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Research Article

Effects of Immobilization on the Kinetic Parameters of Partially Purified Cellulase from Termites (*Macrotermes bellicosus*)

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Abstract

Cellulase is a class of hydrolase enzymes with commercial and industrial value, it is commonly produced by the microorganism such as fungi, protozoans, bacteria and even insect that survives on cellulose. In this study, we evaluated the effect of immobilization on the kinetic parameters of partially purified cellulase from *Macrotermes bellicosus*. The cellulase was extracted from matured *M. bellicosus* and subjected the supernatant of the crude extract that has cellulase activity to gel filtration and ion-exchange chromatography. The enzyme was partially purified 2.0 fold with an overall yield of 40.4% on DEAE- cellulose column and a final specific activity of 51.0U/mg. The partially purified cellulase was immobilized by entrapment on calcium alginate beads. The free and immobilized enzyme showed an optimum temperature of 50°C and 60°C and optimum pH of 6.0 and 8.0 respectively. Initial velocity studies for the determination of kinetic constants with cellulose as a substrate revealed a K_m value of 7.9 mg/ml and 3.4mg/ml with a V_{max} value of 1.59 unit/mg and 1.15 unit/mg for the free and immobilized enzyme respectively. Both the free and immobilized cellulase activity was enhanced by Ca^{2+}, and Mn^{2+} but slightly decreased by Na^{+}. While Mg^{2+} and Zn^{2+} were found to be strong inhibitors of both the free and immobilized enzyme. This research shows that cellulase from *M. bellicosus* could be immobilized and utilized for degradation of cellulose-containing materials because of their high catalytic activity, thermostability and acid-base stability, which reflect the potential industrial significance of the enzyme.

Keywords: Cellulase; *Macrotermes bellicosus*; Partial Purification; Immobilization

Introduction

Cellulose is the most abundant biomaterial (Carbohydrate) on earth produced by plants. It is a linear polymer of glucose units, which are linked together by β-1,4 glycosidic bonds [1]. Cellulose is considered as the most important source of raw material for the production of renewable energy such as bioethanol, as its β-D-(1, 4) glucosidic linkages could be hydrolyzed to glucose units by an enzymatic system referred to as cellulases [2].

Cellulase is a class of enzyme that catalyzes the hydrolysis of cellulose to glucose units. It comprises of three groups of individual

enzymes that work synergistically to depolymerize the 1,4-glycosidic bonds [3]. These enzymes are endoglucanase (Endo-1,4-*β*-D-glucanase (EG), EC 3.2.1.4) which acts on the amorphous region and breaks the cellulose chain with formation of cello-oligosaccharides, cellobiohydrolase (Exo-1,4- *β*-D-glucanase (CBH), EC 3.2.1.91) acts on microcrystalline cellulose converting it to cellobiose as the primary product and *β*-glucosidase (1,4-*β*-D-glucosidase (BG), EC 3.2.1.21) which causes the hydrolysis of cellobiose to glucose [4].

Cellulase has attracted many scientific researchers because of its biotechnological potentials in various industries such as pulp and paper, detergent, textile, food and biofuel production [5]. The cost of production and low yield of this enzyme has been a major problem for industrial applications.

Immobilization of enzyme is the attachment of soluble enzyme onto an insoluble material {support) which enables retention of catalytic activity, resulting in the possibility of reusing the biocatalyst. Moreover, easier and faster separation of immobilized enzymes from the reaction mixture makes it more attractive for industrial applications [6]. Calcium alginate beads entrapment is a method used for the immobilization of enzyme. This technique increases the efficiency of catalytic processes, reduce the associated costs for the reproduction of soluble enzyme, provide large surface area for catalytic reaction together with least diffusion limitation in the transport of substrate and product for an enzyme reaction. Furthermore, improve its stability at harsh reaction conditions, as variable reaction parameters might affect enzymes [7].

Cellulase from microorganism such as bacteria and fungi has been produced and characterize [8]. Some bacterial cellulases are produce in low yield with no exoglucanase activities detected, whereas fungal cellulase are produced in large amount which includes all the enzyme components (endoglucanase, exoglucanase and β-glucosidase) but the high cost of production has been an area of concern [9]. However, the production and purification of these multi-enzyme complexes (cellulosome) have been a difficult task and laborious to researchers, to carry out without a noticeable loss of enzyme activity. Apart from bacterial and fungal cellulase, cellulolytic enzymes from insects such as, termites, beetle and snails have been investigated [10,11].

Termites are among the most significant wood-feeders on earth, they utilize cellulosic materials as their food source for survival apart from been regarded as a structural pest because of their ability to destroy all materials containing cellulose, but termite still has a positive impact to the terrestrial ecological processes. Termites are divided into two groups these are; the lower termites which do not produce sufficient cellulase for survival, but contain protozoans in their gut that aid in cellulose breakdown, this interaction is an obligate mutualism for both termite and protozoan [12], and the higher termites (Termitidae) that do produce sufficient cellulase endogenously in their mid-gut to digest and obtain adequate nutrition from cellulose [13].

Macrotemes bellicosus is an African mound-building termite that mainly feeds on wood and dry plant matter (Lignocellulosic matter). This species of termites has a highly developed social caste system which includes workers, soldiers and reproductive [14]. *M. bellicosus* worker termites that strive for food and feed the colony were used for this study. The present study aimed to evaluate the effects of immobilization on the catalytic function and biochemical characterization of partially purified cellulase extracted from *M. bellicosus* as the source of catalyst for further industrial applications.

Materials and Methods

Materials

All chemicals used were obtained from the Departmental laboratory of Biochemistry Bayero University Kano and Sigma Aldrich Chemical Company. These includes; Bovine serum albumin, Coomasie brilliant Blue G-250, Sephadex G-75, DEAE-cellulose, dinitrosalicyclic acid (DNS), sodium alginate (Sigma Aldrich) Sodium chloride, manganese, zinc, magnesium, calcium.

Methods

Sample collection and preparation.

Macrotermes bellicosus worker termites were obtained by field trapping technique from termites mound located at Biological Science garden, Department of Biological Science Bayero University, Kano. The sample was washed with distilled water and gently homogenized using an electric blender with ice-cold 10 mM sodium acetate buffer at pH 5.0, containing 1mM EDTA. The homogenate was centrifuged at 15,000 rpm for 15 minutes at 4°C. The supernatant served as the crude extract. Protein concentration was determined based on Bradford method [15] while cellulase activity of the crude protein was evaluated as described earlier by Zhang [16].

Determination of protein concentration and cellulase activity

Method of Bradford [15] was employed for the determination of cellulase from *M. bellicosus* using Bovine Serum Albumin as a standard. The absorbance of sample and Bradford reagent (Coomasie brilliant Blue G-250) were taken at 595 after 5min of incubation.

Cellulase assay was conducted according to Zhang., *et al.* protocol [16], a mixture containing 450µl of 1%CMC substrate, 50µl cellulose for the free cellulase, and 0.5g for immobilized cellulose, was incubated in 10mM citrate buffer, pH 5 at 50ºc for 30minutes. The reaction mixture was stopped by the addition of 2ml DNSA reagent and boiled for 30min. The absorbance was taken at 540nm. 1unit of enzyme was defined as the amount of cellulase that catalyzes the hydrolysis of cellulose to produce 1µmol of reducing sugar in 1minute.

Partial purification of cellulase using size-exclusion chromatography

Sephadex G-75 was packed into 10nm 30cm column and equilibrated with 50mM sodium acetate buffer, pH 5. The Recovered supernatant was loaded onto the glass column, and the column was eluted with the same buffer. 35 fractions (of 3ml) each were collected from the column at a flow rate of 1ml/2minutes. The fraction with the highest cellulase activity was selected and used for the next purification step.

Ion-exchange chromatography

DEAE-Cellulose anion exchanger was equilibrated with 50mM sodium acetate buffer, pH of 5.0. A fraction with the highest activity was poured onto the 10mm × 30cm column without disturbing the bed. The column was then washed with buffer to removed unbound protein, elution was done with a gradient of 1M salt concentration solution. 30 fractions were collected each (of 3ml) from the column at a flow rate of 1ml/minute. Cellulase activity of the fractions was determined using the DNSA method and protein concentration using Bradford assay at an optical density of 595nm.

Enzyme immobilization

Sodium alginate (3%) was prepared by dissolving 3g of sodium alginate in 100ml of sodium acetate buffer, pH 5.0 and autoclaved at 121^0c for 15 minutes. the mixture was allowed to cool at room temperature, after which 5ml of the partially purified cellulase was added. The alginate beads were formed by dropping the mixture from a height of approximately 20cm drop wise using a sterile 10ml syringe into a 250ml beaker containing (0.2M) CaCl solution with continuous shaking to form beads, thereby entrapping the enzyme. The beads were washed 3times with distilled water and then the immobilized enzyme beads were left in 200mM sodium acetate buffer at 4^0C for further studies.

Effect of pH on the activity of free and immobilize cellulase

The effect of pH on the activity of free and immobilized cellulase were determined by assaying the enzyme activity using sodium acetate buffer at different pH values (2-14). Cellulase activity was determined as previously explained.

Effect of pH on the stability of free and immobilize cellulase

The immobilized and free cellulases were incubated at different pH values from 2 to 14 at room temperature for 30minutes. The residual enzyme activities were then determined using the method of enzyme assay described above.

Effect of temperature on the activity of free and immobilize cellulase

The effect of temperature on the activity of free and immobilized cellulase was determined by assaying the activity of the enzyme at different incubation temperatures. The reactions were stopped and absorbencies were taken at 540nm. The activity of cellulase was plotted against different temperatures.

Effect of temperature on the stability of free and immobilize cellulase

The thermal stability was determined by incubating the free and immobilized cellulase in a water bath at different temperatures (10, 20, 30, 40, 50, 70, 80, 90 and 100 ºC) for 30minutes, then immediately transferred into an ice bath. The activity was assayed for each treatment. The remaining enzyme activity for cellulase was plotted against the temperature.

Effect of metal ion

The effect of divalent and monovalent metal ions on the activity of partially purified cellulase was assayed by incubating the free and immobilized cellulase with sodium acetate buffer, pH 5.0 containing 10mM concentration of Na^+, Mg^{++}, Ca^{++}, Mn^{++}, and Zn^{++} for 1hr at 4ºC. Activities of the enzyme were assayed after incubation. Cellulase activity in the absence of metal ions was used as control.

Effect of substrate concentration

A reaction mixture containing free cellulase and immobilize cellulase with variable substrate concentrations ranging from (0.2, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5%) respectively were incubated in a water bath at 50 ºC for 30min. Absorbance was taken at 540nm. The K_m and V_{max} parameters were calculated using Lineweaver-Burk plot.

Data analysis

Results were represented as mean ± SD of triplicate determinations. Statistical analysis for optimum temperature and pH results were performed using student t test at (P<0.05). While the test for level of significance for reusability of immobilized cellulase was perform by ANOVA (with Turkey post-test) at P <0.05 level of significance. All statistical analyses were carried out using GraphPad Instat Software version 3.05.

Results and Discussion

Results

Partial purification of cellulase from *M. bellicosus*

Cellulase from *M. bellicosus* was successfully extracted by differential centrifugation of the crude extract and partially purified using size exclusion and ion-exchange chromatography (Table 1). CM Cellulose was used as the substrate to monitor the enzymatic activity.

Purification Step	Volume (ml)	Total protein (mg/ml)	Total activity (U/ml)	Specific Activity U/mg	Purification Fold	Percentage Yield
Crude	15	0.155	2.315	14.87	1	100
Size-exclusion chromatography on Sephadex G-75 column	3	0.082	2.157	26.34	1.7	93
Ion-exchange chromatography on DEAE-cellulose column	3	0.018	0.935	51.00	2.0	40.4

Table 1: Purification profile of cellulase enzyme from *M. bellicosus.*

The elution profile of size exclusion chromatography revealed some protein peaks with fraction 9 having the highest cellulase activity (Figure 1). Furthermore, a single peak with the highest enzymatic activity was obtained following the subsequent purification step by ion-exchange chromatography (Figure 2).

Figure 1: Elution profile of cellulase from *M. bellicosus* on Sephadex G-75 Column. The column (300mm) was eluted with 95ml of 50mM sodium acetate buffer pH 5.0. Elution was carried out at a flow rate of 1ml/120sec at 25^{O}c and 3ml of 30 fractions were collected.

Figure 2: Elution profile of cellulase from *M. bellicosus* on DEAE Cellulose Column. The column (300mm) was initially washed with 50mM sodium acetate buffer pH 5.0 and then eluted with a linear gradient of 1.0M NaCl Elution was carried out at a flow rate of 1ml/60sec at 25^{O}c and 3ml of 30 fractions were collected.

Figure 3 (a and b) shows Calcium-alginate bead before and after immobilization with the enzyme solution. Cellulase was effectively immobilized on calcium alginate beads as observed by scanning electron microscope (figure 3b).

Figure 3: Scanning electron microscopy at 15000x magnification. (a) Calcium-alginate bead before immobilization with soluble enzyme (cellulase). (b) Immobilized cellulase on the calcium-alginate bead.

Characterizations of free and immobilized partially purified Cellulase

Cellulase has very high recycling efficiency as it retained more than 50% of its enzymatic activity on calcium-alginate bead after 5 cycles (Figure 4).

Figure 4: Recycling efficiency of immobilized cellulase on calcium-alginate bead. The point represents mean ± S.E (n = 3) *= significant.

The optimum temperature for the activity and stability for free and immobilized cellulose were found to be 50ºc and 60ºc respectively as shown in figure 5 and 6.

An optimum pH of 6 and 8 were observed for the activity and stability of free and immobilized cellulose as shown in figure 7 and 8.

Figure 5: Effect of temperature on the activity of free and immobilized cellulase from *M. bellicosus*. The point represents mean ± S.E (n = 3) *= significant.

Figure 6: Effect of temperature on the stability of free and immobilized cellulase from *M. bellicosus*. The point represents mean ± S.E (n = 3) *= significant.

Effects of metal ions on cellulase revealed that, both free and immobilized enzyme activity were enhanced by Ca^{2+}, and Mn^{2+}, but

Figure 7: Effect of pH on the activity of free and immobilized cellulase from *M. bellicosus*. The point represents mean ± S.E (n = 3) *= significant.

Figure 8: Effect of pH on the stability of free and immobilized cellulase from *M. bellicosus.* The point represent mean ± S.E (n = 3).

slightly decreased by Na^{+}. While Mg^{2+} and Zn^{2+} were found to be strong inhibitors of both the free and immobilized cellulase (figure 9).

Figure 9: Effect of metal ion on the activity of free and immobilized cellulase from *M. Bellicosus*. The point represent mean ±S.E (n = 3). *= significance difference with respect to the control.

The enzyme kinetic studies revealed Km values of 7.9 mg/ml and 3.4mg/ml with a V_{max} values of 1.59 unit/mg and 1.15 unit/mg for free and immobilized cellulase Respectively (Table 2).

Cellulase	K_m(mg/ml)	V_{max}(U/ml)
Free	7.953 ± 2.4	1.593 ± 0.1
Immobilized	3.455 ± 0.4	1.154 ± 0.03

Table 2: Kinetic study of free and immobilized cellulase from *M. bellicosus* worker termite.

Discussion

An increase in demand of cellulase, high cost of production and low product yields has become a global issue to many industrialists. Immobilization of cellulase is an efficient method which makes it easier to restore and reuse the enzyme, consequently reducing the cost of bioethanol production [6]. Studies had been carried out on the purification and characterization of cellulase from termites *Reticulitermess peratus* [17], *Ametermes eveuncifer* [18] *Hodotermopsiss jostesti* and *Nasutitermes takasagoensis* [19] but a limited study on the kinetic studies of immobilized cellulase from termites has been done. This study revealed and confirms the presence of cellulase in *Macrotermes bellicosus* and could be used as a local source of the enzyme. The result obtained for partial purification of cellulase by size-exclusion chromatography (Figure 1) agrees with the work of Paul., *et al.* [11] who partially purified and characterized cellulase from the digestive track of African giant snail. A single protein peak was observed on ion-exchange elution profile (Figure 2) with decrease in total protein from 0.082 to 0.018mg, thus a decrease in the total Enzyme activity by 25.0% (Table 1). This result is comparable with the work of Fagbohunka., *et al.* [20] who reported the purification and characterization of cellulase from *Ametermes eveuncifer* with decrease in enzyme activity.

Calcium alginate beads is an excellent substrate for immobilization. Scanning electron microscope revealed a compact pores on the surface of the beads having no entrapped enzyme as shown in figure 3a, whereas, for the cellulase immobilized beads morphological changes and presence of small particles with irregular ridges were observed on the surface as shown in figure 4.3b**.** This result also corroborates with the work of Muhammad., *et al.* [21] who reported some morphological changes on the surface of immobilized maltase on calcium alginate bead. Immobilized cellulase retained more than 80% and 70% recycling efficiency after 3rd and 4th cycle. And also more than 65% was observed even after 5th recycle as shown in figure 4. Therefore this reusability process reduces the associate cost of low amount of enzymes used in industries [22]. This result is similar to the finding of Viet., *et al.* [23] who also immobilized cellulase on calcium alginate.

The production of different compounds from raw materials requires a proper choice of process and optimal enzyme reaction conditions. In this study we found out that free cellulase from *M. bellicosus* has an optimum pH of 6 which increased to pH 8 when the enzyme is immobilized on calcium-alginate beads (Figure 5), this finding corroborates with the work of Watanabe [17] who reported cellulase of Japanese subterranean termite (*Reticultermes speratus)* with an optimum pH of 6 and Viet., *et al.* [23] who reported immobilized cellulase with an optimum pH 8. In contrast, Simon., *et al.* [24] reported that optimal pH of both free and immobilized cellulase remained the same when poly (methyl methacrylate) nanoparticles were employed for immobization of cellulase. The higher pH value of the immobilized enzymes is caused by residual charges on the solid matrix and the nature of the bound enzyme; this could be utilized by textile industries due to its high alkalinity [25].

Free and immobilized cellulase are more stable at pH 6 and 8 respectively, as such, immobilized cellulase exhibited better stability in the neutral and basic medium than free enzyme. This result agreed with the work of Rahnama., *et al.* [26] who reported that cellulase is more stable at pH 5.8 and Zhou., *et al.* [27] who reported greater stability of the immobilized cellulase at pH 8. Therefore, immobilized cellulase is more suitable for industrial applications.

The optimum temperature of 50ºc and 60ºc for free and immobilized *M. bellicosus* cellulase (Figure 3) are within the range of 50^0C-70^0C reported earlier for immobilized cellulase [23]. The higher temperature profile of the immobilized cellulase could be as the result of lower temperature in the gel microenvironment compared to the bulk solution [28].

Similarly, both free and immobilized cellulase are more stable at their respective optimum temperatures of 50ºC and 60ºC-70ºC as shown in figure 4. The immobilized cellulase retained 100% of its initial activity at 60°C while free cellulase maintained not more than 84% of its initial activity at the same condition but decreases slowly when the enzyme was incubated at temperature values higher or lower than the optimum temperature for the enzyme stability.

The effect of metal ion on the free and immobilized cellulase was investigated. The results showed that $Ca^{2+}$and Mn^{2+} stimulated cellulase activity, while Na^{2+} slightly decrease its activity, but Zn^{2+} and Mg^{2+} were found to be strong inhibitors of free and immobilized cellulase respectively as compared with control (Figure 9). The results obtained in this work corroborate with the work of Fagbohunka., *et al.* [20] who reported that chloride salts of Mg^{+}, Na^{+}, Zn^{+}, greatly inhibited cellulase activity from *A. eveuncifer* soldiers at 10Mm concentration.

The initial velocity using cellulose as substrate at different concentration revealed that Vmax value for the free cellulase was 1.593 unit/ml whereas its K_m value was 7.953 mg/ml while the V_{max} value for the immobilized cellulase was 1.154 unit/ml and K_m is 3.455 mg/ml (Table 2). The decrease in V_{max} of the immobilized cellulase enzyme may be due to the lower accessibility of the substrate to the active site of the immobilized cellulase.

Conclusion

Immobilization of partially purified cellulase from *M. bellicosus* extracted by entrapment in calcium alginate beads enabled enzyme reusability for up to five (5) cycles, without losing significant catalytic activity. The characterization of free and immobilized partially purified cellulase revealed the optimum working conditions of the enzyme with better stability when immobilized on calcium alginate beads which would help in the use of the enzyme in various industries such as food, textile, detergent industries, etc.

Recommendation

The results obtained shows partially purified cellulase from *M. bellicosus* when immobilized on calcium alginate could be used for the degradation of cellulosic materials. Therefore further investigation should be carried on the purification and characterization of cellulase from different species of termites. The ability of immobilize cellulase from *M. bellicosus* to be used in bioethanol production should be evaluated.

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Volume 2 Issue 6 June 2021