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*Full Length Research Paper*

# Production, purification and characterisation of a β-mannanase by *Aspergillus niger* through solid state fermentation (SSF) of *Gmelina arborea* shavings

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**Mannanase production by a wild-type *Aspergillus niger*, isolated from spontaneously-degrading wood from South Western Nigeria was monitored at three day intervals for a period of 15 days in media containing pretreated wood shavings of *Anogeissus leiocarpus, Gmelina arborea* and *Terminalia superba* as carbon sources. Highest mannanase activity of 25.938 U/g of dry wood shavings was obtained on the 9th day in a medium containing *G. arborea* as sole carbon source. Maximum activity value for the medium containing *A. leiocarpus* was 14.110 U/g obtained on the 9th day. On the medium containing *T. superba*, a maximum activity value of 10.148 U/g was obtained on the 12th day of incubation. *G. arborea* substrate gave the highest total protein yield of 18.146 mg/ml and the specific mannanase activity of 1.201 U/mg on the crude enzyme extracts. A mannanase activity of 19.091 U/mg was subsequently achieved after purification on ion exchange chromatography with a purification fold of 15.894. Enzyme activities were at their peaks at pH 5.5 (0.4331 U/ml) and at temperature 30°C (1.8617 U/ml). A $K_m$ value of 0.754 mg/ml and $V_{max}$ value of 1.364 U/mg/min was achieved for the enzyme which was thermally stable up to 65°C. *G. arborea* wood shavings hold tremendous potential in mannanase production for several industrial uses by the fungus.**

**Key words:** *Aspergillus niger,* enzyme characterisation, *Gmelina arborea,* mannanase, solid state fermentation, purification.

## INTRODUCTION

Hemicellulose is a complex group of heterogenous polymers and are structural polysaccharides of the plant cell wall found in close association with lignin and cellulose, forming the lignocellulosic biomass. Hemicellulase are the third most abundant constituents of plant cell found in nature and represents 20 to 30% of lignocellulosic dry mass, which makes them one of the major sources of renewable organic matter. Wastes generated from timber and other pulp industries are lignocellulosic in nature and thus forms part of the lignicellulosic biomass (Sjostrom, 1993; Moreira and Filho, 2008).

Mannan, which forms one of the major constituent groups of hemicelluloses in the wall of higher plants, is composed of linear or branched polymers derived from hexose sugars such as D-mannose, D-glucose and D-galactose, from pentoses such as D-xylose, L-arabinose and also from sugar acids. These are linked together by β-1,4-glycosidic bonds but other bonds such as β-1,3-, β-1,6-, α-1, 3-, α-1, 6- glycosidic bonds have also been

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reported (Sjostrom, 1993; Moreira and Filho, 2008). Endo-1,4-D-mannanases initiates depolymerisation by random hydrolysis of the D-1,4-mannopyranosyl linkages within the main chain of mannan backbone releasing manno-oligisaccharides of various length (Franco et al., 2004).

A wide variety of fungi which including *Aspergillus niger* form part of a large group of microorganisms which produce plant polysaccharide-degrading enzymes which are responsible for the spontaneous degradation of plant polysaccharide in the environment. β-mannanases are hemicellulases which degrade mannan-based plant polysaccharides, and it has several existing and potential industrial applications, such as increasing the brightness of pulps in pulp bleaching experiments, production of fruit juices, soluble coffee and preparation of poultry diets (Kobayashi et al., 1984; Gubitz et al., 1996; Nicholas, 1998). They also show a strong potential as viscosity reducers of hydraulic fracturing fluids in oil and gas production and also recycling of copra and coffee wastes (Grassin and Fauquembergue, 1996; Jackson et al., 1999; Clarke et al., 2000). Extraction of lignin can be improved by pretreatment of the pulp with β -mannanase alone or in combination with cellulase-free xylanase (Gubitz et al., 1996). Little is known about the potential and characteristics of mannan-degrading enzymes produced by *A. niger*, which is easily grown fungus in solid substrates. The ability of *A. niger* to grow in palm kernel cake, waste containing mannan-based polysaccharides, for manannase production has been reported (Abd-Aziz et al., 2008). This work is aimed at using *A. niger* to produce β-mannanase using wood shavings as substrate in solid state fermentation (SSF) of and characterising the enzyme biochemically.

## MATERIALS AND METHODS

### Sample collection and pretreatment

Wood samples of *Gmelina arborea*, *Terminalia superba* and *Anogeissus leiocarpus* were obtained from a Sawmill at Old-Ife Road in Egbeda Local Government Area, Ibadan, Oyo State, Nigeria and their identification were verified at the Forestry Research Institute of Nigeria, Idi-ishin, Ibadan, Oyo State in Nigeria. The wood samples were milled into about 3 × 5 mm size. This was because the highest Mannanase activity for the isolate was obtained at this particle size when production parameters were tested. The substrates were subjected to thermal pretreatment at 121°C for one hour (Goyal et al., 2008) to make the wood components more readily available for hydrolysis by the fungal enzyme (Zeng et al., 2007).The samples were prepared in triplicates.

### Source of fungal isolate and maintenance

*A. niger* used in this work was selected for its relatively high mannanase activity among 20 screened fungal isolates obtained from local wood samples subjected to spontaneous degradation. It was maintained on Potato Dextrose Agar, PDA (Oxoid) slants at 4°C in the refrigerator. This isolate was identified using Domsch et al.1980) and Kiffer Morrelle (2000).

### Inoculum Preparation

#### *Preparations of fungal inoculum*

Inocula were prepared by pouring 20 ml of Tween 80 solution (0.1%) into a sporulating 5 day old growth of organism on Potato Dextrose Agar slants that had been incubated at 28°C. The suspension was kept under agitation with a magnetic stirrer and counted in a Neubauer chamber until the amount in ml of fungal suspension that will give $10^6$ spore/ml was determined.

### Solid state fermentation for β - mannanase production

15 g of each pretreated wood sample was measured in triplicates into separate 250 ml Erlenmeyer flasks The samples were moistened with a moistening medium having the following composition in g/l of 0.1 M citrate buffer (pH 5.6), $K_2HPO_4$ – 0.23 g, $MgSO_4.7H_2O$ – 0.05 g, $CaCl_2$ – 0.005 g, $NaNO_3$ – 0.05 g, $FeSO_4.7H_2O$ – 0.009 g, $ZnSO_4$ – 0.002 g, $MnSO_4$ – 0.012 g, yeast extract (LAB M) –7 g using Mandel and Webber, (1969) medium as modified by Chahal (1985). The samples were autoclaved at 121°C for 15 min, cooled and inoculated with spore number of about $10^6$ spores per ml of *A. niger* culture (5 days old). Incubation was carried out at 28°C at 60% initial moisture content. Each fermented sample was harvested at 3 day interval over a period of 15 days using Simple Contact Method (Krishna, 2005). Extracts were centrifuged at 5000 rev/min at 4°C for 10 min and supernatants were used as the crude enzyme preparations for enzyme activity and total soluble protein assay.

### Mannanase activity assay

Mannanase activity was determined based on the method of Xu et al. (2002) using a substrate solution of 0.5% locust bean gum (Sigma) in 0.1 M citrate buffer (pH 5.6). The reaction mixture is made up of 0.3 ml of appropriately mixed enzyme filtrate and 2.7 ml of the substrate solutions and incubated for 30 min in a water bath at 50°C. The reducing sugar released was measured as mannose equivalents by the method of Miller (1959). Absorbance of the reaction mixture was measured at 540 nm using a spectrophotometer. One unit of mannanase was defined as the amount of the enzyme that released 1µmol of mannose per minute under the assay condition. The data presented are averages and standard errors of three independent experiments.

### Partial purification of β-mannanase

All purification steps were carried out at 4°C, unless otherwise stated. The cell free crude enzyme filtrates were precipitated using ammonium sulphate. The precipitates were collected by centrifugation at 8000 rpm at 4°C for 15 min and resuspended in sodium citrate buffer (0.1 M) pH 5.6 followed by dialysis against the same for 18 h at 4°C using dialysis tubing of 10 to 100 Millipore size.

### Determination of total soluble protein

Total soluble protein in the sample was determined using the method of Lowry et al. (1951) using Bovine serum albumin as standard. Protein in the column effluents was monitored by measuring absorbance at 680 nm in a colorimeter.

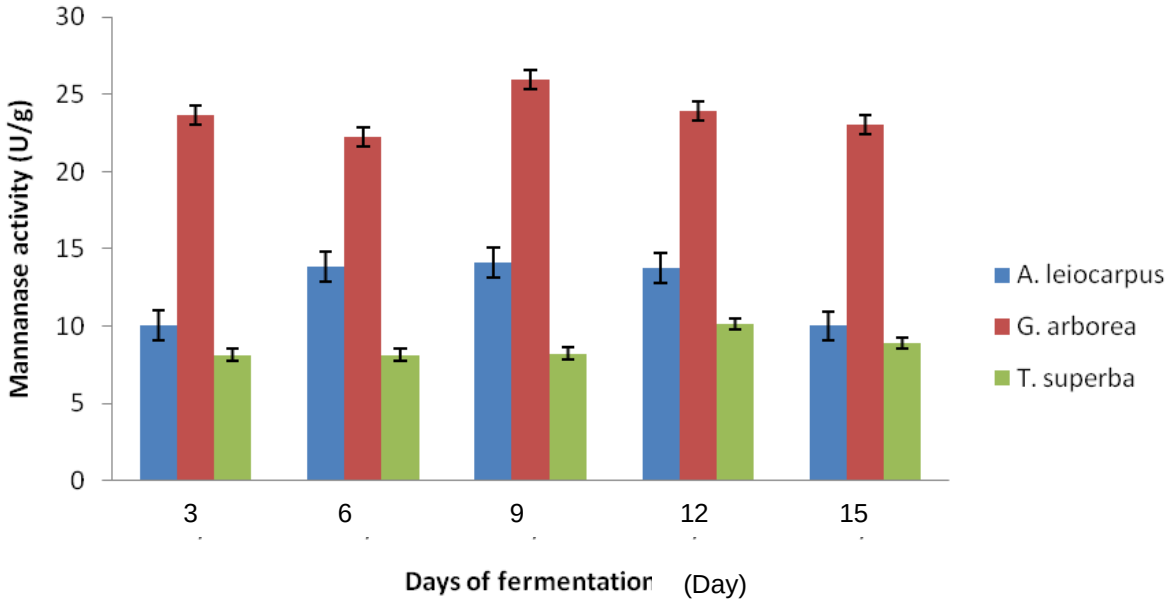


**Figure 1.** Mannanase activities of A. niger on the wood substrates on different days of fermentation.

**Purification of β-mannanase**

The ammonium sulphate precipitated enzyme sample was fractionated on a column packed in 2.5 × 70 cm internal dimension) (Pharmacia) of Sephadex G – 100 (Sigma) surrounded by a water jacket maintained at 20°C was prepared and callibrated according to the method described by Olutiola and Cole (1980). The column was pre-equilibrated with 0.1 M citrate buffer (pH 5.6). Elution was performed with the same buffer at a flow rate of 1.67 ml $min^{-1}$. The pooled enzyme fractions were further fractionated on a C- 75 Sephadex (Sigma) packed in a 2.5 × 30 cm dimension Pharmacia column equilibrated with citrate buffer (pH 5.6) at flow rate of 0.87 ml $min^{-1}$. Bound proteins were eluted by a linear gradient of 0 to 0.5 M NaCl in citrate buffer (pH 5.6). Active fractions containing enzyme activity were pooled and dialyzed overnight. Total proteins and β-mannanase activities were determined after each purification step.

**Biochemical characterisation of the β-Mannanase**

Biochemical characterization of the Mannanase produced by the isolate using *G. arborea* as substrate was further characterized biochemically as described below:

***Effect of varying temperature on enzyme activity***

Enzyme-substrate mixture made by adding 0.3 ml of enzyme filtrate to 2.7 of 0.5% LBG in 0.1M sodium citrate buffer (pH 5.6) was incubated at temperatures 25, 28, 30, 35, 40 and 44°C for 30 min. Mannanase activity was determined according to the method of Miller et al. (1959).

***Effect of varying pH on enzyme activity***

Enzyme –substrate mixture was prepared as described above in 0.1 M sodium citrate buffer at pH 4, 4.5, 5, 5.5, 6 and 6.5 and incubated at 28°C for 30 min. Mannanase activity was determined according to the method of Miller et al. (1959).

***Effect of different mannan concentrations on enzyme activities***

Michaelis-Menten kinetic constants ($V_{max}$ and $K_m$) were determined by assaying the enzyme at different mannan concentrations ranging from 0.25 to 1.25% (w/v) and the double reciprocal plot method of Lineweaver-Burke (Dixon and Webb, 1971) was used to plot the graph.

***Effect of different cations on enzyme activity***

1 ml each of 0.1, 0.2, 0.3, 0.4 and 0.5 M solution of $MgCl_2$, NaCl, $FeCl_3$ and $MnCl_2$ were added to 1.5 ml each of enzyme-substrate mixture prepared by adding 0.3 ml of enzyme to 2.7 ml of 0.5% LBG in 0.1 M sodium citrate buffer (pH 5.6). These were incubated at 28°C for 30 min. Mannanase activity was determined according to the method of Miller et al. (1959).

**Statistical analysis**

Statistical analysis was carried out on results obtained in this work using one-way Anova test while means were separated using Duncan's Multiple Range Test (Duncan, 1955).

## RESULTS AND DISCUSSION

Figure 1 shows the mannanase activities of the fungus on each wood substrate on the different days of fermentation. On *A. leiocarpus* a peak mannanase activity of 14.110 units/g was demonstrated on the 9$^{th}$

**Table 1.** Result of purification of mannanase from *Aspergillus niger* produced by solid state fermentation.

| Form of enzyme | Total volume (ml) | Protein content (mg/ml) | Total protein (mg) | Mannanase activity (units) | Total activity (units/ml) | Specific mannanase activity (units/mg) | Purification fold | % yield |
|---|---|---|---|---|---|---|---|---|
| Crude | 100 | 18.146 | 1814.60 | 21.788 | 2178.8 | 1.201 | 1 | 100 |
| $(NH_4)_2SO_4$ Precipitated | 100 | 15.417 | 1541.7 | 11.571 | 1157.1 | 0.751 | 0.6253 | 53.1 |
| Gel filtration chromatography | 100 | 4.746 | 4746 | 6.72 | 672 | 1.415 | 1.1782 | 30.8 |
| Ion exchange chromatography | 100 | 0.264 | 26.4 | 5.04 | 504 | 19.091 | 15.894 | 23.1 |

(Each value is a mean of triplicate values).

day of fermentation, while on *G.arborea*, a peak of 25.938 Units/g on the 9$^{th}$ day of fermentation and on *T. superba*, the highest mannanase activity of 10.148 Units/g was recorded on the 12$^{th}$ day of fermentation. The results showed that *A. niger* when cultured in the medium containing wood shavings of *A. leiocarpus, G. arborea* and *T. superba* as sole carbon source in SSF, produced extracellular proteins with significant mannanase activity. This is in line with various authors who have reported the use of *Aspergillus* sp. in the production of various bioproteins, both enzymic and non-enzymicsuch as mannanases, cellulases, xylanases and organic acids have been produced using different lignocellulosic substrates (Pentilla, 1998; Viikari et al., 1998; Fadel, 2001; Immanuel et al., 2007).

Among the wood substrates used for this work, *A niger* had the highest mannanase-producing activities on *G. arborea*. This might possibly be related to *G. arborea* being a low density wood and therefore has as its hemicelluloses component consisting of more glucomannan than other tropical wood (Moreira and Filho, 2008; RMRDC, 2010). *A. leiocarpus* and *T. superba* are high density woods with their hemicellulose component consisting mainly of glucuronoxylan (Hagglund, 2002).

The differences in enzyme activities recorded on the different substrates may be as a result of variation in lignin, hemicellulose and cellulose composition of the wood shavings which affected the degree of resistance to fungal biodegradation (Donnelly et al., 2007). As a result of this, high early production of lignocellulosic enzymes enabled *A. niger* to rapidly colonize *G. arborea* which is a low density wood and successfully utilize the available nutrients.

Table 1 shows the result of purification of mannanase from Aspergillus niger produced by solid state fermentation. The crude enzyme extract had a total protein of 18.146 mg/ml, demonstrated a Mannanase activity of 21.788 Units and a corresponding specific Mannanase activity of 1.201 Units/mg, while the partially purified enzyme extract had a total protein of 15.417 mg/ml, demonstrated a Mannanase activity of 11.571 Units and a corresponding specific activity 0.751 Units/mg.

Mannanase was purified by a combination of ammonium sulfate precipitation, gel filtration chromatography and ion exchange chromatography. Purification of 15.894 fold was obtained for the enzyme produced by *A. niger* in this work, as shown in Table 1. This result is in variance with those obtained by Puchart et al. (2000) who purified a mannanase produced by a thermotolerant strain of *A. fumigatus.*

## Characterisation results

### *Effect of varying temperature on mannanase activity*

The optimum activity for this enzyme was achieved at 30°C (11.103 U/ml) while the least activity was at 65°C (1.003) as shown in Figure 2. There was a downturn of enzyme activity from 45 to 65°C a point where the activity was virtually nil indicating that the enzyme may lose its activity at such temperature range. These findings are important in the view of possible industrial applications of this enzyme. The thermostability of the enzyme is higher than that obtained by Puchart et al. (2004) but lower than what was observed by McCutchen et al. (1998).

### *Effect of varying pH on mannanase activity*

Influence of varying pH on the activity of the enzyme is shown on Figure 3. The enzyme was most active at pH 5.5 (10.111 U/ml, the pH at which the enzyme activity was lowest was 4 (5.0617 U/ml), however there was a significant rise in the activity at pH 6.5 after a downward turn from pH 6.0. This may be an indication that two distinct endo mannanases may have been

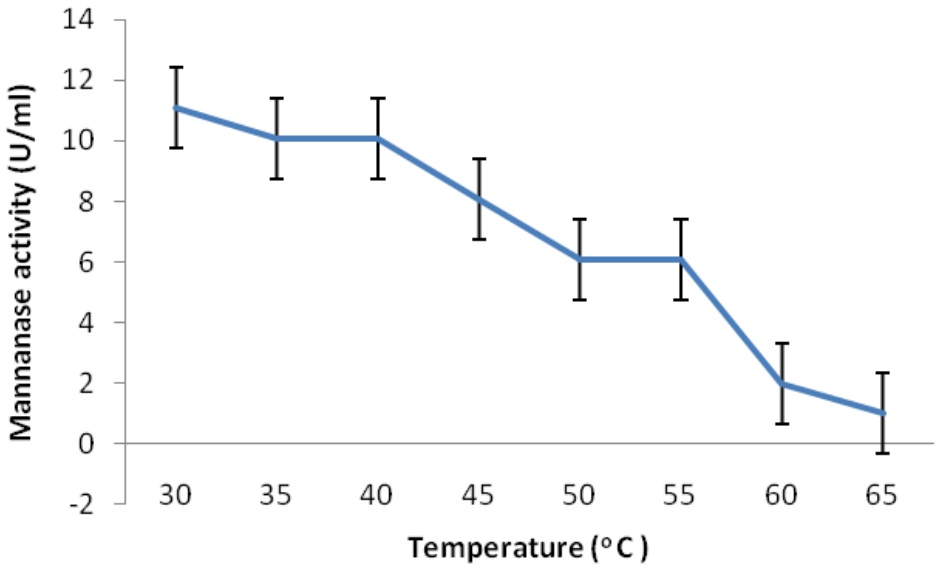


**Figure 2** Effect of different temperatures on Mannanase activity.

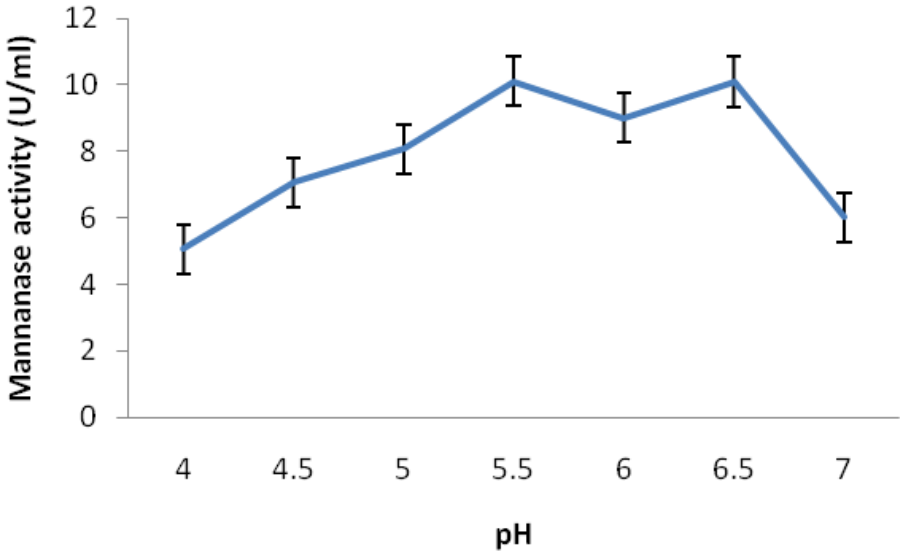


**Figure 3**. Effect of different pH on mannanase activity.

secreted by the fungus Viikari et al. (1993) reported that mannanases were usually secreted into the culture fluid as multiple enzyme form. The enzyme was more stable between pH 4.5 to 6.5 but the activity was low at pH 7 and 4. The pH optimum obtained for this enzyme is in it is in accordance with the acidic pH of up to 5.5 reported for fungal mannanases (Christgau et al., 1994; Ademark et al., 2001; Sachslehner and Haltrich, 1999). The optimum pH was lower than those reported for bacterial mannanases which have pH optima close to neutral (Viikari et al., 1993). Recently, Mannanase produced by *Scopulariopsis candida,* most active at pH 6 was reported (Mudau and Setati, 2008). However, the relatively high activity at pH 6.5 makes the enzyme a good candidate for some industrial applications.

### *Effect of different mannan concentrations on mannanase activity*

This result is shown on Figure 4. The enzyme activity was very low at a concentration of 0.25% mannan (0.162 U/ml) but at 0.5% mannan concentration, the activity became relatively high (10.190 U/ml) and remained

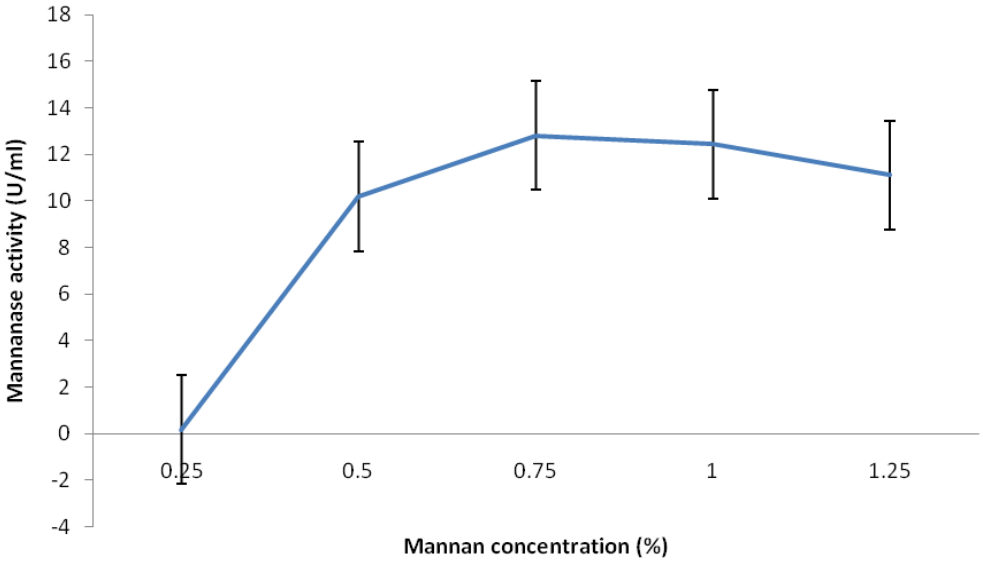


**Figure 4.** Effect of different mannan concentrations on mannanase activity.

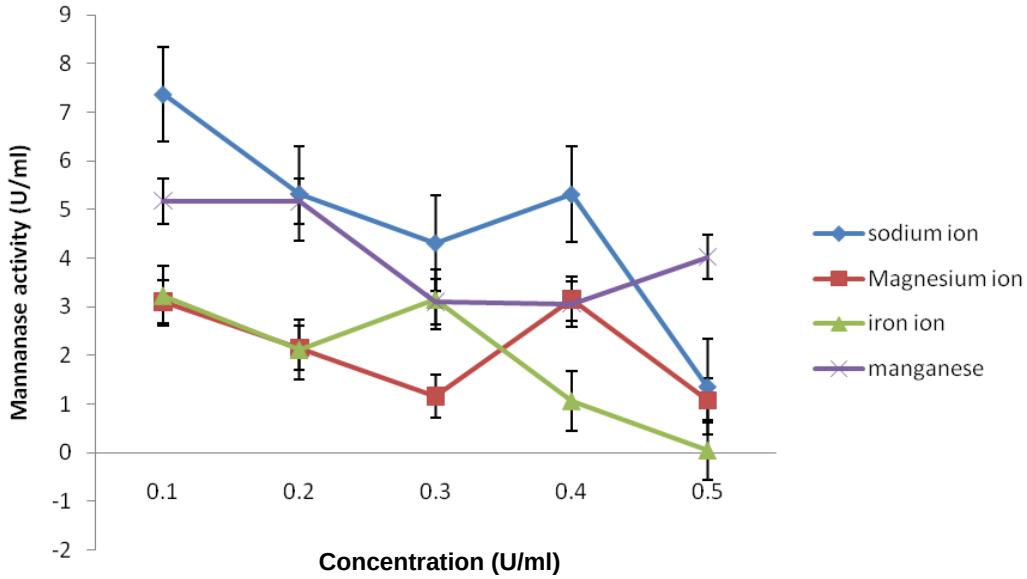


**Figure 5.** Effect of different cations concentrations on mannanase activity.

higher even at high concentration but the rate of increase was lower as compared to the increment recorded between 0.25 and 0.5%.The Lineweaver Burk plot obtained using these figures revealed that the $V_m$ and $K_m$ values for the enzyme were 1.364 and 0.75 mg/ml repectively. *Km* values for different galactomannan substrates have been determined for the *Candida. saccharolyticum* b-mannanase and reported to be 0.127 mg/ml for Locust Bean Gum (Bicho et al.,1991) while *Termomyces neapolitana* mannanases had Km value range of 0.23 to 0.55 mg/ml.

**Effect of different cations concentrations on mannanase activity**

Figure 5 shows the effect of different cation concentrations on the enzyme activity. Generally, the cations did not induce increase Mannanase activity because they all caused lowered enzyme activity as shown in the figure. However sodium ion seems to have the least effect on Mannanase activity of the isolate. For all the cations used, the enzyme activity appears to be stabble at first but reduces again and then there was an

increase from the progressive fall, this observation may be pointing to the fact that more than one Mannanase was released by the isolate. The chloride of these cations were used for this test, Mudau and Setati (2008) also recorded relatively good Mannanase activities at up to 2% NaCl concentration using a yeast strain on locust bean gum substrate. The level of halotolerance observed with the enzyme is similar to other polysaccharide hydrolysing enzymes from halotolerant fungus *A. oryzae*, they include b-xylanases and b-xylosidases (Hashimoto and Nakata, 2003).

## Conclusion

The data obtained in this research has shown that wild-type *A. niger* is able to produce mannanase in significant amounts when cultivated in media containing sawdusts as sole carbon source and has also shown that *G. arborea* has proven to be a very promising substrate for the production of this enzyme.

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