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Research Article

Effect of Metal Ions on the Activity of Cellulase Produced by *Aspergillus niger* Using *Arachis hypogaea* Shells

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ABSTRACT

Cellulases have attracted much interest because of the diversity of their applications but the major constraint against the use of cellulase is the high cost of production. There is need for certain parameter to be optimized as well as some cofactors that will enhance cellulase production. Therefore, this study investigated the effect of metal ions on the activity of cellulase produced by *Aspergillus niger* using *Arachis hypogaea* shells with a view to increase cellulase production. The kinetic properties (K_m and V_{max}) of cellulase in the presence of some cations (Na^+, K^+, Mg^{2+}, Cu^{2+}, Zn^{2+}, Ca^{2+}, Fe^{2+}, Mn^{2+}, Co^{2+}) and anions (CO_3^{2-}, Cl^-, and SO_4^{2-}) were investigated. The presence of anions (CO_3^{2-}, Cl^- and SO_4^{2-}) decreased the activity of cellulase. Cations like Na^+ activated cellulase activity at a concentration above 1 mM while K^+ did not affect the cellulase activity. Divalent cations such as Mg^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+} and Fe^{2+} inhibited the activity of enzyme. Mn^{2+} and Co^{2+} enhanced the activity of purified cellulase at all the concentrations investigated. Fitting of the data to Michaelis-Menten kinetics showed that Mg^{2+} competitively inhibited cellulase (K_m = 0.12 mg/ml and V_{max} = 1.66 U/ml) while Zn^{2+}, Cu^{2+}, Ca^{2+} and Fe^{2+} noncompetitively inhibited cellulase. Cellulase was also inhibited by mercaptoethanol and some surfactants such as DMSO, Triton X and Tween 20. The present study has shown that divalent cations such as Mg^{2+}, Zn^{2+} and Cu^{2+}, Ca^{2+} and Fe^{2+} inhibited the activity of enzyme while Mn^{2+} and Co^{2+} enhanced the activity of purified cellulase at all the concentrations investigated.

Keywords: *Cellulase, Aspergillus niger, Arachis hypogaea, Metal ions, Characterization*

INTRODUCTION

Cellulases (EC 3.2.1.21) refer to a class of enzymes that catalyze hydrolysis of cellulose and related cellu-oligosaccharide derivatives (Ire and Berebon, 2016). It is a multienzyme complex composed of endoglucanase, exoglucanase and β-glucosidase (Singh *et al*., 2019). Cellulases have a wide range of applications in the textile, detergent, food, pulp and paper industry as well as in biofuel production (Abushe, 2018). The high cost of enzyme production is the main constraint against comprehensive application of cellulase. Cellulase complex act serially or synergistically to hydrolyzes cellulose. Currently, there are two main methods by which cellulose can be converted to glucose: chemical and enzymatic methods. Unlike chemical methods, enzymatic hydrolysis seems to be preferable for hydrolysis of lignocelluloses because it offers an attractive method and relatively pure products can be obtained from the hydrolytic process (Adeleke *et al*., 2012). Several celluloses exist as wastes and such include wheat straw, corn cobs, wood wastes, peat, bagasse and waste paper (Quadri *et al*., 2017). Current research on cellulase is mainly focused on characterization of cellulases. Mawadza *et al*. (2000) reported that metal ions K^+, Na^+, Mg^{2+}, Cu^{2+}, Ca^{2+}, Ni^{2+}, Zn^{2+} and Fe^{2+} had little effect on the activity of cellulase at 1 mmol/l whereas the effect of Co^{2+} was significant at the same concentration. Cellulase activity was inhibited by 10 mmol/l of Cu^{2+}, Zn^{2+}, Co^{2+} or Pb^{2+} when carboxymethyl cellulose (CMC) was used as substrate (Murashima *et al*., 2002). In addition, metal ions, notably Ag^+, Hg^{2+} and Mn^{2+}

showed a tendency to inhibit cellulase activity (Tovar-Herrera *et al*., 2018). In the present study, the effects of these metal ions and anions on the activity of purified cellulase produced by *Aspergillus niger* cultured on *Arachis hypogaea* shells were investigated at different concentrations, and the enzyme kinetics parameters were also determined so as to postulate the possible mechanism of action.

MATERIALS AND METHODS

Materials

The substrates (*Arachis hypogaea* shells) were collected from Oja-Tuntun, Ilorin, Kwara State, Nigeria and it was identified and authenticated at the herbarium unit of the Botany Department, University of Ilorin, where a voucher number of UILH/001/156 was assigned. *Aspergillus niger* was obtained from microbial culture collection of Microbiology Department, University of Ilorin, Nigeria.

Carboxymethyl cellulose (CMC) was obtained from BDA Chemicals Ltd., Poole, England. 3,5-Dinitrosalicyclic acid (DNS) was obtained from Lab. Tech. Chemicals, Avighkar, India; Sodium hydroxide, Sodium Potassium Tatarate (Rochelle salt), Sodium dihydrogen phosphate, disodium hydrogen phosphate, NaCl, KI, $MgSO_4$, $ZnSO_4$, $CuSO_4.5H_2O$, $CaCl_2$, $FeSO_4$, $MnCl_2.H_2O$ and $CoCl_2.6H_2O$ were of analytical grade and were ordered from Santa Crux Biotechnology (Germany). Triton X and Tween-20 were obtained from Sigma-Aldrich Chemical Co., Uk.

Production and Purification of Cellulase

The optimization parameters for cellulase production, purification and characterization of cellulase were carried out as reported in Sulyman *et al*. (2020).

Effect of Cations on the Activity of Purified Cellulase

The effect of various cations on activity of the purified cellulase was studied using the various concentrations of salts in the reaction systems. Each salt of the following cations i.e. are NaCl, KI, $MgSO_4$, $ZnSO_4$, $CuSO_4.5H_2O$, $CaCl_2$, $FeSO_4$, $MnCl_2.H_2O$ and $CoCl_2.6H_2O$ was prepared in the concentrations of 0.05, 0.1, 0.2, 0.5, 0.75 and 1 mM.

Briefly, 0.5 ml of varying concentrations (0.05 – 1 mM) of each cation was incubated with 0.1 ml of purified cellulase. The mixture was incubated for 30 minutes at 50°C. Thereafter, 3 ml of 3,5-dinitrosalicyclic acid (DNS) solution was added and the mixture was placed in boiling water for 5 minutes. The mixture was then allowed to cool and 5 ml of distilled water was added. The absorbance was measured at 540 nm.

Effect of Anions on the Activity of Purified Cellulase

The effect of some anions like CO_3^{2-}, Cl^- and SO_4^{2-} were also investigated on the activity of purified cellulase. Briefly, 0.5 ml of varying concentrations (0.05 – 1 mM) of each salt of anion was incubated with 0.1 ml of purified cellulase. The mixture was incubated for 30 minutes at 50°C. Thereafter, 3 ml of 3,5-dinitrosalicyclic acid (DNS) solution was added and the mixture was placed in boiling water for 5 minutes. The mixture was then allowed to cool and 5 ml of distilled water was added. The absorbance was measured at 540 nm. The blank was set up by replacing the enzyme with equal volume of distilled water.

Effect of Some Surfactants on the Activity of Purified Cellulase

The effect of some surfactants such as DMSO, Triton X, Tween 20 and Mercaptoethanol at the concentrations of 0.05, 0.10, 0.20, 0.50, 0.75, and 1.0 mM were investigated on the activity of purified cellulase. Briefly, 0.1 ml of varying concentrations of each of the surfactants was added to 0.1 ml of purified cellulase. The reaction mixture was incubated for 15 min at 55°C. Then, 3 ml of 3,5-dinitrosalicyclic acid (DNS) solution was added to stop the reaction and the mixture was placed in boiling water for 5 min. The mixture was then allowed to cool and 5 ml of distilled water was added. The absorbance was measured at 540 nm.

Assay of Cellulase Activity

Briefly, 0.5 ml of substrate solution (1 % CMC) prepared in 0.5M citrate buffer (pH 4.8) was added to 0.1 ml of enzyme source in a test-tube and mixed well. The tubes were incubated at 50 °C for 30 minutes. Then 3 ml of 3,5-dinitrosalicyclic acid (DNS) solution was added to each tub to stop the reaction and the tubes containing the mixture were placed in boiling water for 5 min. The tubes were then allowed to cool, and 5 ml of distilled water was added. The absorbance was measured at 540 nm. The amount of reducing sugar obtained was extrapolated from glucose standard curve. One unit of endo-β-1, 4-glucanase activity is defined as the amount of enzyme that could hydrolyze CMC and release 1 µg of glucose within 1minute reaction at 50 °C (Abdelraof *et al*., 2019).

Statistical Analysis of Data

All experiments and enzyme assays were performed in triplicates and the results were expressed as mean ± SEM. Data obtained were subjected to one-way analysis of variance and means found to be significantly different at $P < 0.05$ were separated by Duncan Multiple Range Test. Graphpad prism version 6.02 was used to plot all the graphs

RESULTS AND DISCUSSION

Results

The effect of monovalent cations (Na^+ and K^+) is presented in Figure 1. The Na^+ at concentrations between 0.75 – 1.00 mM stimulated the activity of purified cellulase with about 1.48 folds increase when compared to control while K^+ at all the concentrations investigated (0.05, 0.1, 0.2, 0.5, 0.75 and 1.0 mM) inhibited endoglucanase activity of purified cellulase. Divalent metal ions such as Mg^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+} and Fe^{2+} inhibited the activity of purified cellulase at all the concentrations investigated as compared to the control while Mn^{2+} and Co^{2+} stimulate the activity of purified cellulase at all the concentrations investigated (Figure 2). Addition of Mg^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+} and Fe^{2+} to the enzyme solution at 1.0 mM resulted to 83 %, 64 %, 64 %, 97 % and 99 % loss of activity respectively.

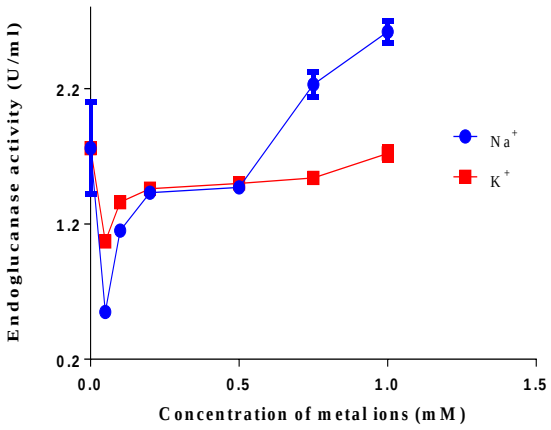

Figure 1. The Influence of Na^+ and K^+ on Endoglucanase Activity.

Each value is expressed as mean ± SEM of three different determinations.

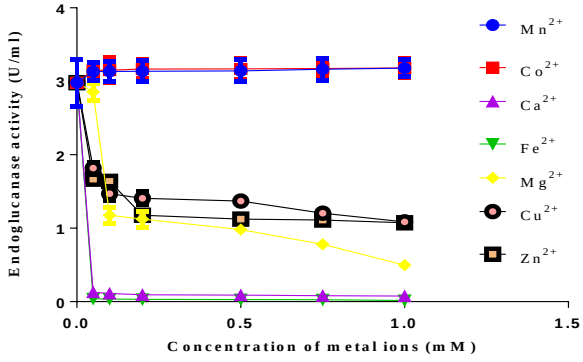

Figure 2. Effect of Divalent Cations (Mn^{2+}, Co^{2+}, Ca^{2+}, Fe^{2+}, Mg^{2+}, Cu^{2+}, and Zn^{2+}) on the Activity of Purified Cellulase.

Each value is expressed as mean ± SEM of three different determinations.

The Lineweaver-Burk plot of purified cellulase catalyzed hydrolysis of carboxylmethylcellulose in the presence and absence of Mg^{2+} is shown in Figure 3. The value of K_m in the presence of Mg^{2+} was 0.1176 mM which was greater than the value obtained in the absence of Mg^{2+} (0.0212 mM). The V_{max} of the purified cellulase in the presence and absence of Mg^{2+} was approximately 1.66 U/ml.

The maximum rate of reaction (V_{max}) was 1.418 U/ml in the presence of Zn^{2+} with K_m of approximately 0.02 mM. However, the V_{max} of purified cellulase was found to be 1.66 U/ml with K_m of 0.02 mM in the absence of Zn^{2+} (Figure 4). The Lineweaver-Burk plot of purified cellulase in the presence and absence of Cu^{2+} is presented in Figure 5. The V_{max} in the presence of Cu^{2+} was 1.478 U/ml with a K_m value of 0.02 mM. The absence of Cu^{2+} resulted in an increase in V_{max} value to 1.620 U/ml and the K_m value remained unaffected.

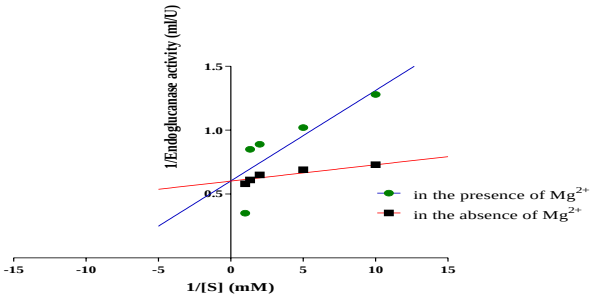

Figure 3. Lineweaver-Burk Plot of Cellulase Catalyzing the Hydrolysis of Carboxylmethylcellulose in the Presence of Mg^{2+}.

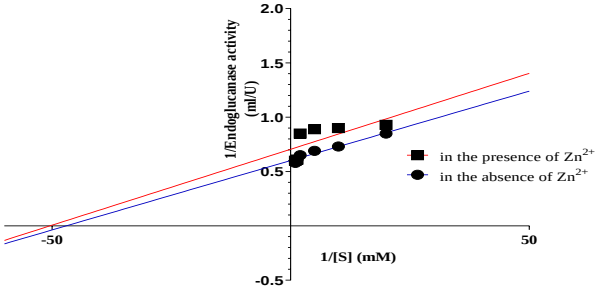

Figure 4: Lineweaver-Burk Plot of Cellulase Catalyzing the Hydrolysis of Carboxylmethylcellulose in the Presence of Zn^{2+}.

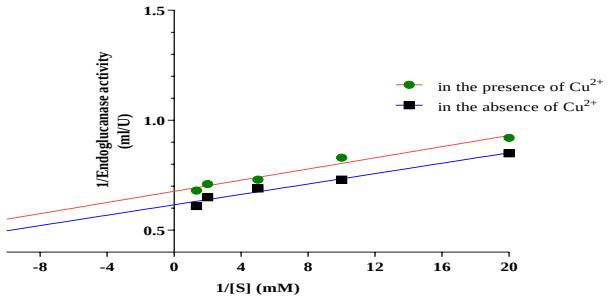

Figure 5. Lineweaver-Burk Plot of Cellulase Catalyzing Hydrolysis of Carboxyl methylcellulose in the Presence of Cu^{2+}.

Similarly, the V_{max} of purified cellulase in the presence of Ca^{2+} (0.095 U/ml) was found to be lower than the value of 1.590 U/ml obtained in the absence of Ca^{2+}. The K_m of purified cellulase in the presence and absence of Ca^{2+} was calculated to be 0.012 and 0.017 mM respectively (Figure 6). Figure 7 shows the Lineweaver-Burk plot of purified cellulase in the presence and absence of Fe^{2+}. The same value (0.02 mM) of K_m in the presence and absence of Fe^{2+} was obtained. However, the V_{max} of the purified cellulase in the presence and absence of Fe^{2+} differs with values of 0.039 and 1.663 U/ml respectively. The summary of K_m and V_{max} for each of the metal ions was presented in Table 1.

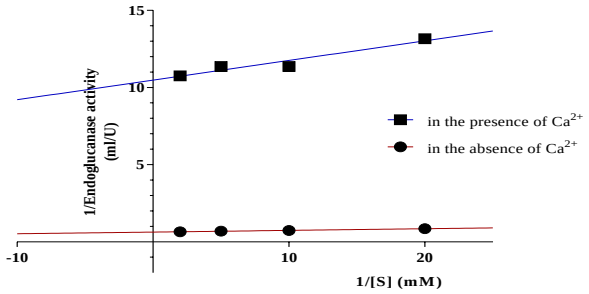

Figure 6. Lineweaver-Burk Plot of Cellulase Catalyzing the Hydrolysis of Carboxylmethylcellulose in the Presence of Ca^{2+}.

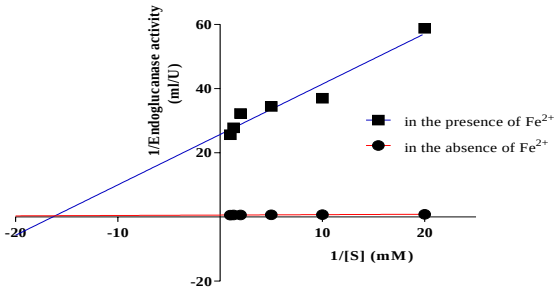

Figure 7: Lineweaver-Burk Plot of Cellulase Catalyzing the Hydrolysis of Carboxylmethylcellulose in the Presence of Fe^{2+}.

Table 1. Effect of Cations on Kinetic Parameters of Purified Cellulase.

Cations	V_{max} (U/ml)	K_m (mM)
Absence of Mg^{2+}	1.663	0.0212
1 mM Mg^{2+}	1.660	0.1176
Absence of Zn^{2+}	1.660	0.020
1 mM Zn^{2+}	1.418	0.020
Absence of Cu^{2+}	1.620	0.020
1 mM Cu^{2+}	1.478	0.024
Absence of Ca^{2+}	1.590	0.017
1 mM Ca^{2+}	0.095	0.012
Absence of Fe^{2+}	1.663	0.020
1 mM Fe^{2+}	0.039	0.0612

The effects of some anions (CO_3^{2-}, Cl^- and SO_4^{2-}) on the activity of purified cellulase are presented in Figures 8. The presence of Cl^- and SO_4^{2-} at 0.1 and 0.2 mM respectively activated cellulase enzyme with about 2.9 and 3.2 folds increase in the activity though the activity of cellulase dropped drastically as concentration of Cl^- and SO_4^{2-} increased. There was a loss of activity of about 32 % in the presence of 1 mM Cl^-. As observed from the result, there was a decrease in the activity of cellulase in the presence of CO_3^{2-} at all the concentrations investigated. Addition of CO_3^{2-} inhibited the activity of partially purified cellulase with about 95 % loss of activity. Also, the concentrations above 0.2 and 0.5 mM in the presence of Cl^- and SO_4^{2-} respectively inhibited the cellulase (Figure 8).

The effect of DMSO, Triton X-100, Tween-20 and Mercaptoethanol on endoglucanase activity of purified cellulase is shown in Figure 9. The addition of DMSO decreased the activity of purified cellulase and this resulted in about 49 % loss of activity. Also, addition of Triton X-100, Tween-20 and Mercaptoethanol inhibited the activity of purified cellulase at 0.1 to 1.0 mM concentrations which resulted to 31 %, 85 % and 98 % loss of activity respectively.

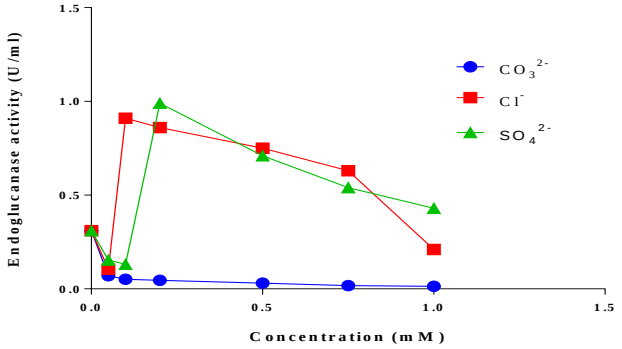

Figure 8: Effect of Some Anions on Endoglucanase Activity of Purified Cellulase.

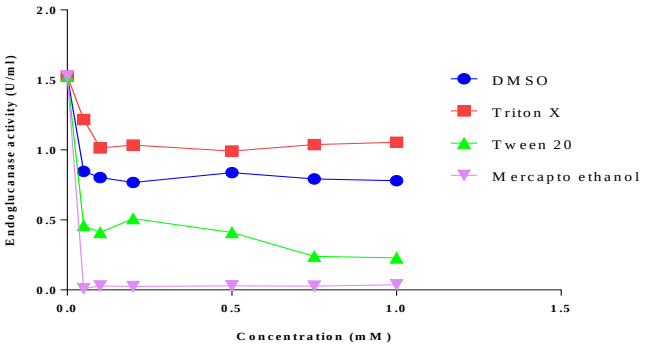

Figure 9. Effect of DMSO, Triton X-100, Tween-20 and Mercaptoethanol on the Activity of Purified Cellulase.

Discussion

Cations may influence the activity of enzymes positively or negatively. Some metal ions may act as cofactors that stimulate the activity of enzymes while some may act as inhibitors. For example, pyruvate kinase, dialkyllglycine decarboxylase, diol dehydrogenase, inosine monophosphate dehydrogenase are all potassium-activated enzymes. Also, alkaline phosphatase is a magnesium-activated enzyme. In some enzymes, the activated cation is near the active site of the enzyme while in others, it is distant. The activity of purified cellulase was inhibited by K^+ at all the concentrations investigated. Also, Na^+ at a concentration above 0.5 mM, stimulated the activity of purified cellulase. This implies that for effective catalysis, concentrations above 0.5 mM will be required for cellulase obtained from *A. niger* cultured on *A. hypogaea* shells. This result contradicts the report of Wang *et al*. (2012) who reported that Na^+ and K^+ showed little or no effect on the activity of purified cellulase. The activity of purified cellulase was inhibited by Mg^{2+}, Cu^{2+} and Zn^{2+}. Findings in this study agreed with Sanwal (1999) and Wang *et al*. (2012) who both reported inhibitory effect of Mg^{2+}, Cu^{2+} and Zn^{2+} on the activity of cellulase. Also, Ca^{2+} and Fe^{2+} inhibited the activity of cellulase while Mn^{2+} and Co^{2+} stimulated cellulase activity. Stimulatory actions of Mn^{2+} and Co^{2+} have been reported (Irfan *et al*., 2012). The kinetic properties and pattern of inhibition of Mg^{2+} differs from other divalent cations like Cu^{2+}, Zn^{2+}, Ca^{2+} and Fe^{2+}. Based on the results obtained in this study, Mg^{2+} inhibited cellulase competitively. The active site of an endoglucanase contains two conserved glutamate residues at the active site (Tambalo *et al*., 2020). Divalent cation such as Mg^{2+} may bind to this conserved glutamate residue to influence cellulase activity. Mg^{2+} may bind with electronegative glutamate residues in a reversible manner, giving rise to changes in the enzymatic catalytic activity and compete with substrate for the active site. Unlike Mg^{2+}, other divalent cations like Cu^{2+}, Zn^{2+} and Fe^{2+} inhibited the purified cellulase in a non-competitive manner. In the presence of Cu^{2+}, Zn^{2+} and Fe^{2+}, the V_{max} values were lowered while K_m values remained approximately unchanged. In a noncompetitive inhibition, the inhibitor binds to an enzyme at a site other than the active site (Amaly *et al*., 2018). Ca^{2+} inhibited the purified cellulase in an uncompetitive manner. In uncompetitive inhibition, the inhibitor binds to enzyme-substrate complex without binding to free enzyme.

The results obtained from this study revealed that the activity of cellulase was increased at low concentrations of Cl^- and SO_4^{2-}, enzyme activity was completely inhibited in the presence of CO_3^{2-}. It can be proposed that Na_2CO_3 might not be suitable for pretreating lignocellulosic biomass. The inactivation of cellulase by anioins can simply be attributed to the binding of these anioins to some critical catalytic site of the cellulase. The result obtained from this study agreed with the findings of Wang *et al*. (2012) who reported a decreased in cellulase activity in the presence of an increased concentrations of Cl^-, SO_4^{2-}, and CO_3^{2-}.

The surfactants are believed to be a significant environmental factor that affects the activity of enzymes. Surfactants are used extensively for solubilizing protein from lipid membranes and other biological materials, and for maintaining the solubility of certain proteins in the solution (Ibraheem *et al*., 2017). Surfactants are routinely used in cell lysis buffers to inhibit unwanted proteolysis and facilitate membrane protein solubilization in protein purification procedures. This study reveals the effect of non-ionic surfactants on the activity of purified cellulase from *A. niger* cultured on *A. hypogaea* shells. Thus, Triton X-100 and Tween-20 inhibited the activity of cellulase at all concentrations investigated. The implication of this finding is that the purified cellulase may not be stable in the presence of these surfactants. Different effect of anionic surfactants on the activity of enzymes has been reported (Shaheen *et al*., 2017). Anionic surfactants such as DMSO and mercaptoethanol showed inhibitory effect on the activity of purified cellulase.

CONCLUSION

The present study has shown that divalent cations such as Mg^{2+}, Zn^{2+} and Cu^{2+}, Ca^{2+} and Fe^{2+} and CO_3^{2-} inhibited the activity of enzyme while Mn^{2+} and Co^{2+} enhanced the activity of purified cellulase at all the concentrations investigated. Also, non-ionic detergents such as triton X and tween-20 inhibited the activity of cellulase at all concentrations investigated.

AUTHORS' CONTRIBUTIONS

All authors contributed to the study conception and design. The material preparation, data collection and analysis were performed by author AOS. The first draft of the manuscript was written by author AI and the design was done by author SOM. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declared no conflict of interest

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