtained either directly or indirectly as forest production or in wastes such as straw, paper waste, municipal solid waste and other industrial wastes (Ekperigin, 2007).

Recently, cellulases have been exploited on an increasing scale in food, pharmaceutical and chemical industries. This is due to the growing need for energy, food and chemicals and also for more acceptable means of municipal waste treatment (Thatheyus and Vidhya, 2013).

The biodegradation of cellulose is an essential step in the carbon cycle by which the carbon balance of the biosphere is maintained. The group of hydrolytic enzymes, which degrades cellulose to glucose by breaking both the hydrogen bonds and β-1, 4-glycosidic linkages are collectively known as cellulases. Their structural features determine the susceptibility of cellulose to such degradation. Native, crystalline cellulose is an insoluble β-glucose, which occurs in fibres of densely,

packed linear unbranched anhydro-glucose units in β-1, 4-glycosidic linkages. The molecular weight of cellulose varies from 100,000-2,000,000 (Almin and Eriksson, 1968). The most common sources are wood, pulp, cotton lint, agricultural waste material (stalks, stems and husks) and waste products from nature and man-made activities.

The shortage of oil and fossil material has resulted in the interest of using renewable resources; for example wood and woody plants (biomasses), either as such or from residues, for the production of energy and organic chemicals (Nevelle and Zeronian, 1985). Cellulase has been employed for its application in industries for the degradation of cellulose and celluloytic materials into glucose (Lambert *et al.*, 2013).

A lot of work had been done on the enzyme from different sources of microorganisms. However, limited work has been done on the enzyme from plant and other sources. Studies on cellulases from the snail species, *Helix pomatia* have been reported (Myers and Northcote, 1959).

The objective of the present study was to examine the properties of crude and partially purified cellulase from the common African giant land snail species *A. marginata* and to compare with the results obtained from other sources.

MATERIALS AND METHODS

Materials: The common African giant land snails, *A. marginata*, were bought from local markets in Akure, Ondo State, Nigeria. All chemicals used for this work are products of BDH Chemicals limited. Some of the chemicals used were: Sodium acetate trihydrate, carboxymethyl cellulose, sephadex G-200, sodium hydroxide pellets, sodium-potassium tartarate, dinitrosalycylic acid (DNS), d-glucose, 98% ethanol, blue dextran, bovine serum albumin (BSA), cytochrome c, sodium citrate, hydrated and anhydrous sodium carbonate, copper sulphate, pepsin and trypsin.

Methods
Collection of snail haemolymph: The shell of the snail (*A. marginata*) was cleaned with detergent, sponge and distilled water. It was then swabbed with cotton wool in 98% ethanol. The pointed tips of the shell of two snails were carefully broken, so as not to puncture the intestines. The haemolymph was drained into a sterilized beaker and was centrifuged at 5000 g for 30 min. The supernatant (about 40 mL) was transferred to a clean reagent bottle. This served as the crude extract of the cellulase enzyme and this was stored inside the refrigerator.

Quantitative assay: The 0.1 g of CM-Cellulose was weighed and dissolved in 10 mL sodium acetate buffer pH 5.0, containing 1 mM EDTA. The cellulase enzyme activity was analyzed by measuring the release of reducing sugar (glucose) from CM-cellulose (Perner *et al.*, 2012).

The CM-cellulose was properly stirred with the acetate buffer to obtain a homogeneous substrate solution. 0.5 mL of the substrate was measured into a clean test tube and 0.5 mL

of crude haemolymph or purified fraction was added to make a total volume of 1 mL. This reaction mixture was incubated at 40°C for 15 min. The glucose formed in the reaction was determined using the dinitrosalicylic acid (DNS) method (Perner *et al.*, 2012). The amount of glucose liberated (μmol mL^{-1}) was determined by extrapolation on the glucose standard curve and the enzyme activity determined. The protein concentration was determined by Biuret method.

Gel filtration: The 6.6 g of Sephadex G 200 was weighed and dissolved in 200 mL of distilled water. This was allowed to swell. This swollen gel was gently let down into the cleaned column with a glass rod. The column was calibrated as (1.5×95) cm. The properly packed column was equilibrated with 0.05 m sodium acetate buffer pH 5.0 that contained 1mM EDTA. The crude cellulosic enzyme was applied to the top of the column and 2 mL fractions were collected in numbered tubes. The flow rate was 1 mL per 3.5 min and the absorbance of the fractions were read at a wavelength of 280 nm. A graph of absorbance versus tube number was plotted. The activity of each fraction under the two peaks from the graph was assayed for.

Molecular weight determination: The Void volume (V_0) of the column was first determined. The 2 mg mL^{-1} of blue dextran was loaded on the column. The column was set for the partial of the crude extract. The elutes of the loaded column were collected and the absorbance of the fractions (2 mL each) was read at a wavelength of 620 nm. The graph of absorbance against tube numbers was plotted and the void volume (V_0) was determined.

An equilibrated sephadex G-200 column (1.5×95) cm was loaded with four protein markers. The protein markers used were; Bovine serum albumin (Molecular weight-67,000), pepsin (Molecular weight-35,000), trypsin (Molecular weight-23,000) and papain (Molecular weight-20,800). For each of the standard protein marker, 5 mg mL^{-1} was prepared and the protein mixture applied to the equilibrated column. The flow rate was 1 mL per 3.5 min. The 2 mL fractions were collected in 82 numbered tubes and their absorbance was read at 280 nm.

The graph of absorbance against the tube number was plotted. This gave elution volume of the marker proteins. Subsequently, the plot of log of molecular weight of the marker proteins against dissociation constant (Kd) was plotted to give a molecular weight standard curve from which the molecular weight of the crude cellulose enzymes was extrapolated.

Michealis menten constant (Km) determination: This was determined using CM-cellulose as a substrate. The concentration of the CMC used ranged from 1-10 mg mL^{-1}. The pooled fractions of the two cellulose activity peaks were assayed at 8-10 different CMC concentrations using the DNS method (Miller, 1959). The amount of glucose liberated in μmol mL^{-1} was determined by extrapolation on the glucose

standard curve. A graph of V (μmol min^{-1}) against (S) (mM) and the double reciprocal plot (line weaver Burk) were plotted for each sample to determine Vmax and Km.

Purification of cellulase in snail haemolymph on sephadex G-200: The cellulase complex in the giant snail haemolymph was resolved into two molecular weight fractions, which were represented by two distinct peaks tagged fractions 1 and II.

RESULTS

Protein determination: The protein concentration was determined by Biuret method gave the value of 8.0 mg mL^{-1}. From the result obtained, the number of enzymes units per enzyme sample was calculated. For the second enzyme peak, fraction II, the fractions tubes that had activity were pooled, the protein concentration determined by Biuret method gave the value of 0.8 mg mL^{-1}.

Figure 1 represents the protein standard curve determined by the Biuret method. In addition, the total unit determined by DNS method was 0.256 U mL^{-1}. The specific activity gave the value of 0.002 U mg^{-1} protein. The protein concentration determined by Biuret method gave the value of 8.0 mg mL^{-1}. In addition, the total unit determined by DNS method was 0.256 U mL^{-1}.

Activity of cellulase in *Archachatina marginata* haemolymph: From the result obtained, the number of enzymes units per enzyme sample was calculated from the first enzyme peak, fraction I and second enzyme peak, fraction II, respectively. The fraction tubes that had activity were pooled, the protein concentration determined by Biuret method. Figure 2 and 3 represent the graph of V against [S] for the first enzyme fraction and the second enzyme fraction, respectively. It depicts the enzyme activity of cellulase. The total unit of the enzyme determined by DNS method was 1.13 U mL^{-1}. The specific activity had the value of 0.007-U mg^{-1} protein. The specific activity gave the value of 0.002 U mg^{-1} protein. The total unit determined by DNS method was 0.048 units. The specific activity was 0.01 U mg^{-1} protein.
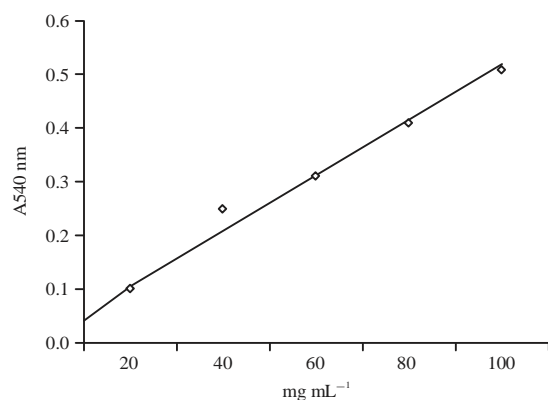
Determination of V$_{max}$ and K$_M$ by double-reciprocal plots: A plot of $1/V_0$ versus $1/[S]$, called a Lineweaver-Burk or double-reciprocal plot, yields a straight line with an intercept of $1/V_{max}$ and a slope of K_M/V_{max}. The intercept on the x-axis is $-1/K_M$. (Fig. 4) depicts the double reciprocal plot for the first enzyme fraction while, Fig. 5 represents the double reciprocal curve for the second enzyme fraction.
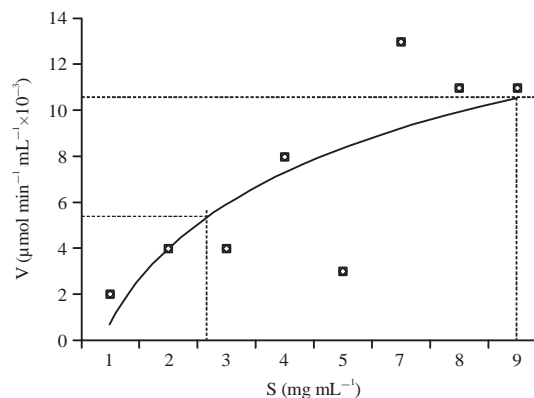

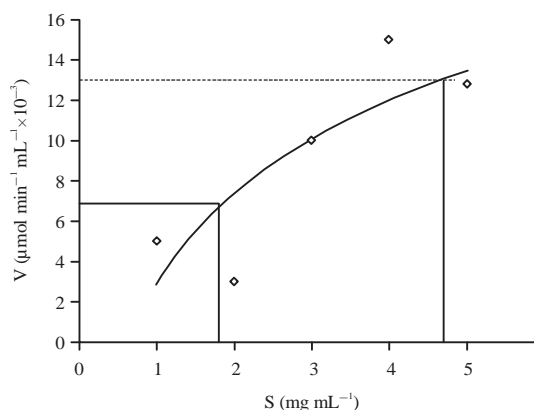Fig. 2: Plot V against (S) for the first enzyme fraction

Fig. 3: Plot of V against (S) for the second enzyme
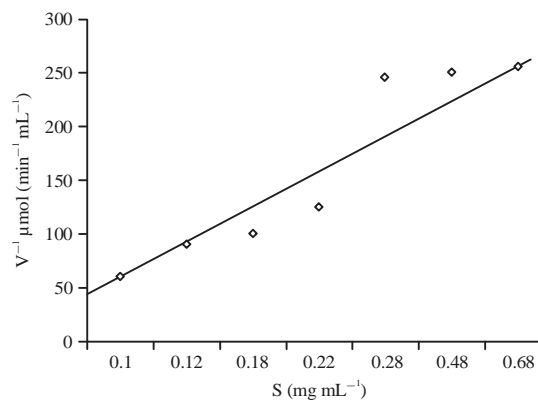

Fig. 1: Protein standard curve

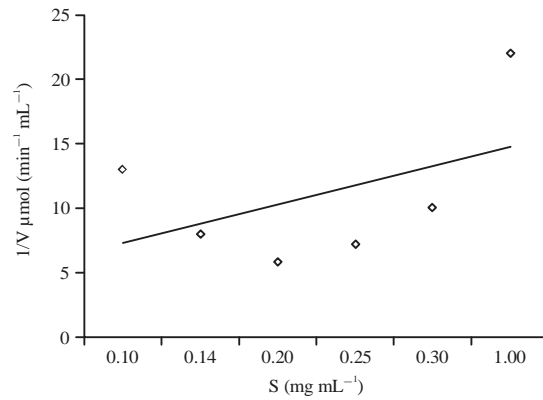Fig. 4: Double reciprocal plot for the first enzyme fraction

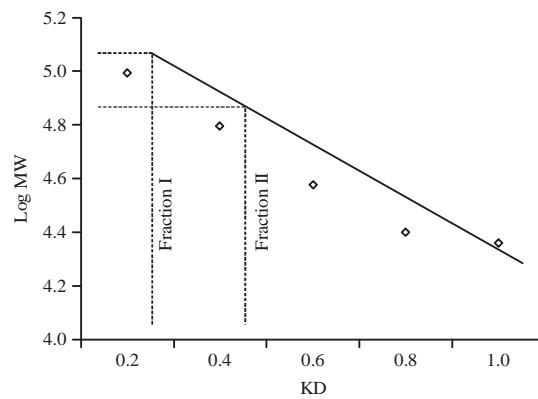Fig. 5: Double reciprocal curve for the second enzyme fraction

Fig. 6: Calibration curve on sephadex G-200 for molecular weight determination
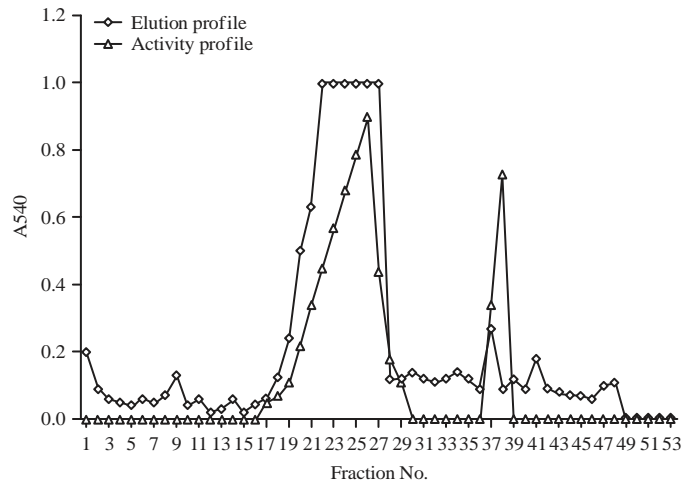

Fig. 7: Gel filtration of snail haemolymph on sephadex G-200

Molecular weight determination and partial purification of *Archachatina marginata* snail haemolymph: Figure 6 represents the calibration curve for the molecular weight determination. This is shown as the log of Molecular weights versus kd. The first fraction came out with the haemocyanin, while the second fraction weight represented by the second peak was colourless. Figure 7 represents gel filtration of snail haemolymph on sephadex G-200. The fraction I was pooled from tube numbers 17-29, while fraction II was pooled from tube numbers 36-38. From the line-weaver Burk plot (Fig. 6 and 7) the km for the higher molecular weight cellulose activity on CM-cellulose was found to be 4.3 mg mL^{-1}
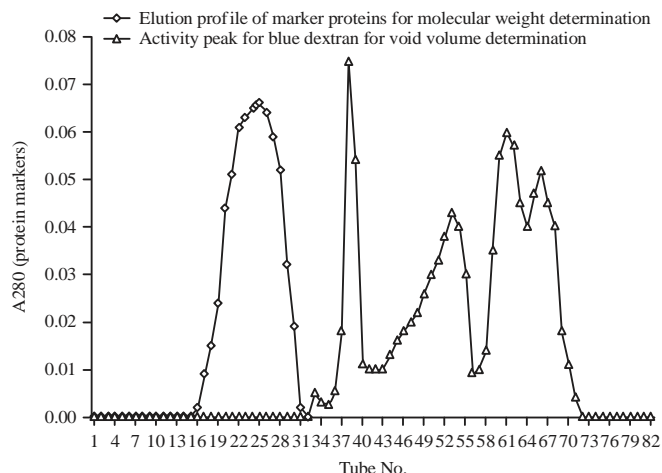
Fig. 8: Plot of the protein markers used

Table 1: Purification of snail haemolymph

Fractions	Volume (mL)	Total unit (U mL^{-1})	Total protein (mg mL^{-1})	Specific activity (U mg^{-1} protein)	Purification fold	Yield (%)
Crude extract	30.0	1.130	168.0	0.007	1.0	100.0
Gel-filtration on G-200						
Fraction 1	16	0.256	8.0	0.002	ND	45.6
Fraction II	6	0.048	0.8	0.010	1.40	8.8

ND: Not defined

(0.14 mM), while the km for the lower molecular weight enzyme activity was found to be 2.8 mg mL^{-1} (0.09 mM).

Fraction I gave a total volume of 16 mL, total protein of 8.0 mg mL^{-1}, while fraction II gave a total volume of 6 mL and total protein of 0.8 mg mL^{-1}. The percentage yield of the fraction I is 45.6 while that of fraction II is 8.8. Table1 shows the purification of snail haemolymph.

The graph in Fig. 8 depicts the plots of protein markers used {bovine serum albumin (Molecular weight-67,000), pepsin (Molecular weight-35,000), trypsin (Molecular weight-23,000) and papain (Molecular weight-20,800)} and the loading of the partially purified cellulase enzyme from *A. marginata* snail haemolymph. The molecular weights of the two cellulases in the snail haemolymph by gel filtration (Sephadex G-200) was found to be 112,202 and 67,000 for Fractions I and II, respectively.

DISCUSSION

The African giant land Snail, *A. marginata* produces substantial extracellular cellulases. It feeds mainly on cellulosic materials hence its choice as source of cellulose enzyme. The protein concentration in the crude haemolymph determined by Biuret method was 1.13 U mL^{-1}. The specific activity, using carboxymethyl cellulose (CMC) as substrate was determined to be 0.007-U mg^{-1} proteins. The specific activity value is comparable to the observed activity obtained in a similar study, Meyer *et al.* (1997) using the enzyme from haemolymph.

The purification of the crude enzyme on sephadex G-200 resulted in two activity peaks. One of specific activity of

0.002 U mg^{-1} protein was recorded for the first activity peak-(fraction 1), when compared to the specific activity of crude enzyme value implies that the enzyme was not purified. But the second activity peak (fraction II) was purified 1.40 fold. The application of the undiluted snail haemolymph on the sephadex G-200 gave no resolution (results not shown). About 100% recovery was observed during the purification process, when the crude enzyme was applied to the column indicated that there was no much loss of activity in the purification process.

From the specific activity results of fraction 1 and II, Sephadex G-200 does not serve as an effective purification step. The use of the gel has allowed the resolutions of two molecular forms of cellulose in the snail haemolymph. A gel filtration while the second activity peak has a molecular weight of 67,000. Gong and George (1979) reported, the molecular weight of cellulose of *T. reesii* to be 72,000 from the report of Afolayan *et al.* (1997).

Alan (1985) reported that β-glucosidase could exist as low molecular weight monomeric forms in *Aspergillus fumigates* with molecular weight of 40,805 and in *T. virdes*, as extracellular enzyme molecular weight of 47,805 was obtained. Ekperigin (2007) studied the cellulase production by *Acinetobacter anitratus* and *Branhamella* sp. from snail. Similarly, the purification of the β-glucosidase from *Selerotium rolfsii* in different molecular range of 90,000-107,000. The molecular weights are catalytically active monomers. By Afolayan *et al.* (1997) the various molecular range of 90,000-107,000 the various molecular forms of β-glucosidase were detected either in the haemolymph or in the partially purified enzyme. It was reported that three

molecular forms of β-glucosidase of snail haemolymph as monomers (98,000-115,000), the dimmer (190,000) and high molecular weight specie (>400,000). The physiological significance of the ability of the haemolymph β-glucosidase to exist in multiple forms, was known by the fact that haemolymph β-glucosidase may be part of multiple complex. Therefore, this work suggests that the higher molecular weight species (67,000) is a monomer.

The Km values for the faction I and II were 4.3 mg mL^{-1} (0.14 mM) and 2.8 mg mL^{-1} (0.09 mM), respectively. The affinity of the higher molecular weight species for CM- cellulose is lower compared to lower molecular species on a distinctively different enzyme.

Umezurike (1976) reported compable km values (0.09-0.16 nM) for the unpurified molecular forms of snail gut β-glucosidase, while Afolayan *et al.* (1997) reported the Km of the pure β-glucosidase for nitrophenyl β-D-glucopyranoside to be 0.05 mM and the partially purified β-glucosidase had a km of 0.11 mM. Components of the cellulase complex in the gel filtration peaks could not be identified due to lack of reagents and equipment.

CONCLUSION

Cellulose is a very important industrial raw material and a source of renewable energy. It is used as a source of fuel e.g., ethanol and a source of food e.g., glucose and single cell protein. The present commercial applications of cellulase include its use, as digestive aids to degrade cellulose in foodstuffs for instance garlic, mushrooms and domestic wastes.

The cellulase of the giant African *A. marginata* might be a cellulase that contains two forms of cellulases that can qualitatively convert derived cellulose to glucose. These two different types of cellulase multi enzyme complex in snail haemolymph can be further investigated.

REFERENCES

Afolayan, A., F.K. Agboola and A.O. Akinloye, 1997. Some properties of β-glucosidase from haemolymph of the giant African Snail. Niger. J. Biochem. Mol. Biol., 12: 43-50.

Alan, W., 1985. Handbook of Enzyme Biotechnology. 2nd Edn., Handbook of enzyme biotechnology, USA., pp: 26-28.

Almin, K.E. and K.E. Eriksson, 1968. Influence of carboxymethyl cellulose properties on the determination of cellulase activity in absolute terms. Arch. Biochem. Biophys., 124: 129-134.

Ekperigin, M.M., 2007. Preliminary studies of cellulase production by *Acinetobacter anitratus* and *Branhamella* sp. Afr. J. Biotechnol., 6: 28-33.

Gong, C.S. and T.T. George, 1979. Cellulase and Biosynthesis Regulation. Academic Press Inc., West Lafayette, pp: 111-140.

Lambert, J.C., C.A. Ibrahim-Verbaas, D. Harold, A.C. Naj and R. Sims *et al.*, 2013. Meta-analysis of 74,046 individuals identifies 11 new susceptibility loci for Alzheimer's disease. Nat. Genet., 45: 1452-1458.

Meyer, J.J.M., A.J. Afolayan, M.B. Taylor and D. Erasmus, 1997. Antiviral activity of galangin isolated from the aerial parts of *Helichrysum aureonitens*. J. Ethnopharmacol., 56: 165-169.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem., 31: 426-428.

Myers, F.L. and D.H. Northcote, 1959. Partial purification and some properties of a cellulase from *Helix pomatia*. Biochem. J., 71: 749-775.

Nevelle, T.P. and S.H. Zeronian, 1985. Cellulose Chemistry and its Applications. In: Radiation Chemistry, Radiochemistry and Nuclear Chemistry, Nevell, T.P. and S.H. Zeronian (Eds.). Ellis Horwood Series Chemical Science, England, pp: 312-337.

Perner, A., N. Haase, A.B. Guttormsen, J. Tenhunen and K. Gudmundur *et al.*, 2012. Hydroxyethyl starch 130/0.42 versus ringer's acetate in severe sepsis. N. Engl. J. Med., 367: 124-134.

Thatheyus, A.J. and R. Vidhya, 2013. Biodegradation of carboxymethyl cellulose employing *Cheatomium globosum* MTCC 2193. Sci. Int., 1: 92-97.

Topakas, E. and P. Christakopoulos, 2007. Microbial Xylanolytic Carbohydrate Esterases. In: Industrial Enyzmes: Structure, Function and Applications, Polaina, J. and A.P. MacCabe (Eds.). Springer, New York, pp: 83-97.

Umezurike, G.M., 1976. Metabolic regulation of beta-glucosidase in the gut content of the snail *Achatina achatina*. Biochim. Biophys. Acta, 438: 333-338.