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Isolation, Partial Purification and Characterization of Endoglucanase (EC.3.2.1.4) from *Aspergillus niger* S.L.1 Using Corn Cobs as Carbon Source

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ABSTRACT

Endoglucanase (EC3.2.1.4) from *Aspergillus niger* s.I.1 was isolated, partially purified and characterized. *Aspergillus niger* was cultivated for endoglucanase production by the submerged culture technique using processed corn cobs as carbon source. Xylose was added to the media for cellulose induction. Partial purification was conducted using ammonium sulphate precipitation, and anion exchange chromatography on DEAE-Sephadex A-50 column. The enzyme was purified 5.26-fold from the crude extract. It migrated homogenously and had a relative molecular weight of 46 KDa as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). It had an optimum pH and temperature of 4.5 and 60^{o}C, respectively. It also had the K_m of 0.014 g/ml and V_{max} of 0.377µmoles/hr. The Arrhenius plot of the enzyme activity gave activation energy (E_a) of 22.79 kJ/mole, Dixon plot gave extrapolated pK_a values of 3.0 and 6.2 suggesting aspartate, glutamate and histidine as possible amino acids in the catalysis of the enzyme. The activity of endoglucanase was enhanced in the presence of $MgCl_2$, $CoCl_2$, $FeCl_3$, $CaCl_2$ and $FeCl_2$. This indicates that the enzyme may be a metalloprotein and that it requires metal ions for its optimum activity. Finally, it was observed that cellulose acetate inhibited the activity of the enzyme on carboxymethylcelluloe(CMC) in a competitive manner, with an inhibition constant Ki $1.33x10^{-6}$ µmole/ml and $5.33x10^{-6}$ µmole/ml at the concentration of 0.01 g/ml and 0.04 g/ml respectively. The significance of the findings has been discussed.

Keywords: Endoglucanase, *Aspergillus niger,* carbon source.

INTRODUCTION

Natural cellulosic substrates, primarily plant cell materials are composed of heteregenous interwined polysaccharide chains with varying degrees of crystallinity, hemicelluloses and pectins, embedded in lignin. Cellulose, the most abundant natural resource available to man on earth is found almost exclusively in plant cell walls and it comprises about 95% of earth's land-based biomass (Udotong, 1997).

Cellulose is an important material that can be converted into valuable products, for example, fermentable sugars for the production of ethanol and other chemicals (Brown, et al., 1987). The problem is that cellulose is not in a form suited for direct human needs, that is, for food or fuel unless it is modified. This is because the β-1-4 linkages of cellulose are not hydrolyzed by α-amylases which hydrolyze α-1-4 linkages in humans (Carpita, 1996). Cellulases are involved in this modification.

Cellulases are extracellular enzyme mixtures produced by fungi and bacteria, insects and lower animals (all of which use cellulose nutritionally as carbon source) which can hydrolyze cellulose to smaller sugar component like glucose (Shin and Yanz, 1996). Generally, microorganisms produce three major cellulolytic enzymes, i.e. endoglucanase, exoglucanase and β-glycosidase which all act synergistically on cellulose generating low molecular weight reducing sugars (Liu, *et al*, 1996).

Endoglucanases cut at random at internal amorphous sites in the cellulose polysaccharide chain generating oligosaccharides of various lengths and consequently new chain ends. Exoglucanases act in a processive manner on the reducing or non-reducing ends of cellulose polysaccharide chains liberating either glucose or cellobiose as major products while β-glycosidase hydrolyzes cellodextrins and cellobiose to glucose (Liu *et al*, 2002). It is documented that these enzymes are produced in varying quantities by different microorganisms. *Aspergillus niger* has been reported to produce large amount of endoglucanase but low quantities of exoglucanase (Liu *et al*, 2002).

Therefore, the study of endoglucanase is of practical importance because of its potential role in the conversion of cellulosic substrates into food materials, chemicals or fuel. A better result will be obtained if the various enzymes are studied separately in details and then put together to hydrolyze cellulose.

The ojectives of this study are to (a) cultivate *Aspergillus niger* S.L.1 in appropriate media

with corn cobs as carbon source, (b) induce the microorganism using xylose for the production of endoglucanase, (c) isolate and partially purify the endoglucanase, and (d) determine the kinetic properties of the partially purified enzyme.

MATERIALS AND METHODS

Cellulosic Substrates

Corn cobs were obtained from indiscriminately disposed households waste in Samaru Zaria, Kaduna State, Nigeria.

Organism

The organism, *Aspergillus niger* S.L.1 used was isolated from the soil. It was purified, characterized and identified at the Department of Microbiology, Ahmadu Bello University, Zaria, Kaduna State, Nigeria.

Culture Medium

The following ingredients (g/l) were used for the preparation of culture medium as reported by Abu et al, (2003), KH_2PO_4 – 10; $(NH_4)_2\ SO_4$ – 10.5; $MgSO_4\ 7H_2O$–0.33; $CaCl_2$-0.5; $FeSO_4\ 7H_2O$-0.013; $MnSO_4\ H_2O$-0.004; $ZnSO_4\ 7H_20$-0.004; $CoCl_2\ 6H_2O$-0.0067 and yeast extract -0.5. The pH of the culture medium was adjusted to 6.0 using 0.1N HCl.

Cultivation of *Aspergillus niger* for Endoglucanase Production

Exactly 4.0g of alkaline-pretreated corn cobs were taken into 250ml conical flasks containing 100ml of the mineral salt media. The solutions were mixed thoroughly by shaking and the pH adjusted using 0.IN HCl to pH 6.0. The flasks were plugged with cotton wool and then autoclaved at 121^{o}C for 15 minutes. The sterilized culture cooled to room temperature was inoculated with 2.0 x 10^{-4} spores of *A. niger* S.L.1spore suspension, and then 0.01g of xylose was added to the media for induction. The flasks were incubated in an orbital cooled shaking incubator (CA NO. IH460; APP NO. IB, 2621 C40 Gallenkamp) at 28^{o}C adjusted at 100 rpm for 6 days. Exactly 5.0ml of samples were withdrawn from each of the fermentation medium every 24 hours for the determination of crude protein concentration and endoglucanase activity.

Enzyme Assay

Endoglucanase activity was assayed colorimetrically according to the method described by Ali et al., (1991). Endoglucanase was investigated for CMC-saccharifying activity by incubating 1.0 ml crude or partially purified enzyme with 1.0 ml of CMC in 10 mM phosphate buffer, pH 5.6. The mixture was incubated at 37^{o}C for 15 minutes and the reducing sugar was determined using the DNS method (Miller, 1959).

Activity was defined as the amount of endoglucanase that released 1µmole of reducing sugar per hour.

Protein Concentration

Protein content was determined using the biuret method (Plummer, 1987).

Ammonium Sulphate Precipitation

The enzyme culture was centrifuged at 8000 rpm for 10 min at 10^{o}C to remove the cells. Crude endoglucanase in the culture supernatant fluid was precipitated by adding solid $(NH_4)_2SO_4$ at 40 -80% saturation. The solution was kept at 0-4^{o}C overnight and the precipitated proteins were then collected by centrifugation at 26000 rpm for 20 min. The resultant pellet was redissolved in approximately 20 ml of 20 mM phosphate buffer (pH 7.0) and dialyzed against same buffer for 2 days with three changes of buffer. Insoluble proteins were removed by centrifugation at 8000 rpm for 10 min. This method is as described by Li *et al* (1998).

Anion Exchange Chromatography

Exactly 5 ml of the dialyzed sample was loaded onto DEAE – Sephadex A-50 column equilibrated with 20 mM phosphate buffer (pH 7.0). The column was eluted with a linear gradient (0.05 M - 0.75 M) NaCl in phosphate buffer at pH 7.0 during washing, at a flow rate of 26 ml/h. The endoglucanase activity in each 5 ml fraction was determined. The active fractions were pooled, concentrated and dialyzed extensively against the same buffer as described by Li et al, (1998).

Characterization of Partially Purified Endoglucanase

pH and Temperature Optima

The optimum pH and temperature were determined by measuring the activity as described in 2.5 at pH ranging from pH 2.0 – 9.5 and temperatures ranging from 10 – 90^{o}C.

Kinetic Constants (K_m and V_{max})

The substrate concentration was varied over the range of 0.01 – 0.14 g/ml of CMC. The kinetic constants, K_m and V_{max} for the enzyme were determined from a Lineweaver-Burk plot.

Thermostability

Equal volumes (1.0 ml) of endoglucanase enzyme were incubated at intervals of 10^{o}C for 30 min at a temperature range of 20 – 90^{o}C as described by Stauffer (1969) Plots of logarithm of enzyme activity (LogV) versus reciprocal of temperature (K^{-1}) were done to determine Arrhenius plot of energy of activation. The residual activity of the

enzyme was also determined.

Estimation of Amino acid on the Catalytic Site of Endoglucanase

The activity of endoglucanase was determined while varying CMC concentration from 0.01 – 0.14 g/ml for different pH values (pH 2.0 – 9.0). inhibitor (0.01 and 0.04 g/ml) were incubated with substrate of varying concentrations (0.01 – 0.14 g/ml). Endoglucanase activity was determined and the inhibition constant Ki, calculated from the Lineweaver-Burk plot.(Dixon and Webb, 1964).

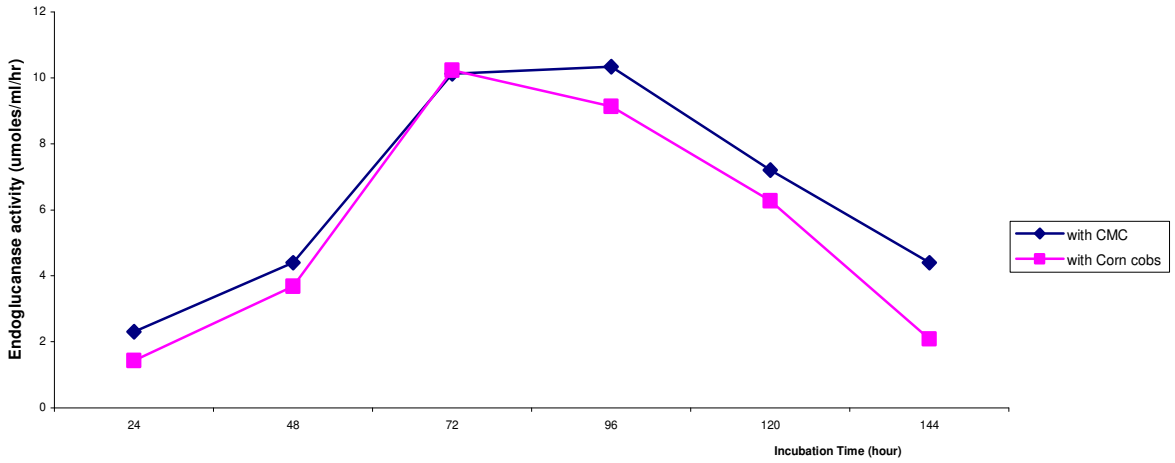

Fig. 1: Time Course for endoglucanase production by *A. niger* in media supplemented with corn cobs and CMC

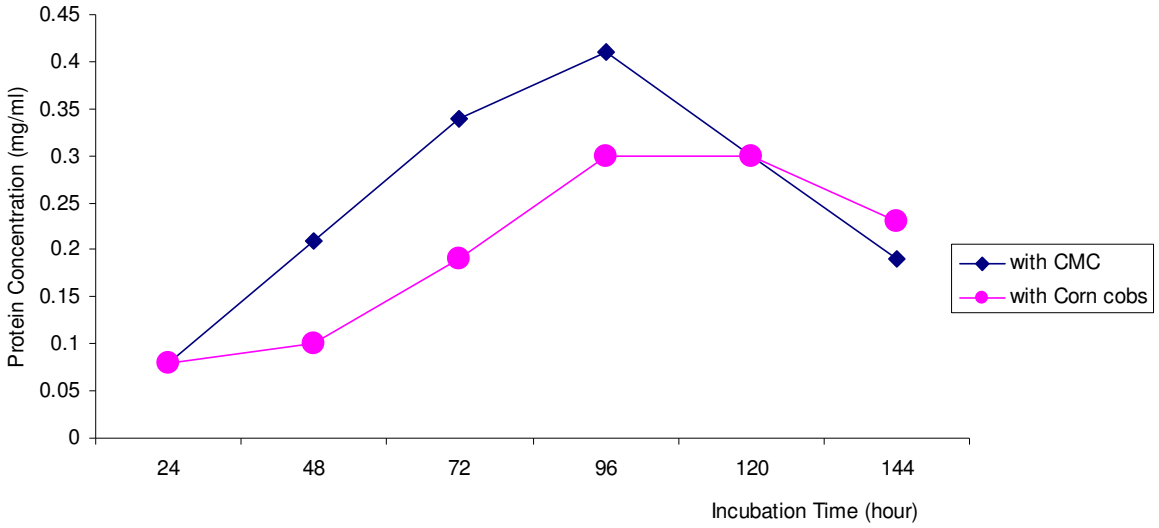

Fig. 2: Protein level in culture of A. niger supplemented with corn cobs and CMC

K_m and V_{max} at each pH value were determined using the Lineweaver-Burk plot. pH values (Pk_a) were determined using the Dixon plot.

Effect of Cations on Endoglucanase Activity

Exactly 1.0 ml of the enzyme and 0.2 ml of 0.02 M of the cations ($MgCl_2$, $CoCl_2$, $FeCl_3$, $CaCl_2$, $FeCl_2$ and $HgCl_2$) were incubated with 1.0 ml of CMC. The activity of the enzyme was then determined.

Effect of Cellulose Acetate (Inhibitor) on endoglucanase activity

Aliquots of the enzyme (1.0 ml) and 0.2 ml of the

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE of the partially purified endoglucanase enzyme was performed as described by Webster and Campbell (1972), in polyacrylamide slab gels made up of 7% stacking gel and 12% running gel. The proteins were stained with 0.25% Coomasie Brilliant Blue R250 overnight, rinsed and then destained in 7% acetic acid. (Laemli, 1970)

Determination of Molecular Weight

The relative mobility (R_f) of endoglucanase and

the standard proteins were calculated by measuring the distance migrated by the protein divided by the distance migrated by the tracker dye. The relative molecular weight of the partially purified enzyme was calculated by proportion. (Weber *et. al.,* 1972)

RESULTS AND DISCUSSION.

Figure 1.0 shows the time course of endoglucanase production by *Aspergillus niger* S.L.1 using corn cobs and CMC as the respective carbon sources. In both media, endoglucanase production increased until it reached optima on the third and fourth day for corn cobs and CMC medium respectively. The decline in activity after the third and the fourth day may in addition to other factors be due to presence of inhibitors (Lee et al, 1994). The presence of glucose, a preferred and more easily metabolizable carbon source in the substrate may also inhibit the enzyme production (Smith, 1999).

substrate may also inhibit the enzyme production (Smith, 1999).

The pilot run conducted to establish the appropriate percentage of ammonium sulphate $(NH_4)_2SO_4$ saturation to precipitate the enzyme endoglucanase shows that the enzyme suspension was precipitated between 40 - 80% $(NH_4)_2SO_4$) saturation (fig. 3). At this stage, a purification fold of 1.64 was achieved. This result is anticipated because $(NH_4)_2SO_4$ has been reported to be the best protein precipitant without having effect on the active site or biological activity of the enzyme. It also has high solubility characteristic and changes of temperature have little or no effect on its solu-

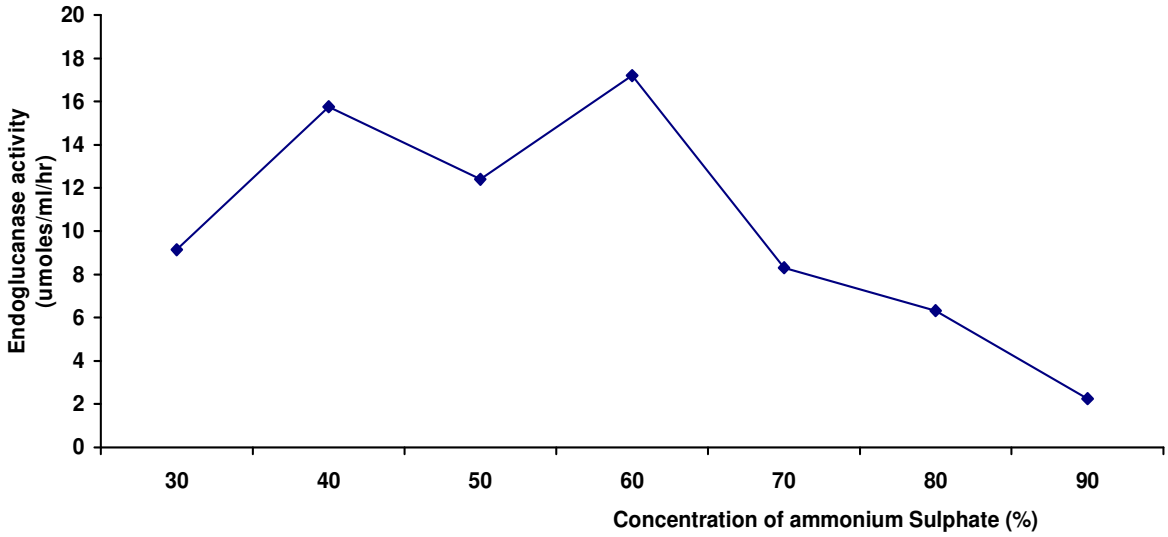

Fig. 3: Ammonium sulphate precipitation curve of endoglucanase in corn cobs supplemented media

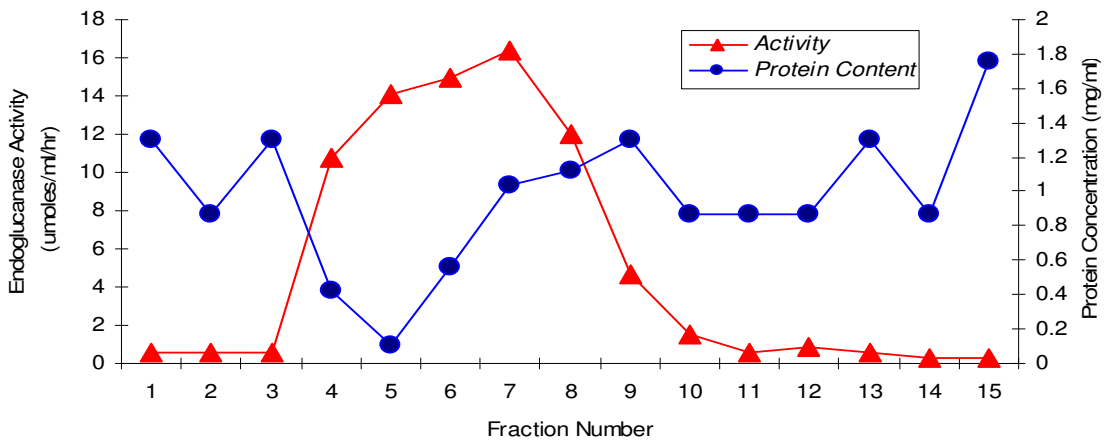

Fig. 4: Bution profile of endoglucanase from DEAE-Sephade x A50 anion exchange column

Table 1: Purification Profile Of Endoglucanase From *Aspergillus Niger Sl.1*

	Total protein (mg)	**Total Activity (µ/mole/ml/ hr)**	**Specific Activity (µmole/ml/hr/mg protein)**	**Purification fold**	**Yield (%)**
Culture Filtrate (Crude)	1.30	14.08	10.83	1	100
$(NH_4)_2SO_4$ Precipitation	0.62	11.02	17.74	1.64	78.27
Dialysis	0.51	10.71	21	1.94	76.07
DEAE- Sephadex A - 50	0.11	6.27	57	5.26	44.53

bility (Dixon and Webb, 1964). Dialysis produced a purification fold of 1.94 over the crude extract. The increase in purification fold is due to the removal of low molecular weight contaminants and desalting.

The anion exchange chromatographic separation of the partially purified endoglucanase by elution on DEAE-sephadex A-50 column showed a single broad peak (Fig.4) suggesting the presence of a single active protein in the eluate. This purification step produced the final purification fold of 5.26 with 44.53% recovery of the enzyme activity. Li et al (1998), reported 1.61 purification fold with 30.1% recovery of endoglucanase activity, isolated from *streptomyces species*. The difference in the percentage yield of endoglucanase activity in this work and others is probably due to the difference in source of the enzyme. The purification of endoglucanase is summarized in Table 1.

The partially purified enzyme was found to be stable from pH 3.0 – 6.5 with an optimum pH of 4.5 (Fig. 5). This result is in agreement with that reported from other fungal sources, pH 5.0 (Howard *et al*, 2003). *streptomyces omiyaensis*. Reports have shown that maximum activity at an acidic pH is a common property of endoglucanase from fungal sources which differentiates it from the endoglucanase obtained from *streptomyces omiyaensis,* which has optimum activity within the neutral pH (Alam *et al*, 2004). Endoglucanase had a broad temperature range with

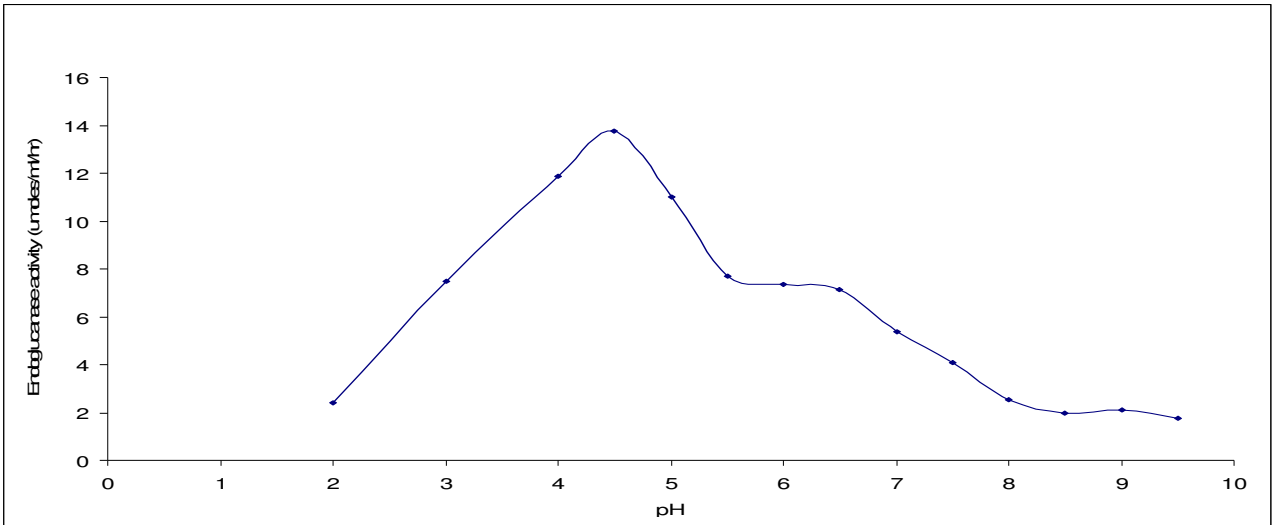

Fig. 5: Effect of pH on Endoglucanase activity

a highest value of CMC liquefying activity at 60°C (Fig.6). This value is higher than that reported for *streptomyces species* (Li et al, 1998; Alam et al, 2004) with optimum temperature of 50ºC , but lower than that reported for endoglucanase from *Aspergillus niger* (Howard et al, 2003) with optimum temperature of 70ºC. The value obtained in this work is in agreement with that reported by Rov et al (1990), which has optimum temperature of 65°C.

The Michael's – Menten constant (K_m) for the substrate CMC of the partially purified endoglucanase was calculated from the Lineweaver-Burk plot to be 0.014 g/ml (Fig. 8). This value is slightly higher than the K_m of 0.013 g/ml reported by Wittman et al (1994) and slightly lower than

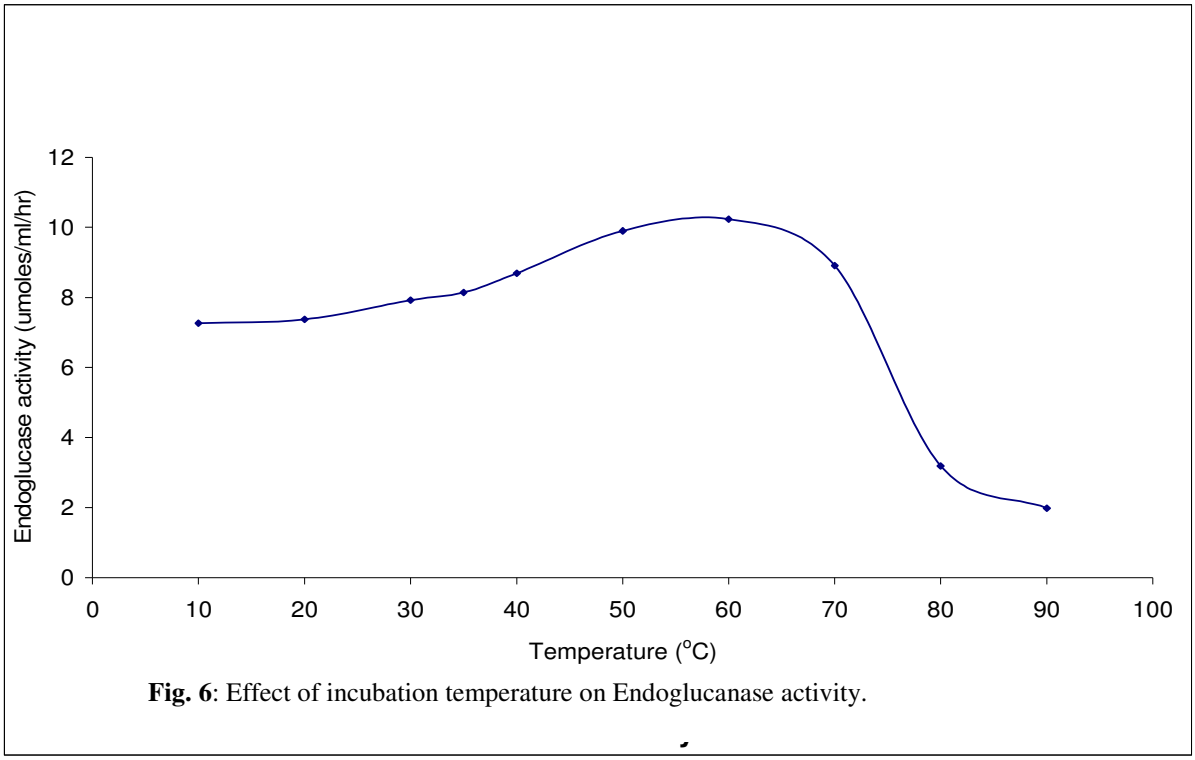

Fig. 6: Effect of incubation temperature on Endoglucanase activity.

the Km of 0.0173 g/ml reported by Pardo and Forchiassin (1999). Similarly, the V_{max} for the partially purified enzyme gave a V_{max} of 0.377 µmole/hr reducing sugar. A higher value of 0.45 µmole/hr has been reported by Pardo and Forchiassin (1999).

The Arrhenius plot on the effect of temperature on endoglucanase activity produced activation energy (Ea) of 22.79 KJ/mole over the temperature range of 20 – 70°C (Fig. 10). This value is higher than the value of 20.80 KJ/mole reported by Pardo and Forchiassin (1999). The Ea ob-

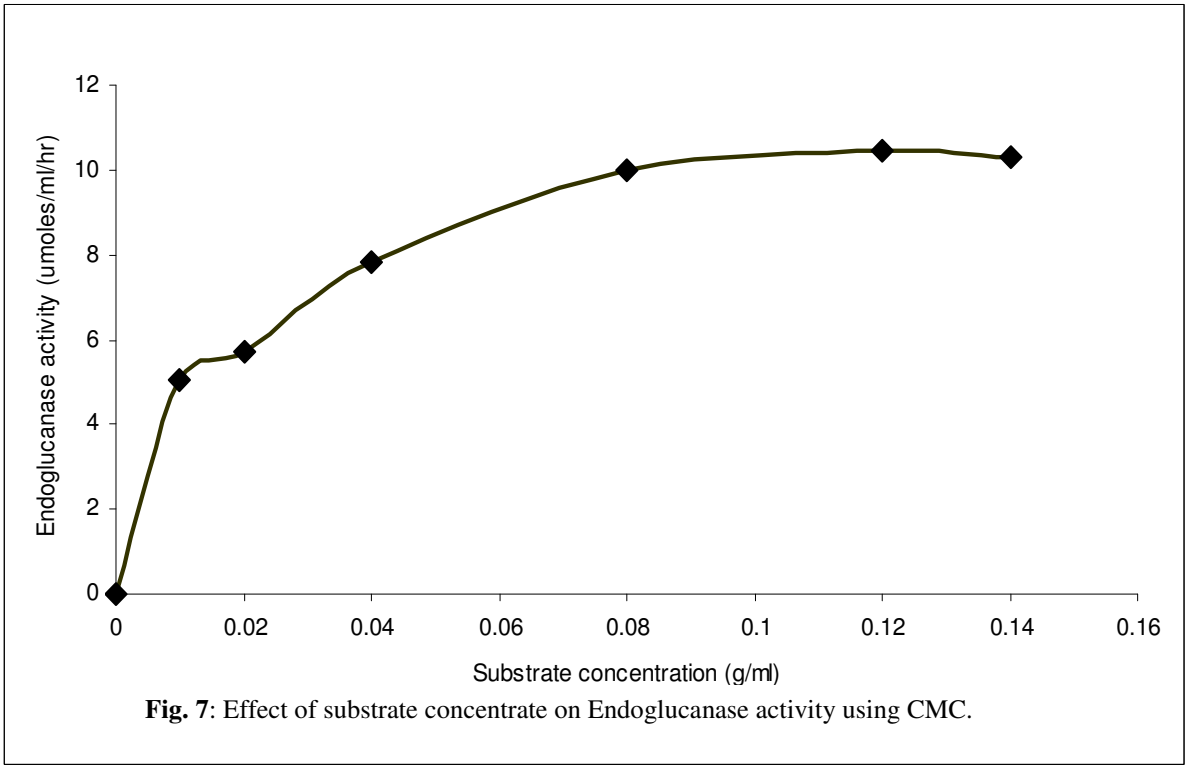

Fig. 7: Effect of substrate concentrate on Endoglucanase activity using CMC.

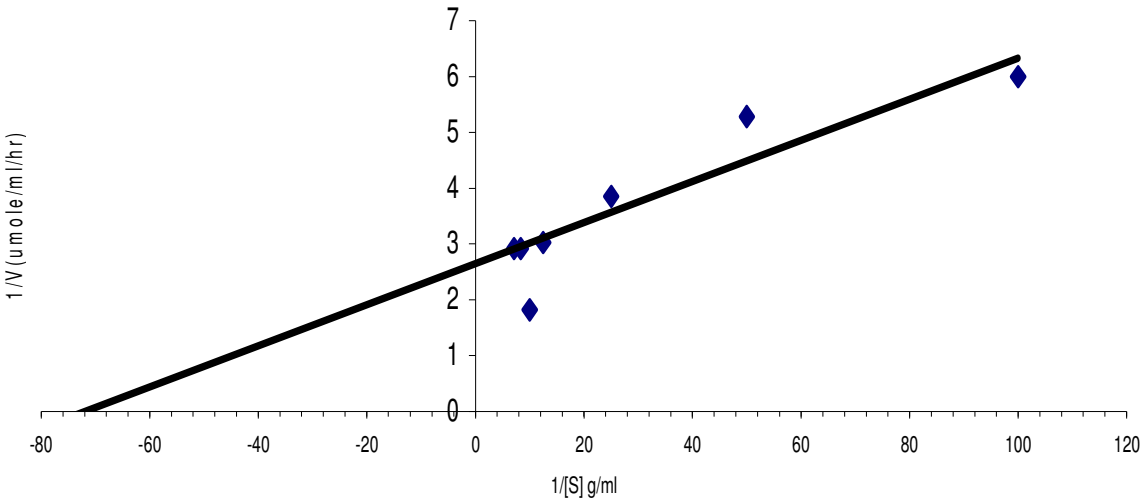

Fig. 8: Double reciprocal plot of Endoglucanase using CMC as substrate

tained in this work falls within the range of Ea of many glycosidases. According to Dixon and Webb (1964), for transformation of substrates to products, the energy of activation ranges from 20.92 – 62.76KJ/mole in enzyme catalyzed reaction, increase in temperature will affect the reaction with subsequent inactivation of the enzyme at high temperatures, in which the secondary and tertiary conformations of the enzyme is destroyed which affects the catalytic functions of the enzyme.

Dixon plot was done to determine the probable amino acid type in the active site of the enzyme. The extrapolated values from Fig. 11 gave pKa values of 3.0 and 6.2. Similar results have been reported by Petrova *et al* (2005) where endoglu-

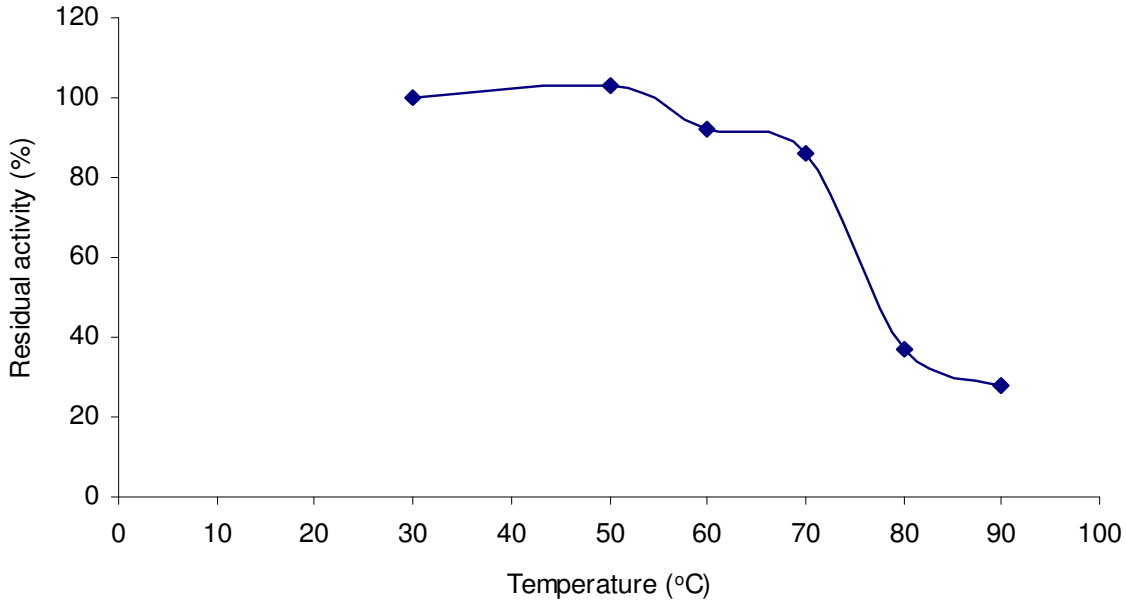

Fig. 9: Residual activity of Endoglucanase

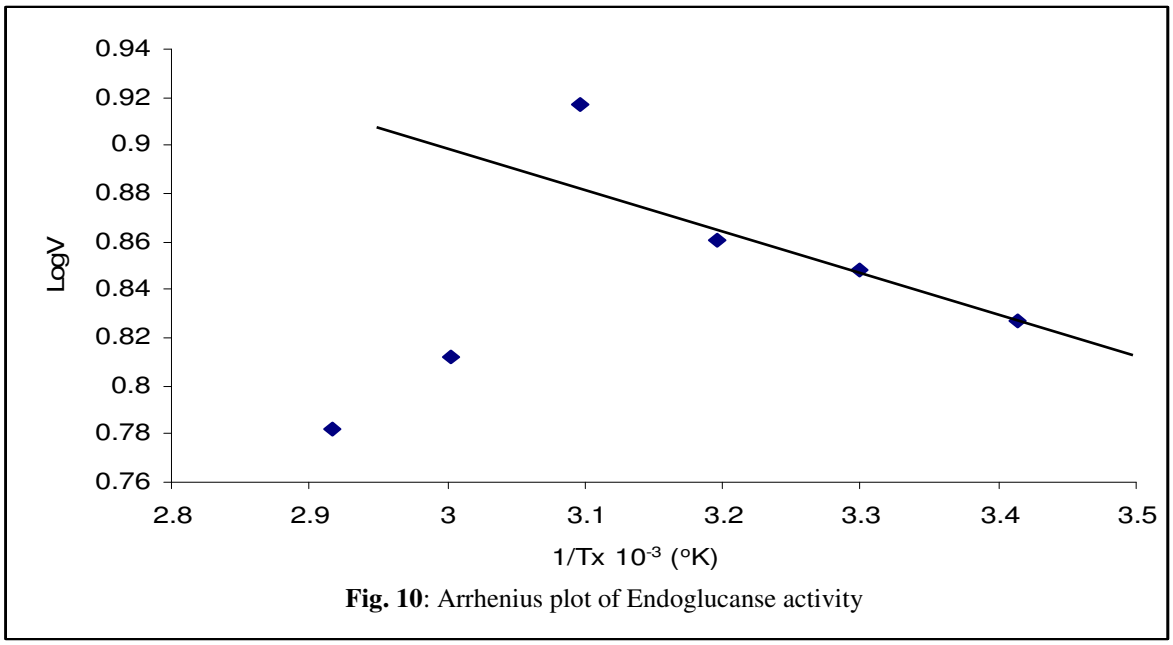

Fig. 10: Arrhenius plot of Endoglucanse activity

canase from a mutant strain of *trichoderma specie* gave extrapolated pka values of 4.6 and 5.5. These values appear to implicate acidic groups in the catalytic site of endoglucanase which may suggest the involvement of aspartate, glutamate and histidine residues (Dixon and Webb, 1964). The effect of cations on the partially purified endoglucanase as indicated in Fig. 12 shows that the activity of the enzyme was enhanced in the presence of $MgCl_2$, $FeCl_3$, $CoCl_2$, $CaCl_2$ and to some extent by $FeCl_2$. These studies indicate that the enzyme may be a metalloprotein and that it requires metal ions for its optimum activity. The result also shows that endoglucanase was insensitive to $HgCl_2$. The insensitivity of the enzyme to mercury ions also suggest that SH-groups may not be involved in the enzyme catalyzed reaction mechanism. This is because heavy metals like mercuric chloride would selectively react with –SH residue of the enzyme thereby inactivating it (Dixon and Webb, 1964).

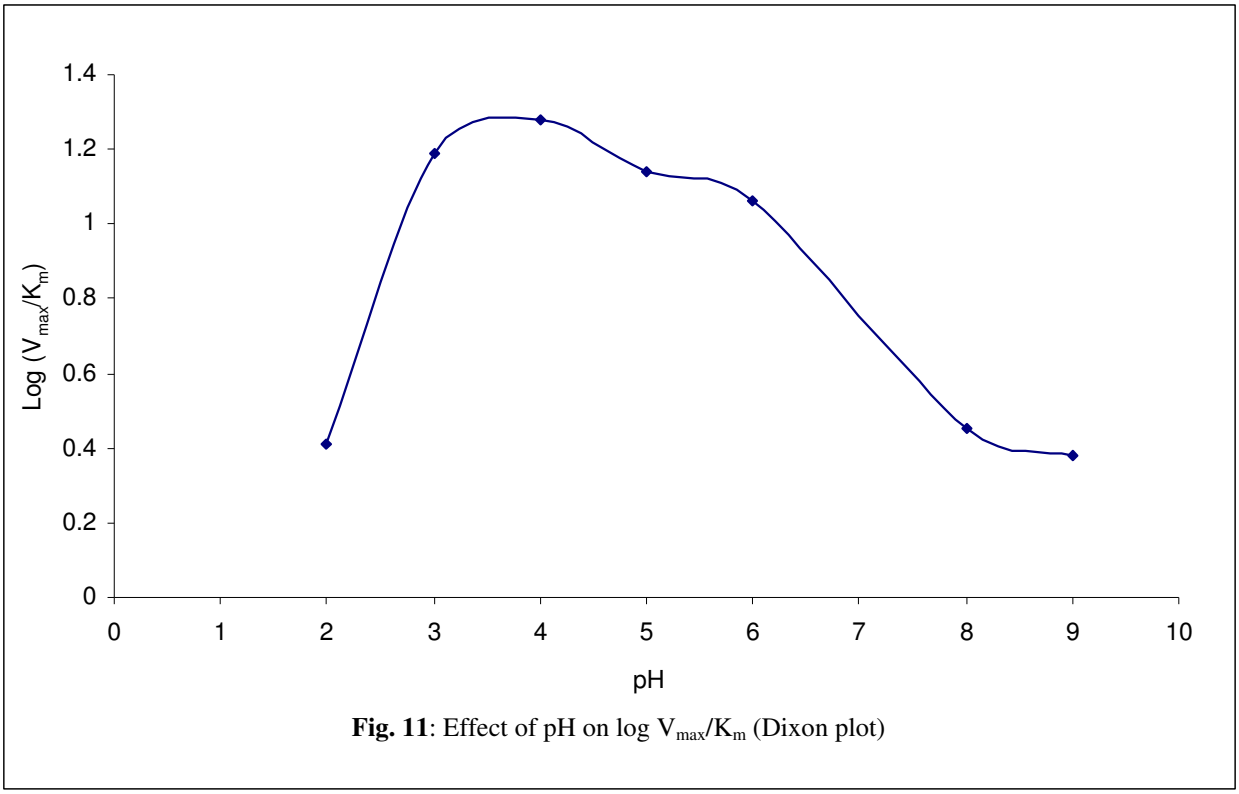

Fig. 11: Effect of pH on log V_{max}/K_m (Dixon plot)

The partially purified enzyme was found to be inhibited by cellulose acetate (Fig. 13) at a concentration of 0.01 g/ml and 0.04 g/ml with Ki of 1.33 x 10^{-6} µmole/ml and 5.33 x 10^{-6} µmole/ml respectively. The type of inhibition was competitive in nature.

Sodium dodecyl sulphate electrophoresis on polyacrylamide gel gave a single protein band for the partially purified enzyme (Fig. 14). The occurrence of the single band of the enzyme suggests apparent homogeneity. When the band was estimated for molecular weight, it was found to be 46kDa. This molecular weight correlate with 48 KDa being molecular weight of endoglucanase purified from culture supernatant fluids of isolated *streptomyces specie* (Li et al, 1998).

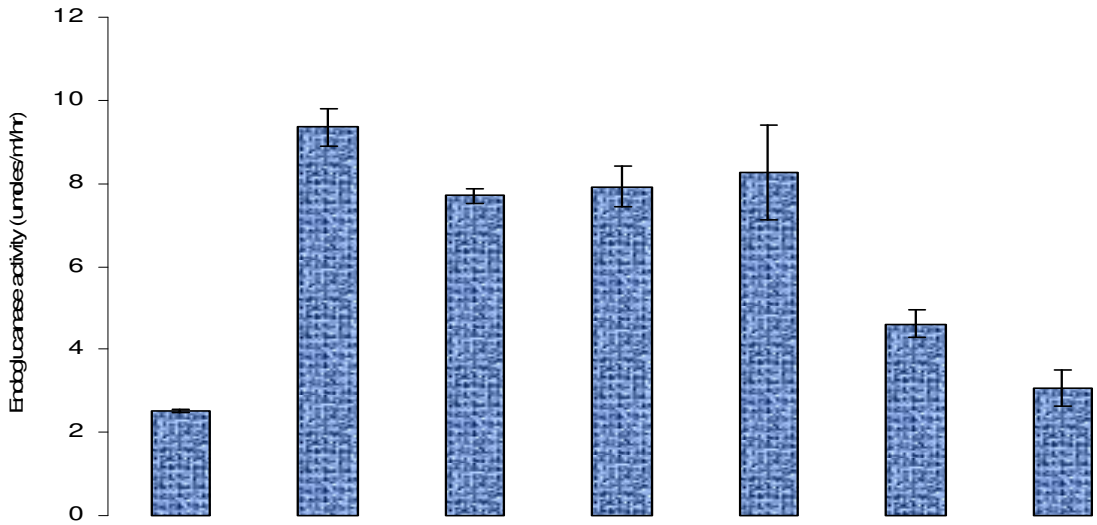

Fig. 12: Effect of cation on Endoglucanase activity
Bars are mean values of duplicate

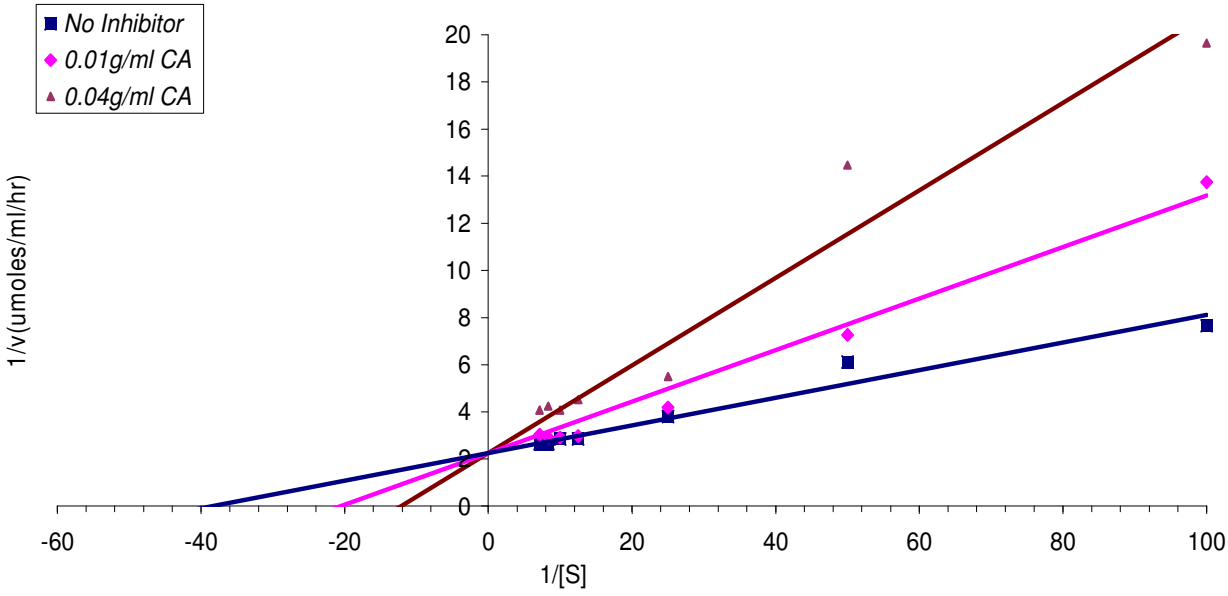

Fig. 13: Inhibition studies on Endoglucanse using cellulose acetate (CA) as inhibitor

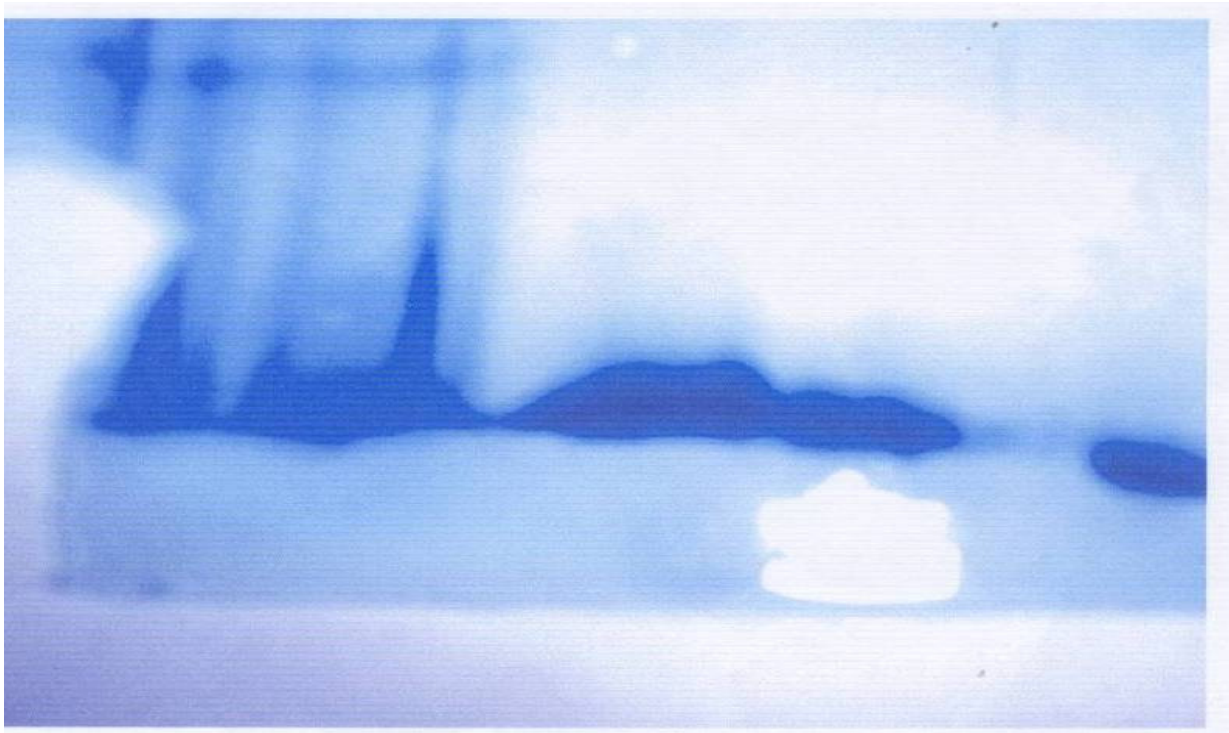

Fig. 14: SDS-PAGE of endoglucanase from *Aspergillus niger s.l.*1

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