Purification and Characterization of Cellulase from Termite *Ametermes eveuncifer* (Silverstri) Soldiers

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Abstract

Cellulase enzyme was purified and characterized from termite soldiers (*Ametermes eveuncifer*) using 70% ammonium sulphate precipitation, ion exchange chromatography and affinity chromatography. The enzyme isolated had a specific activity of 5.04 U/mg with a percentage yield of 11.7%. The enzyme showed maximum activity at 50^0C and pH 8. The enzyme was not inhibited by Ba^{2+} at a concentration of 1mM and Pb^{2+} at 10 mM concentration but was inhibited by other metal ions at 1 mM and 10 mM concentrations of their salts (NaCl, KCl, $MnCl_2$, and $NiCl_2$).

Keywords: Cellulase, termite soldier, *Ametermes eveuncifer*, purification

1. Introduction

Termites' ability to digest wood and cause significant structural damage to buildings is what majorly distinguishes them from other insect pests. This ability to digest wood is due mainly to the complex array of carbohydrolases which confer on them the ability to digest cellulose and hemicelluloses such as xylan (Whistler & Chen, 1991). Termites are among the most significant wood-feeders on earth. To efficiently digest cellulose for survival, *Ametermes eveuncifer* (Silverstri) require cellulase for the enzymatic hydrolysis of polysaccharide to glucose (Fagbohunka et al., 2015).

The major nutritional component for wood-feeding termites is cellulose, which is plant's main structural constituent. Apart from being structural pests, termites have a huge impact on terrestrial ecological processes, as they are part of the main terrestrial cellulose recyclers (Inoue et al., 1997). Cellulase activity in the midgut of these insects is very high (Hogan et al., 1988), and studies have shown that they rely solely on their own cellulases (Slaytor, 1992; Slaytor, 2000) rather than the cellulases of symbiotic bacteria present in their lower intestine (Brune & Stingl, 2005).

Based on their mode of action, three varieties of cellulase exist which any highly cellulolytic organism must possess (Breznak & Brune 1994). Exoglucanases (EC 3.2.1.91) are the first class of cellulases. The second class of cellulases consists of the endoglucanases (EC 3.2.1.4) while the third class is the beta-glucosidases (EC 3.2.1.21). Exoglucanases bind to the end of a cellulose chain and breaks it down as it moves along the chain length. Most exoglucanases reduce cellulose to cellobiose (a beta-glucose disaccharide) and are said to be cellobiohydrolases. Endoglucanases, on the other hand, bind anywhere along the cellulose chain and break it up randomly, thus reducing it to cellodextrins (beta-glucose oligosaccharides). Beta-glucosidases break cellodextrins down into glucose. They are the last enzyme in the process of generating glucose from cellulose (Mishra, 1991).

In recent times, there have been studies on the diverse cellulase activity in each gut segments of termites (Willis et al. 2010). Findings by Tokuda et al. (2004, 2005) established that the endogenous cellulase genes were expressed in the salivary glands of lower termites while being expressed in the midgut of higher termites. Mo et al. (2004) and Lu et al. (2010) also independently compared the differences in total cellulase activity among lower termites, while Fujita et al. (2008) reported the differences in cellulase expression between soldier and worker termites of *Hodotermopsis sjostedti* and *Nasutitermes Takasagoensis*.

The use of cellulase in various industrial processes is indispensable. It is used in plants and agricultural waste processing (Mswaka & Magan, 1998; Lu et al., 2004), biofuels (Vaithanomsat et al., 2009), triphase

biomethanation (Chakraborty et al., 2000), chiral separation and ligand binding studies (Nutt et al., 1998). An in-depth study of the enzymes involved in the degradation of cellulose and other polysaccharides in termites will play a key role towards discovering novel approaches in termite control, removal of cellulosic waste, improved knowledge of polysaccharide degradation which might drive the discovery of novel industrial enzymes as well as industrial methods in alternative fuel generation from cellulosic materials. This study reports the purification and physicochemical properties of cellulase from *Ametermes eveuncifer* soldiers.

2. Materials and Methods

2.1 Reagents and Apparatus

Sodium chloride, potassium chloride, lead chloride, barium chloride, manganese chloride, nickel chloride and ammonium sulphate, were purchased from BDH Chemical Limited, Poole England. EDTA and Carboxymethyl-cellulose were purchased from Sigma Chemical Company, St. Louis, USA. All other reagents were of high analytical grade and used without further purification. The agro-industrial wastes (banana peel, rice husk, orange mesocarp, pineapple peel, maize cob) used were obtained locally, ground to powder using mechanical grinder, sieved and dissolved in the assay buffer. These wastes were used without prior pre-treatment. Termite soldiers (*A. eveuncifer*) were collected from South-Western part of Nigeria.

Apparatus used include, mortar and pestle, weighing balance (Mettler PN1210), pH meter (PHS-25), Centrifuge (Helmreasinn 80-2) and UV/VIS Spectrophotometer (Bosch 752N).

2.2 Enzyme Extraction and Purification

Termite soldiers were washed and rinsed with distilled water. The termites were gently homogenized in 10 mM sodium acetate buffer, pH 5.0, containing 1 mM EDTA. The mixture collected was centrifuged at 15,000 rpm for 15 minutes at room temperature. The supernatant, which served as the crude enzyme, was collected and stored in a refrigerator. The supernatant was salted out by bringing the crude extract to 70% (w/v) saturation with Ammonium sulphate.

2.3 Cellulase Assay and Protein Concentration Determination

Bradford method (1976) was used to measure the protein concentration of the enzyme using bovine serum albumin (BSA) as standard. Cellulase activity was assayed according to the method of Zhang et al. (2006), with slight modification, using 1% Carboxymethyl-cellulose (CMC) as the substrate. CMC was dissolved in 10 mM citrate buffer (pH 5.0). The reaction mixture, containing 50 μL of the enzyme and 450 μL of CMC, was incubated at 50 ºC for 30 min. The reaction was terminated by adding 2 ml of 3,5-Dinitrosalicylic acid (DNSA) reagent. The mixture was then boiled for 30 min and optical density reading was taken at 540 nm. One unit of cellulase activity was expressed as the amount of enzyme that released 1μmol 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.

2.4 Ion Exchange Chromatography

A CM-Sephadex C-25 cation exchanger was equilibrated with 10 mM sodium citrate buffer pH 4.8. The precipitated enzyme was dialysed against several changes of buffer before being layered on the column (1.5 x 25 cm). The column was then washed with the same buffer (citrate buffer, pH 4.8) to remove the unbound protein, followed by elution with 0.1 M KC1 in 50 mM sodium citrate buffer, pH 4.8. Fractions (3 ml) were collected from the column at a rate of 60 ml/h. The fractions were assayed for cellulase activity using the DNSA method and active fractions were pooled and immediately dialysed against 50% glycerol in the elution buffer to store the enzyme. Protein was determined by Bradford assay by measuring O.D at 595 nm.

2.5 Affinity Chromatography

Agarose Blue resin was packed into a 1.5 x 10 cm column and equilibrated with 10 mM citrate buffer (pH 4.8). Then the sample was applied on the column. The column was then washed with the buffer to remove unbound protein, followed by elution with 1M KC1 in the same buffer. Fractions of 2 ml were collected from the column at a rate of 16 ml/h. Protein was monitored by Bradford assay at 595 nm and the fractions were assayed for cellulase activity following the assay method described above. The active fractions were pooled and immediately dialysed against 50% glycerol in citrate buffer (pH 4.8) to store the enzyme.

2.6 Effect of Temperature

Cellulase activity was assayed at temperatures between 30 ºC and 100 ºC at pH 5.0 to investigate the effect of temperature on the activity of the enzyme and to determine the optimum temperature of the enzyme. The assay mixture was first incubated at the respective temperature for 10 min before initiating reaction by the addition of an

aliquot of the enzyme which had been equilibrated at the same temperature. The cellulase activity was assayed routinely as previously described in section 2.3.

2.7 Effect of pH

The effect of pH on the enzyme activity was performed by assaying for enzyme activity at a pH range of 3 to 11 at 37 °C using varying buffers of different pH: 50 mM of citrate (pH 3-5); phosphate (6 - 8) and borate (pH 9 - 11). The cellulase activity was assayed as previously described in section 2.3.

2.8 Effect of Salts

The effect of various metal ions on the activity of termite cellulase was studied using the following chloride salts: NaCl, KCl, $BaCl_2$, $MnCl_2$, $PbCl_2$ and $NiCl_2$ at final concentrations of 1.0 mM and 10.0 mM in the enzyme assay.

2.9 Substrate Specificity

The substrate specificity of the enzyme was determined by using various agroindustrial wastes as carbon sources such as banana peel, rice husk, orange mesocarp, pineapple peel and maize cob in a typical cellulase assay mixture and the activity expressed as percentage residual activity of the enzyme.

2.10 Inhibition Studies

The inhibition study was carried out using various chelating agents including EDTA, urea and mercaptoethanol at final concentrations of 1.0 mM and 10.0 mM and the percentage residual activity determined.

3. Results

3.1 Purification

The summary of the purification procedure for cellulase from the soldier termite is shown in Table 1. The elution profile of the enzyme using ion-exchange chromatography on CM-Sephadex C-25 is shown in Figure 1 while the elution profile of the enzyme using affinity chromatography is shown in Figure 2. The enzyme was purified using 70% ammonium sulphate precipitation, ion exchange and affinity chromatography.

Table 1. Purification of Cellulase from Termite, *Ametermes eveuncifer* (Silverstri) Soldiers

PURIFICATION STEPS	TOTAL ACTIVITY (U)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (U/mg protein)	PURIFICATION FOLD	YIELD (%)
CRUDE ENZYME	36850.0	30708.33	1.2	1	100
70% AMMONIUM SULPHATE PRECIPITATION	37697.54	20943.08	1.8	1.5	68.2
ION EXCHANGE CHROMATOGRAPHY	18682.9	7413.85	2.52	2.1	35.4
AFFINITY CHROMATOGRAPHY	18108.09	3592.80	5.04	4.2	11.7

Each of the steps was carried out as described in the methodology. Cellulase activity was measured following a modified method of Zhang et al. (2006). Protein concentration was determined by the Bradford method (Bradford, 1976). One unit (U) of cellulase activity was expressed as the amount of enzyme that released 1μmol of reducing sugars (glucose equivalent) in 1 min under the assay conditions.

3.2 Effect of pH and Temperature on Cellulase

The effect of pH on the rate of activity of cellulase is shown in Fig. 3. An optimum pH of 8 was obtained for cellulase. The assay done to investigate the effect of temperature on the activity of cellulase showed the enzyme to have its optimum temperature at 50 °C as shown in Figure 4.

3.3 Effect of Salts

The results of the effect of various salts on the activity of cellulase from the termite soldier (Table 2) showed that $PbCl_2$ at a concentration of 10 mM had no effect whatsoever on enzyme activity while $BaCl_2$ had little effect on enzyme activity at a concentration of 1 mM. However, NaCl, KCl, $MnCl_2$ and $NiCl_2$ inhibited the activity of the enzyme at concentrations of both 1.0 and 10mM.
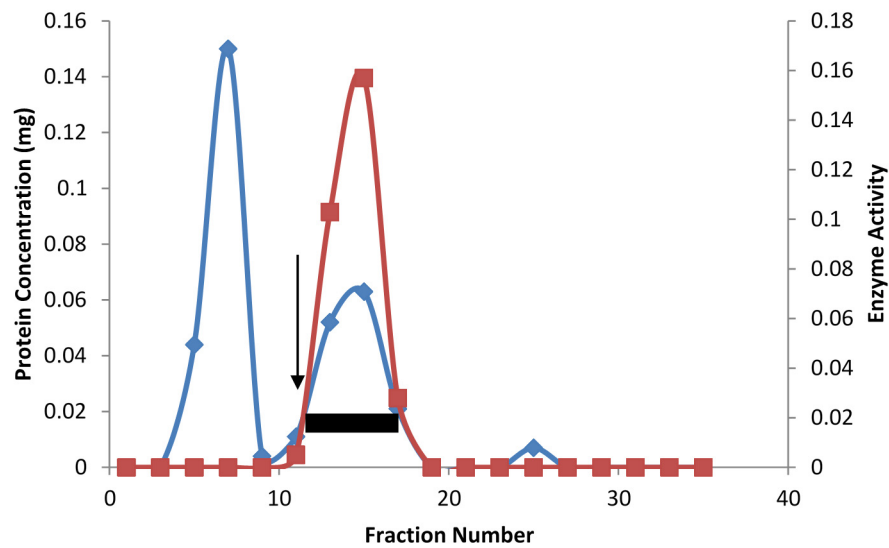
Figure 1. Elution profile of cellulase from termite soldiers using ion-exchange chromatography on a CM-Sephadex C-25 column (1.5 x 25 cm). The column was first washed with 10 mM citrate buffer (pH 4.8) and then eluted with 0.1M KCl (the point of elution is indicated by the arrow). Elution was carried out at a flow rate of 60 ml/h and 3ml fractions were collected. One active peak was obtained. ◆, Protein concentration ($OD_{595\,nm}$); ■, Enzyme Acivity ($OD_{540\,nm}$); ▬, pooled fraction
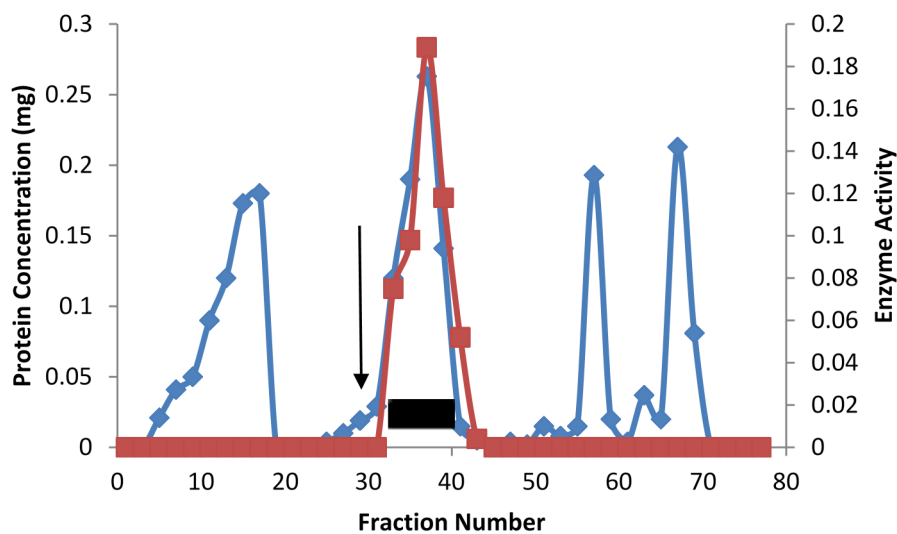

Figure 2. Elution profile of cellulase from termite soldiers using affinity chromatography on an agarose blue resin column (1.5 x 10 cm). The column was first washed with 10 mM citrate buffer (pH 4.8) and then eluted with 1M KCl (the point of elution is indicated by the arrow). Elution was carried out at a flow rate of 16 ml/h and 2ml fractions were collected. ◆, Protein concentration ($OD_{595\,nm}$); ■, Enzyme Acivity (OD_{540nm}); ▬, pooled fraction
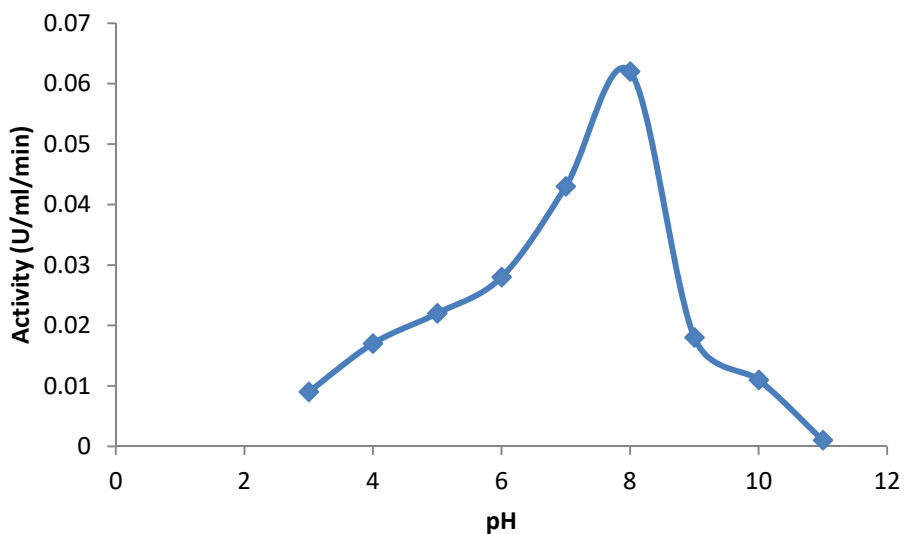
Figure 3. Effect of pH on Cellulase from Termite, *Ametermes eveuncifer* (Silverstri) Soldiers
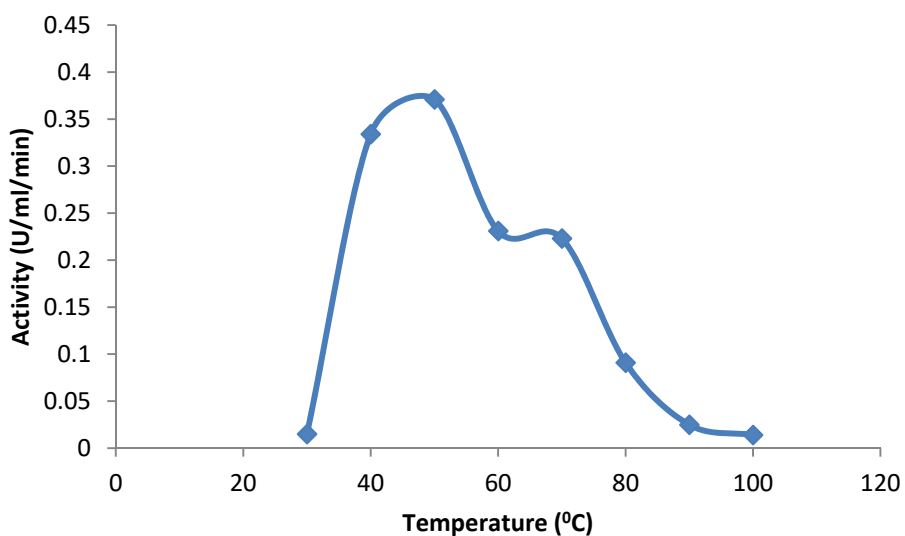

Figure 4. Effect of Temperature on Cellulase from Termite, *Ametermes eveuncifer* (Silverstri) Soldiers

Table 2. Effects of cations on the activity of cellulase from termite soldiers

Salt	% Residual Activity	
	1.0 mM	10.0 mM
NaCl	6.02	44.49
KCl	56.13	40.75
$BaCl_2$	79.0	20.37
$MnCl_2$	23.28	0
$PbCl_2$	19.13	100
$NiCl_2$	0	14.55

Percentage of residual activity is compared with the activity of the enzyme in the absence of metal ions.

3.4 Substrate Specificity

The purified enzyme showed highest activity against maize cob even though this activity was considerably low. There was no hydrolysis ability against banana and rice husk. A summary of this is shown in Table 3.

Table 3. Substrate specificity of the purified cellulase

SUBSTRATES	ACTIVITY (%)
BANANA PEEL	0
RICE HUSK	0
ORANGE MESOCARP	0.416
PINEAPPLE PEEL	0.416
MAIZE COB	3.326

3.5 Effect of Inhibitors

As indicated in Table 4, 10.0 mM β-mercaptoethanol slightly inhibited about 14 % of the enzyme activity while 1 mM β-mercaptoethanol did not inhibit enzyme activity. Similarly, EDTA and Urea at both concentrations of 1.0 and 10.0 mM did not inhibit cellulase activity. This is shown in Table 4.

Table 4. Effect of some inhibitors on cellulase activity from Termite soldiers

Compounds	% Residual Activity	
	1.0 mM	10.0 mM
EDTA	100	100
Urea	100	100
β -Mercaptoethanol	100	86

Percentage of residual activity is compared with the activity of the enzyme in the absence of the chelating agents.

4. Discussion

A greater percentage of cellulotic enzymes have been produced and characterized from fungi, bacteria and several insects including termites. Cellulase is a very important hydrolytic enzyme. Over the years, several authors have purified and reported properties of cellulase from a wide range of termite species including *Nasutitermes walkeri* (Schultz et al., 1986), *Macrotermes mulleri* (Rouland et al., 1988), *Reticulitermes speratus* (Watanabe et al., 1997), *Hodotermopsis sjostesti* and *Nasutitermes takasagoensis* (Fujita et al., 2008). Previously, Fagbohunka et al. (2014) only partially purified cellulase from *Ametermes eveuncifer* and carried out inhibition studies along with other enzymes. In this paper, we report the properties of cellulase from *A. eveuncifer* following the purification and detailed characterization of the enzyme.

Cellulases have been found to be present in both flagellate-harbouring and flagellate-free termites. Findings by Tokuda and Watanabe (2007) showed that cellulases present in the flagellate-free termites, *Nasutitermes takasagoensis* and *Nasutitermes walkeri* were produced by symbiotic bacteria present in the hind gut of these termites, although cellulase activities in the guts of flagellate-free termites were significantly lower than those of flagellate-harbouring termites (Tokuda et al., 2004; 2005). Cellulase characterized from various higher and lower termites have been shown to be similar in a number of properties in some bacteria and fungi (Watanabe et al., 1997). Properties of cellulase from various termites can therefore be compared with those of microorganisms which also play a role in the digestion of cellulose in the gut of termites.

Following the three-step purification of cellulase from *A. eveuncifer*, a specific activity of 5.04 U/mg was obtained. It is often difficult to compare activity assays among studies because various factors such as temperature, substrate, detection methods e.t.c. can influence the activity (Willis et al., 2010). It would be more interesting to make a comparison of cellulase activities among different castes of termites within the same species or different species under similar experimental conditions. Unlike the worker termites which collect cellulose to feed the colony, soldier termites defend the colony from attack because they possess specialized morphology for colony defence, but their feeding activity is dependent on other colony members (Fujita et al., 2008). It is therefore expected that the cellulase enzymatic activities and gene expression of the termites will differ greatly from one caste to another and from species to species.

The cellulase activity was affected by the pH and temperature of the reaction mixture. It was observed that the optimum condition of enzyme activity by the soldier termites was pH 8 and temperature 50 °C which are in agreement with that reported by Lima et al. (2005) for *Bacillus pumilus* strain. The optimum temperature value was the same in comparison to that obtained with the cellulase from the *Reticulitermes speratus* (Kolbe), a Japanese Subterranean Termite (Watanabe et al., 1997) and very close to that from the digestive tract of the termite *Macrotermes mulleri,* in which an optimum temperature of 55 °C was observed (Rouland *et al,* 1988). A similarly high optimum temperature (55 - 60 °C) was observed for the cellulase produced by *Streptomyces malaysiensis* and *Streptomyces* sp. as reported by Nascimento et al. (2009) and Jaradat et al. (2008). A lower optimum temperature of 40 °C was reported by El-Sersy et al. (2010) for cellulase from *Streptomyces ruber*.

Regarding optimum pH for cellulase in other studies, optimum enzyme activity was found at pH 7.0 in *Pseudomonas fluorescence* (Bakare et al., 2005) and *Bacillus amyoliquefaciens* DL3 (Lee et al., 2008). However, lower optimum pH (5.0) for enzyme activity with broad ranges of pH stability was observed in *Thermomonospora* sp. (George et al., 2001), and in *Bacillus* strain M-9 (Bajaj et al., 2009). An optimum pH of 6.0 was reported for the Japanese Subterranean Termite, *Reticulitermes speratus* (Watanabe et al.1997). Cellulase was optimally active at alkaline pH in *Bacillus sphaericus* JS1 (Singh et al., 2004) and *Bacillus* sp. HSH-910 (Kim et al., 2005).

All the metals inhibited cellulase activity except Ba^{2+} at 1 mM and Pb^{2+} at 10 mM. The inhibition of the enzyme by the other metals (Na^+, K^+, Mn^{2+} and Ni^{2+}) to various degrees may be a result of interaction of these metal ions with sulfhydryl groups at the enzyme catalytic site (Yin et al., 2010) or induction of changes in the secondary and tertiary structure of the enzyme. Such structural changes have been reported after incubation of protein with metal ions. According to Stokinger (1981), metal ions showing enzyme inhibition are those that have a strong affinity for ligands such as cysteinyl, phosphate and histidyl side chains of protein. The results obtained in this work are in agreement with that from Fagbohunka et al. (2015) who reported that chloride salts of Hg^+, Mn^{2+}, Na^+, Co^{2+}, NH_4^+, Zn^{2+}, Ba^{2+} and Sn^{2+} greatly inhibited cellulase from *A. eveuncifer* soldiers at 1 mM concentration of the salts. Following the inhibition studies by Fagbohunka et al. (2015), it was recommended that one of the chloride salts of these metals or a combination of at least two can be used for the design of a pesticide for termites such that the organism may not be able to metabolise wood even if it feeds on it. Thus, the organism will be unable to hydrolyze the cellulolytic materials they feed on caused by an inhibition of the metabolic enzymes by chloride salts of the metals. These definitely will result in the death of the organism. Before this study, it had been reported previously that cellulases are inhibited in the presence of heavy metals including Mn^{2+} (Mawadza et al., 2000).

Further studies can elucidate the co-existence of cellulase produced by the different microorganisms present in the digestive tract of these termites and the properties of these can be compared with cellulases in the different regions of the termite guts (mid and hind gut). The kinetic data and the physical properties of the enzyme can be compared to know if the enzymes are identical or not. From such studies, important inferences can be drawn on how the cellulase system of this species accomplish cellulose digestion.

5. Conclusion

Cellulase enzyme was purified from *A. eveuncifer* soldiers and its maximum enzymatic activity was obtained at 50°C and pH 8. The properties of cellulase from the soldier termites are similar to those of the enzyme from other organisms. It is plausible to assume that the enzyme is involved in cellulotic hydrolysis by hydrolyzing the β-1, 4 linkages of cellulose to glucose units. Further inhibition studies of the cellulase system enzymes would reveal the most potent inhibitors and may lead to the development of potential pesticides for termite control.

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