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Effect of Metal Ions and Enzyme Inhibitor on the Activity of Cellulase Enzyme of *Aspergillus flavus*

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Abstract— *Natural by- product of agricultural waste can be turned to products of commercial interests such as glucose, ethanol and single cell protein. Much effort from scientists and researches all around the world has been put to extend the full use of agricultural waste. Reports of cellulase enzyme production from the bioconversion of lignocellulosics materials has much been made. However there is still much space to find the most suitable condition by studying the effect of the various metal ions in the activity of the enzyme. In this research different metal ions were added to the enzyme reaction mixture in 1-5 mM amounts, incubated at room temperature and then used to carry out enzyme assay using CMC, Filter paper and cotton wool. The purpose of this research was to investigate the effect of various metal ions on the activity of the cellulase enzyme. Result shows that only Fe^{2+} had a pronounced stimulating effect ($P<0.05$) on the enzyme activity in all the substrates at 1mM concentration respectively. This was followed by Cu^{2+} in CMC but which was also found to be inhibitory when cotton wool and filter paper were used as substrates. However, other divalent metals were found to have either slight or appreciable inhibitory effects on the enzyme activity. According to these results, these ions must be avoided in future cultivations for a high cellulase production.*

Keywords— Metal Ions, Enzyme Inhibitor, Cellulase Enzyme, Enzyme Activity, Aspergillus flavus.

I. INTRODUCTION

Currently, most commercial cellulases (including β-glucosidase) are produced by Trichoderma species and Aspergillus species (Cherry and Fidantsef, 2003; Esterbauer *et al*., 1991; Kirk *et al*., 2002). Cellulases are used in the detergent market for color care and cleaning, in the food industry for mashing; in the paper and pulp industries, textile industry, drainage improvement, and fiber modification (Cherry and Fidantsef, 2003; Kirk *et al*., 2002). The cellulase market will enlarge when cellulases are used to hydrolyze pretreated cellulosic materials to glucose, which can be fermented to products including bio-based products on a large scale (Cherry and Fidantsef, 2003; Himmel *et al*., 1999; van Beilen and Li, 2002). The large potential and the importance of cellulases in bio-based product industries will stimulate interest for the development of better cellulase preparations for cell wall cellulose hydrolysis. These improved cellulases are expected to have properties necessary for bio-refineries, such as higher catalytic efficiency on insoluble cellulosic substrates, increased stability at elevated temperature and at an optimal pH, and improved tolerance to end-product inhibition.

II. MATERIALS AND METHODS

2.1 Study area

The work is carried out at the Department of Microbiology, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria. Awka is the capital city of Anambra state, which lies within the southern part of Nigeria. The geographical coordinates of Awka corresponds to 6.22 North and 7.07 East and falls within the humid tropics of Nigeria. The town Awka was made after clearing much of the tropical grassland, and outskirts of the city are still covered with grassland. It has a moderate climate with a very high temperature during the dry season and average rainfall during the rainy season. Awka has the mean annual temperature and precipitation of 35°C and 1117mm, respectively (NIMET, 2006).

Materials and Methods

The materials used in this research include reagents, salts, solvents, resins, substrates media among others. Most of the materials were kindly provided by Professor F. J. C. Odibo of Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria, while others were either obtained from the Research Laboratory or purchased from FinLab. Ltd., Enugu.

Experimental Design

Generally, to ensure accuracy, most parameters were measured three times and the mean taken as the value of the parameter. Indices that were measured on graded levels were

statistically analyzed using one way analysis of variance (ANOVA) and the differences between treatment means were separated using Duncan's New Multiple Range Test (DNMRT).

Also, data collected were presented in graphs and histograms to increase clarity. Other descriptive statistics such as range, intervals and the like were employed were necessary.

Methods

Samples of rotten wood and compost were collected from Botanical garden of Nnamdi Azikiwe University, Awka, Anambra State, Nigeria. The samples were pulverized and shaken in distilled water, and filtered using white cloth. A drop of each of the filtrate was placed on Czapex Dox medium to which 1 % carboxymethylcellulose (CMC) of low viscosity (BDH) was incorporated and spread. It was then incubated for 48 h at room temperature (28-30 °C.)

A total of seven dominant colonies were isolated and purified by successive subculture on fresh Czapex Dox medium. The cellulolytic activities of the colonies were determined by point inoculation of each fungal isolate on Czapex Dox-carboxymethyl cellulose medium and incubated for 72 h. After the incubation, zone of clearing which is an indication of cellulolysis was detected by flooding the cultures with 0.5 % Congo- red solution for 15 min. and distaining with 1M sodium chloride for 10 min (Teather and Wood, 1982). The zones were measured and result recorded.

Identification of the Fungal Isolates

The colonies with the highest zone of clearance were observed by slide culture technique under the microscope with the aid of methylene cotton blue stain, for the characteristic morphological features using standard reference manuals (Ellis, 1976; Raper and Fennel, 1965).

Screening for Cellulase Production

The selected isolates were cultivated in a Mandel and Weber (1969) medium containing the following in g/l:

$(NH_4)_2S0_4$	1.4
KH_2P0_4	2.0
Urea	0.3
$MgS0_47H_20$	0.3
$CaCl_2$	0.3
$FeS0_47H_20$	0.005
$ZnS0_47H_20$	0.0014
$MnS0_4H_20$	0.0016
$CoCl_2$	0.002
Tween 80	2.0 ml
Carboxymethylcellulose	10.0
pH	6.8

A loopful of conidia was inoculated into 100 ml of the sterilized medium in a 500 ml flask and incubated at 35 ^{0}C on a Stuart orbital shaker model S150 for 7 days at 200 rpm. After the incubation, the broth culture was subjected to centrifugation at 4000 rpm for 20 min using Centurion Centrifuge to remove the mycelia and other insoluble materials. The supernatant was recovered and used for the enzyme assays. The isolate with the highest enzyme activity was then selected and used for further studies.

Enzyme Purification

Concentration of the Supernatant by Dialysis

A 5 M sucrose solution was prepared and 700 ml of the enzyme supernatant contained in dialysis bag were suspended in the solution and left overnight at 4 ^{0}C until a considerable reduction in the volume of the supernatant was achieved.

The concentrated enzyme solution (100 ml) was recovered and the enzyme assay repeated using CMC, filter paper, cotton wool and crystalline cellulose. The protein content of the concentrated crude enzyme was also determined using Bradford's method.

Ion-exchange Chromatography

A column (18x1.5 cm), packed with Q-Sepharose fast flow was set up and washed with 150 ml of 0.2 M phosphate buffer (pH 6.8) to equilibrate the resin to the buffer pH. Then, 20 ml of the enzyme concentrate was applied and allowed to permeate the column and then washed down with the same buffer. Fractions were then collected with test tubes calibrated to 10 ml volume at the flow rate of 0.5 ml/ min at room temperature. After collecting 17 fractions, a sodium chloride gradient (0-0.5M) was applied and further fractions collection continued until a total of 40 fractions were collected.

The fractions were subjected to protein estimation by measuring absorbance at 280 nm using an Eppendorf biophotometer and the values plotted against the fraction numbers. The fractions were assayed for enzyme activity using CMC, filter paper and cotton wool. The fractions with pronounced activity were pooled and concentrated as earlier described. The enzyme concentrate was assayed again and protein content determined by Bradford (1976) method.

Gel filtration using Sephadex G-200

The enzyme concentrate was subjected to gel filtration using Sephadex G-200 resin packed in a column (1.8 x 44 cm). The column was equilibrated with 50 ml of phosphate buffer pH 6.8. Fractions were then collected in 5 ml volume at the flow

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rate of 0.5 ml/ min and a total of 43 fractions were collected. These were subjected to protein estimation by measuring absorbance at 280 nm using an Eppendorf biophotometer.

The fractions with observable protein peaks were used for enzyme activity on CMC, filter paper and cotton wool.

Metal ions and Enzyme Inhibitor EDTA

The effect of metal ions of the following salts; Magnisum sulphate ($MgS0_4.7H_20$), calcium chloride ($CaCl_2$), zinc sulphate ($ZnS0_47H_20$), manganese sulphate ($MnS0_4H_20$), barium chloride (BaCl), ferrous sulphate ($FeS0_47H_20$), copper sulphate ($CuS0_4$), mercuric sulphate ($HgS0_4$), cobalt chloride ($CoCl_2$) and lead sulphate ($PbS0_4$) on the enzyme activity was investigated.

Equal volumes of the purified enzyme and 1mM solutions of the above salts and EDTA in 0.2M phosphate buffer (pH 6.8), were pre incubated at room temperature for 10 min. To assay for enzyme activity 0.1 ml of various substrates which include Cmc, Cotton wool and Filter paper in 0.2 M phosphate buffer (pH 6.8) was added to pre incubated mixture and then incubated at 40 ^{0}C for 30 min, 1h and 24 h, respectively and the reducing sugars quantitated using DNS. The reducing sugar released was estimated by 3, 5-dinitrosalicylic acid method (Miller, 1959) as follows; at the end of incubation, the enzyme reaction was stopped by adding 0.5 ml of 3, 5- dinitrosalicylic acid reagent (BDH). The mixture was placed in boiling water for 10 min, after which it was cooled, and 5 ml distilled water added. The absorbance was then read at 540 nm using the substrate solution treated in the same way as blank to zero the spectrophotometer (JENWAY), model 6405. One unit (IU) of CMCase activity was defined as the amount of enzyme required to liberate 1 µmol of glucose from the substrate under the assay condition.

The control tube having no metal ion or inhibitor was taken as 100 %.

III. RESULTS AND DISCUSSION

Results of cellulase activity in the presence of metal ions revealed that only Fe^{2+} had a pronounced stimulating effect ($P<0.05$) on the enzyme activity in all the substrates (Figures1-3). This was followed by Cu^{2+} in CMC (Figure 1) but which was also found to be inhibitory when cotton wool (Figure 2) and filter paper (Figure 3) were used as substrates. However, other divalent metals were found to have either slight or appreciable inhibitory effects on the enzyme activity. All ions tested had significant effect on cellulase activity. A considerable decrease (>80% inhibition) in activity was observed in the presence of Cu^{2+}, Pb+, Hg+, Ba^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+},Co^{2+} and Zn^{2+}. These ions are commonly cited in the literature as inhibitors for several microbial cellulases [25–27]. Activity is probably inhibited through the attack of certain groups at the active site of the enzyme, for example, the thiol groups, leading to inactivation [25]. According to these results, these ions must be avoided in future cultivations for a high cellulase production. The effect of balance between different metal ion concentrations could be more.

Metal cations such as Ca^{2+}, Mg^{2+}, Fe^{2+}, Co^{2+} and Zn^{2+} were more important than their individual effects. For example, iron is needed for cellulase production, but it is also inhibitory at higher concentration. It has been hypothesized that the metals may prevent some components necessary for induction from leaking out of the cells.

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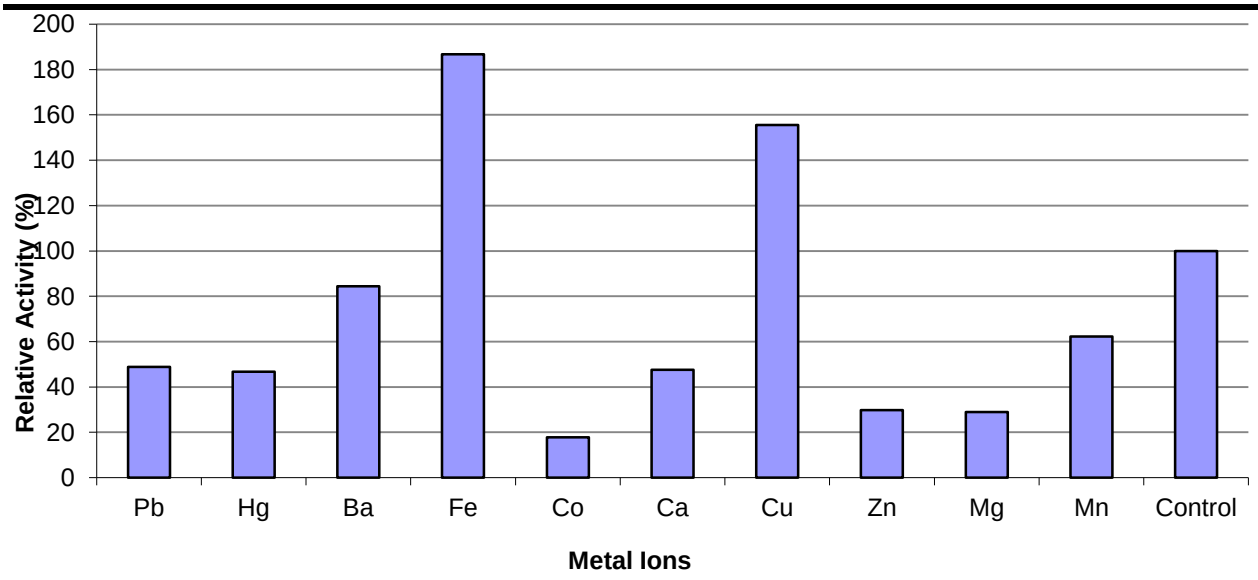

Fig.1: Effect of Metal Ions on the CMC activity of the Cellulase

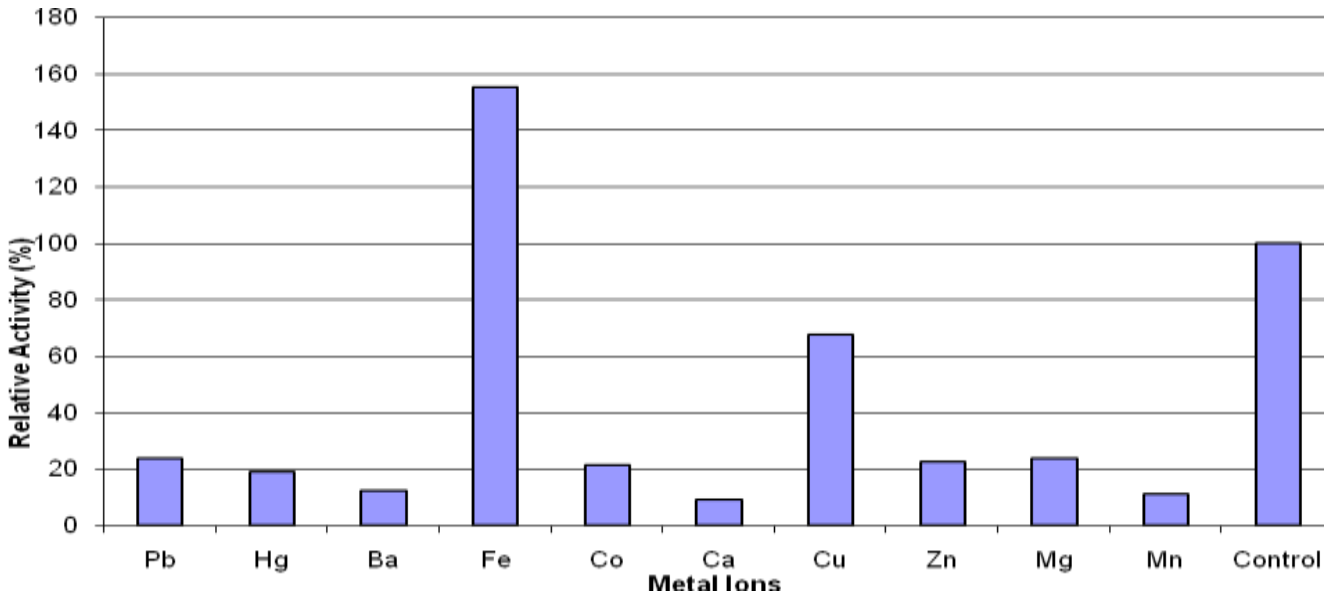

Fig.2: Effect of Metal Ions on Cotton Wool Activity of the Cellulase

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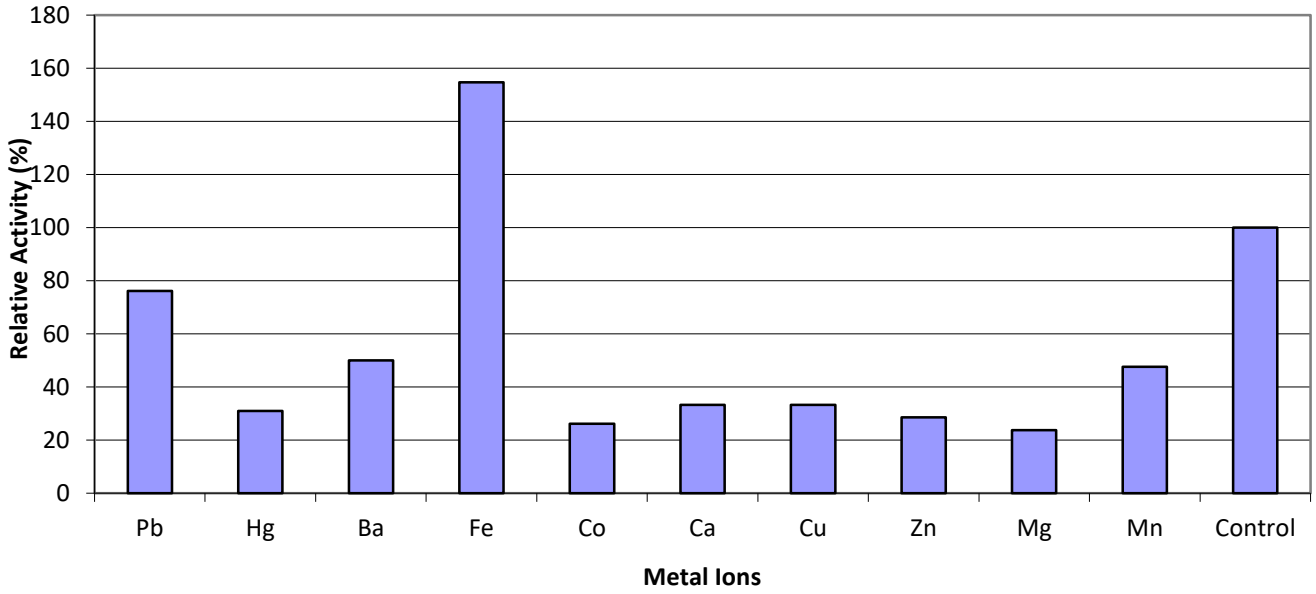

Fig.3: Effect of Metal Ion on Filter Paper Activity of the Cellulase

Effect of different Concentrations of Fe^{2+} on the stability of the enzyme using various Substrates

Varied concentrations of Fe^{2+} exhibited pronounced effects ($P<0.05$) on thermal stability of the *Aspergillus flavus* cellulase both within and across substrates. That is, statistical differences were observed in cellulase activity between various concentrations of Fe^{2+} in a given substrate (Figure 4), and between different substrates (Figure 5). The highest thermal stability of the enzyme was observed at 1 mM concentration of Fe^{2+} for the three substrates studied (CMC, filter paper and cotton wool), and the least thermal stability obtained at 5 mM concentration. In fact, proportionate decline on the stability of the enzyme was recorded as the Fe^{2+} concentration increased, irrespective of the substrate (Figure 4-6).

Again, there was a proportionate increase in thermal stability of the enzyme on filter paper, followed by CMC ($P<0.05$).

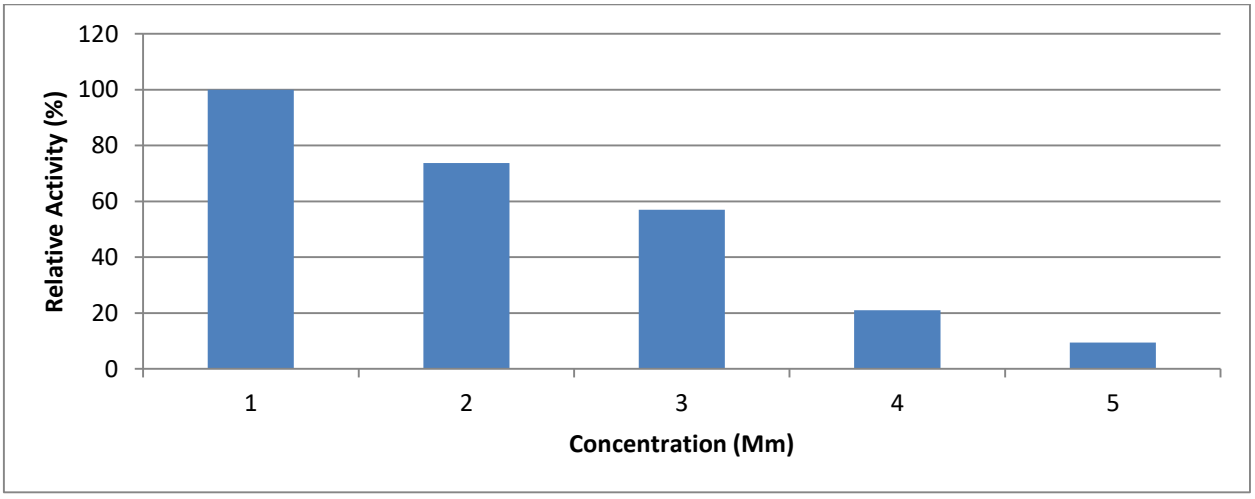

Fig.4: Effect of different Concentration of Fe^{2+} on CMC Activity of the enzyme (Histogram)

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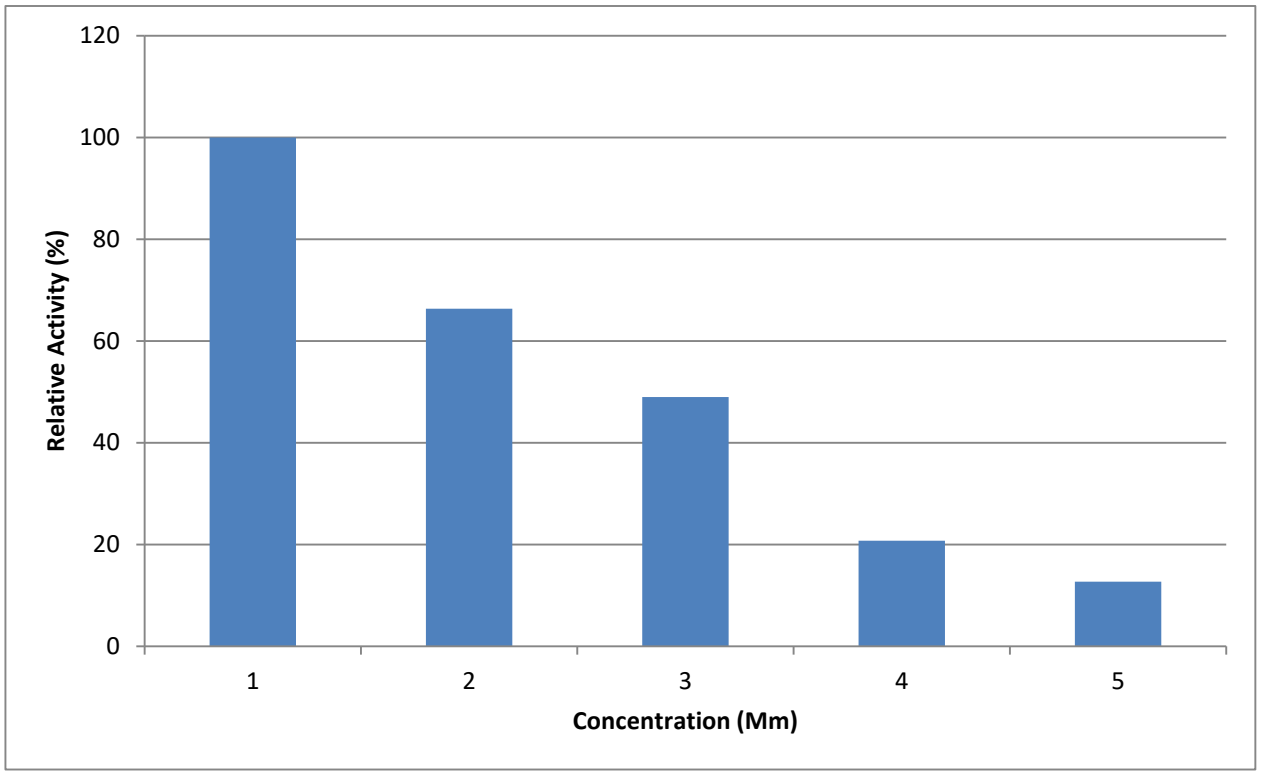

Fig.5: Effect of different concentration of Fe^{2+} on Filter Paper Activity of the enzyme (Histogram)

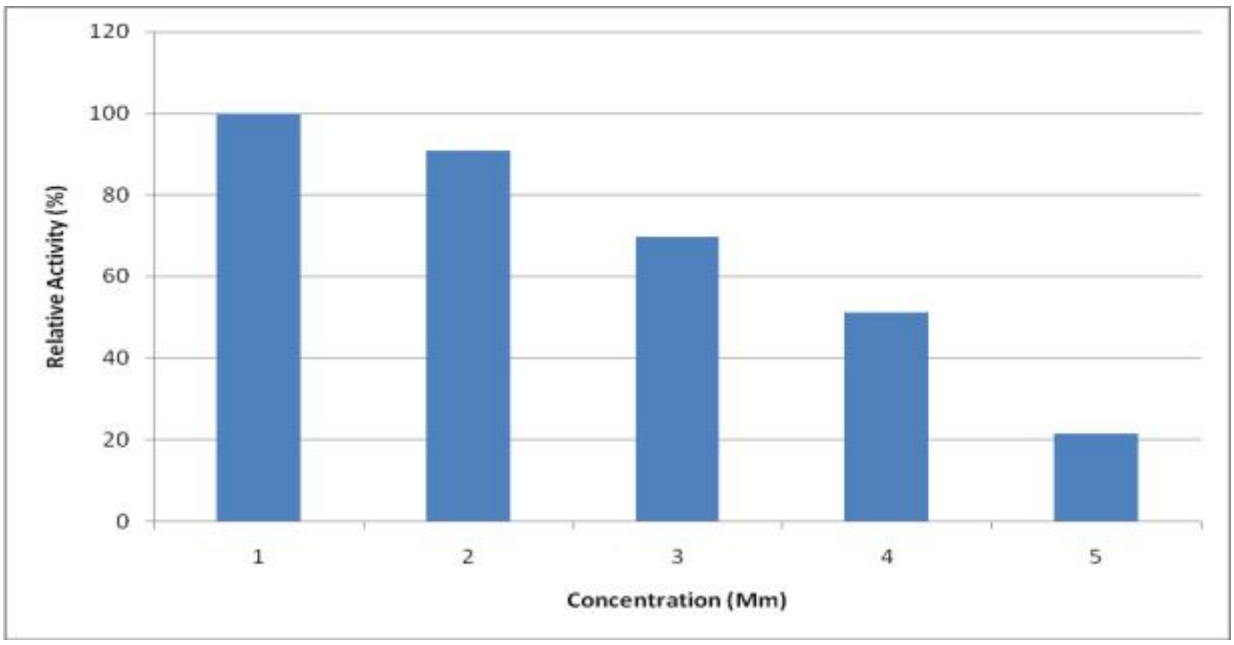

Fig.6: Effect of different concentration of Fe^{2+} on Cotton Wool Activity of the enzyme (Histogram)

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Effect of EDTA on enzyme activity

EDTA had slight inhibitory effect on the cellulase activity (12.4 % in CMC, 38.1 % in filter paper and appreciable effect, 80.9 % in cotton wool) as shown in Figure 7.

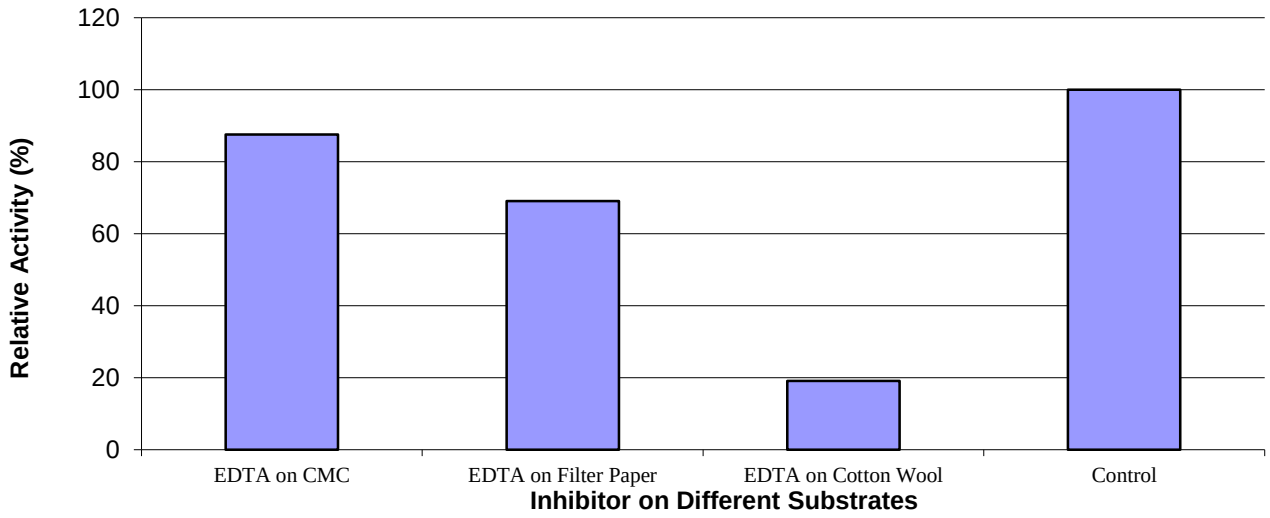

Fig.7: Effect of EDTA on enzyme activity

The role of divalent metals in the stability and activity of this enzyme is confirmed by the reduction of activity in enzyme treated with EDTA which is said to chelate metal ions necessary for enzyme activity and stability. While the exact mechanism of action is still unclear, EDTA is known as an ionic chelator and its inhibition ability indicate that specific ions might be actively involved in the catalytic reaction of the enzyme (Kotchoni *et al*., 2006). It is also deduced that the enzyme sub unit has a structural and catalytic binding sites. The low level activity in EDTA treated samples; probably indicate the important role these metal ions play in the structural integrity and activity of the enzyme. According to Hartley *et al*. (2000) metal ion binding to both sites is essential for the activity of the enzyme.

All the metal ions tested had slight or appreciable inhibitory effects on the purified enzyme which agreed with Liu and Xia (2006) and Petrova *et al*. (2009), except for Fe^{2+} which has the highest stimulatory effect. The implication of Fe^{2+} as a good stimulator of cellulase of *Aspergillus flavus* is in line with the reports of Adams *et al*. (1976) and Shahriarinour1 *et al*. (2011), but disagreed with the finding of El-zawahry *et al*. (2010) who recorded inhibition by Fe^{2+}.

Results suggest that Fe^{2+} apparently protected the enzyme against thermal denaturation and played vital role in maintaining the active form of the enzyme at high temperature (100 ^{0}C). Hence, Fe^{2+} enhances the stability of the enzyme by 55, 86.7 and 54.8 % at 1 mM concentration over values obtained without Fe^{2+}. However, a better understanding of the mechanism of activation of enzyme by divalent ion is needed for clearer understanding of the observation of Fe^{2+} with the cellulase enzyme

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