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Production, Purification, Characterization and Application of Cellulase from *Trichoderma* species

Om Shankar[1]*, M. K. Shrivastava[2], Anoop Batra[3], V. Tripathi[1], Vikas Gupta[4], and Kuldeep Kumar[5]
[1]MJP Rohilkhand University Bareilly, U.P., India
[2]Crop Improvement Division, Indian Grassland and Fodder Research Institute, Jhansi (UP)
[3]Delhi University, Delhi, India
[4]Sam Higginbottom University, Allahabad, U.P., India
[5]HNB Garhwal University, Srinagar Garhwal Uttarakhand, India
*Corresponding Author E-mail: om.biochem@gmail.com

ABSTRACT

In the present study we selected Trichoderma sp. Enzyme production of 4.34 IU/ml (CMCase activity) was obtained using 1% cellulose as substrate at 30 °C and 200 rpm after 6 days of incubation. The enzyme was functional in a pH range of 4.0 to 6.5 with optimum activity at pH 5.0 for crude and 5.0 for partially purified enzyme. Both crude and partially purified cellulase were functional in the range of 30-70°C with the optimum enzyme activity at 60°C. The partially purified cellulase from Trichoderma sp. was stable at pH 5.0 for 24 h retaining. No effect of β-mercaptoethanol, dithiothreitol and urea was found on cellulase activity of Trichoderma sp. Glucose concentrations with 0.025% and above were inhibitory for cellulase activity from Trichoderma sp. Xylose at lower concentrations were found to support cellulase activity. The purified cellulase from Trichoderma sp. showed k_m and V_{max} of 7 mg/ml and 0.45 µmol/ml/min. Modification of tryptophan residues present in the catalytic domain (SBD) using oxidation by N- Bromosuccinimide revealed 2.5 mM was highly inhibitory for cellulase from Trichoderma sp. This clearly suggests that tryptophan is important for the catalytic activity of cellulase. Effect of N-Acetylimidazole, a tyrosine modifiying agent showed that 60% of the native enzyme activity was retained. This result indicates that acetylation of tyrosyl groups changes the enzymatic activity of cellulase. The cellulase from Trichoderma sp. showed a good hydrolytic potential with maximum hydrolysis of 22.76% and 22.14% from Agrowaste1 and Agrowaste2, which was slightly higher as compared to commercial cellulase, thus proving the hydrolytic potential of the selected cellulase.

***Key words**: Cellulose, Trichoderma, Microorganism.*

INTRODUCTION

Plant biomass is the only foreseeable sustainable source of fuels and materials available to humanity. Cellulosic materials are particularly attractive in this context because of their relatively low cost and plentiful supply. Cellulose enzyme has many industrial application in conversions of food industries and agricultures[1,2,3]. The application of cellulase enzyme preparations in food production include the breakdown of the cellulose in citrus products. Cellulases are also used in the pulp paper industry[4,5,6,7]. They are widely applied in textile processing to improve fabric appearance by reducing fuzz, piling, and enhancing the softness, luster and color brightening of cotton fabrics. Cellulases are useful in polishing of jute and its different blends. 'Biopolishing' is the registered trademark of the Novo-Nordisk Industries for the process of cellulase treatments to cotton fibres and their blends[8].

Although a large number of microorganisms are capable of degrading cellulose, only a few of these microorganisms produce significant quantities of cell-free enzymes capable of completely hydrolysing crystalline cellulose *in vitro.* Fungi are the main cellulase-producing microorganisms, though a few

bacteria and actinomycetes have also been recently reported to yield cellulase activity. Microorganisms of the genera *Trichoderma* and *Aspergillus* are thought to be cellulase producers, and crude enzymes produced by these microorganisms are commercially available[9]. Cellulases of the genus *Trichoderma* have received intensive attention due in significant part to the high levels of cellulase secreted. Most commercial cellulases are produced from *Trichoderma* spp., with a few also produced by *Aspergillus niger*[10,11].

Though cellulases have shown potential application in many industries, search for new and more potential source is still an ongoing process. Therefore the present study undertaken with a view to explore potential of microorganisms for cellulase production and evaluation of their properties for their possible use in different industries.

MATERIALS AND METHODS

Production of Cellulose

The source of cellulase was *Trichoderma sp.* The cultures was maintained on Potato Dextrose Agar (PDA) medium and subsequently stored at 4 °C in a BOD incubator. Transfer of fungal culture from master culture tube to the new PDA slants was performed in a complete sterile environment. The cultures were incubated at 30 °C for 6 days and finally were stored at 4° C.

Cellulase production medium was prepared as per the standardized composition and then distributed into 10 conical flasks of 250 ml volume, each one containing 50 ml of medium and autoclaved. 9 ml of normal saline containing few drops of tween-80 was added to slant containing culture. The surface of the culture was gently scratched with the tip of a sterile inoculum needle for release of spores and vortexed. Approximately 1 ml of spore suspension was added into 50 ml of cellulase production medium. For production of cellulase, the inoculated production medium was incubated at 30 °C for 6 days, under shaking conditions (200 rpm). After 6 days the cultures were filtered through whatmann no.1 filter paper.

Determination of cellulose activity

Cellulase activity was determined by using the filter paper assay for saccharifying cellulose and Carboxymethyl cellulase assay for endo-13-1, 4-glucanase given by Ghose, 1987[12]. Finally, enzyme unit was calculated from the obtained glucose concentration. One unit (IU) of enzyme activity is defined as the amount of enzyme required to release one µmole of glucose per ml per min under the standard assay conditions.

Protein Estimation

Protein concentration of the enzyme was estimated using the dye-binding method (Bio-Rad). Bio-Rad Protein Assay/Dye Reagent Concentrate was obtained from Bio-Rad Laboratories, Inc. The reagent was diluted 1:4 with distilled water and filtered. 10 µl of protein (*i.e*. enzyme) sample and 200 µl diluted Bio-Rad reagent was added in the well of microtitre plate. The reaction mixture was incubated for 10 min for binding of the dye. Absorbance of the colored reaction was measured at 595 nm using an ELISA plate reader. Protein concentration was estimated from the standard graph. Each time estimations were conducted in triplicates. Standard graph for estimation of protein concentration was prepared using Bovine Serum Albumin (BSA) as standard. Standard curve was plotted for absorbance (at 595 nm) *vs*. protein concentration (µg/ml), where BSA concentrations of 50, 100, 150, 200, 250, µg / ml were used for the standard assay.

Purification of Cellulose by different methods

Three-Phase Partitioning (MLFTPP)

This approach of affinity precipitation combines the convenience of precipitation with the selectivity of bioaffinity. In MLFTPP, a solution of a smart polymer ("smart" means that the ligand is reversibly soluble and insoluble in response to a stimulus such as pH or temperature) is added to the crude extract of protein(s). Upon addition of optimized amounts of ammonium sulphate and t-butanol, an interfacial precipitate consisting of the smart polymer and the protein(s) having affinity for the polymer is obtained[13]. TPP of cellulase was carried out as follows.

Thus, to a culture filtrate of *Trichoderma sp.* (containing 54 U of cellulase activity), 30% (w/v) ammonium sulphate and tert.-butanol in a ratio of 1:2 (v/v; aqueous solution to tert.-butanol phase) was added and the reaction mixture incubated at 37 ºC for 1 h and formed three phases. The upper t-butanol layer was removed carefully with a pipette. After this, the lower aqueous layer was removed by piercing the interfacial layer using a pipette, and was stored at 4 °C for further analysis. The interfacial precipitate consisting of chitoson bound enzyme was dissolved in 6.115 ml of 0.05 M sodium citrate buffer and incubated at 4 °C for further analysis. Enzyme was then recovered by decreasing the pH because at lower pH chitosan became insoluble. The mixture of enzyme solution was centrifuged at 4000 r.p.m. for 45 min at 4 °C. The supernatant was taken out and subjected to extensive dialysis (for about 12 hours) in 0.05 M Sodium-citrate buffer of pH 4.8 at 4 °C.

Ammonium sulphate Precipitation

All procedures were carried out at 4 °C. For partial purification, solid ammonium sulphate (30 % saturation) was slowly added to the supernatant. The mixture was centrifuged at 10000 x g for 20 min, and discarded the pellet. Solid ammonium sulphate was added under stirring to a final saturation of 80 % saturation under ice cold conditions. The suspension was stirred for 1 h and kept overnight**.** The precipitate was collected by centrifugation at 12,000 X g for 20 min. dissolved the pellet in 0.05m sodium citrate buffer pH 4.8. The enzyme activity and specific activity were measured in both supernatant and pellet.

In order to remove chemical impurities, the dialysis tubing was boiled for 10 min in 10 mM sodium bicarbonate containing 1 mM EDTA. The tubing was washed extensively in distilled water and stored at 4 °C in 1 mM EDTA to prevent microbial contamination. For desalting, dialysis was carried out against 0.05m sodium citrate buffer pH 4.8 overnight at 4 °C under stirring. Finally, the desalted protein solution was centrifuged at 13,000 rpm to remove any undissolved material for 5 min. Afterwards the enzyme activity & specific activity were again measured. Protein content of the culture filtrate, purified cellulase measured by Bio-Rad reagent.

Gel filtration chromatography (Size-exclusion or molecular-sieve chromatography)

This is very effective technique in enzyme purification, is carried out with columns packed with swollen gel which separate components of a sample on the basis of molecular size. Molecules which are larger in size they pass out quickly without entering in pores of gel, whereas those molecules which are smaller in size they enter first in the pores of gel and pass out after the bigger molecule. So when we are collecting the fraction the bigger molecule collect first than smaller molecule .The actual speed of each component being dependent on the ease with which its molecule can pass in to the gels and be thus retarded.

The partially purified cellulase after dialysis was subjected to gel filtration chromatography. 1.5 ml of dialyzed enzyme was added in the column filled with swollen sephadex G-50. Fractions were eluted using 0.05 M sodium-citrate buffer, pH 4.8 with a flow rate of 1 ml /min. 90 fractions of 1.5 ml each were collected and stored at 4°C for further analysis. Protein content of the fractions obtained was measured by Bio-Rad dye reagent. Activity of the fractions of partially purified cellulase showing high protein content was estimated by CMCase assay.

SDS-PAGE Analysis of partially purified fractions

This process allows analytical electrophoresis of proteins in polyacrylamide gel under conditions that ensure dissociation of the proteins into their individual polypeptide subunits. The strongly anionic detergent SDS is used in combination with a reducing agent and heat to dissociate the proteins before they are loaded on the loaded on the gel. The denatured polypeptides bind SDS and become negatively charged. Because the amount of SDS bound is almost always proportional to the molecular weight of the polypeptide and is independent of its sequence, SDS-polypeptide complexes migrate through polypeptide gels in accordance with the size of the polypeptide. By using markers of known molecular weight, it is therefore possible to estimate the molecular weight of the polypeptide chain(s).

The prepared gel was mounted in the electrophoretic apparatus. Samples mixed with 6 x gel loading dye were heated in boiling water for 5 min & thereafter were centrifuged at 3000 r.p.m. for 5 min. About 20 µl (8 µg) of prepared sample was loaded into a well. 5 µl of low molecular weight marker (sigma) was

also loaded in one of the wells. A constant voltage of 200 volt was applied and the gel was allowed to run until the dye reaches almost bottom of the gel. After the completion of electrophoretic run, the gel was immersed in staining solution (Coomassie brilliant blue R-250) and placed on a slowly shaking platform for 2 hours at room temperature. The gel was destained by soaking it in destain solution placed on a slowly shaking platform for about 4-6 hours, changing the destain solution three to four times.

Characterization of Crude and Partially Purified Cellulose

All determinations were performed in duplicates and measured against blank samples.

pH tolerance

Both crude and partially purified cellulase (after gel filtration) from *Trichoderma sp*. were examined for their activity by incubating 0.5ml of the samples with 0.5ml of substrate (CMC; 2%) in buffers of different pH for 30 min. Activity of crude and partially purified cellulase was estimated by CMCase method.

Temperature tolerance

The temperature tolerance of the enzyme was studied by an assay of the enzyme mixture at temperature ranging from 30 -70 °C. 0.5 ml of the enzyme was incubated with the (0.5 ml) substrate solution at different temperatures (30, 40, 50, 60 and 70°C) for 30 min. Activity of culture filtrate and partially purified cellulase was estimated by CMCas assay at different temperatures.

pH stability

pH stability of the partially purified enzyme estimated after incubating the enzyme for different time interval at room temperature (approx 25 °C) mixed with pH 4.8 buffer. Aliquots were withdrawn at regular intervals of 6 h till 24 h and enzyme activity was measured by CMCase assay.

Temperature stability

The temperature stability of partially purified enzyme estimated after incubating the enzyme for different time intervals at 60 °C in sodium citrate buffer, pH 4.8. Aliquots were withdrawn at intervals of 3,6 & 24h and enzyme activity was measured by CMCase assay.

Effect of various chemicals

The effect of various chemical on partially purified cellulase was measured with CMCase method. These compounds change the structure of the protein by breaking the disulphide bonds. 0.5 ml of partially purified *Trichoderma sp*. cellulase & 0.5 ml of test compounds. Enzyme assay was performed as per the protocol described in material & method. Control was taken as the enzyme solution not subjected to treatment with any of the compounds.

Effect of divalent cations

The effect of different divalent cations at a final concentration of 10 mM was studied. 0.5 ml of purified enzyme & 0.5 ml of divalent cation solution were mixed to achieve a final metal ion concentration of 10 mM and incubated for 30 minutes. The enzyme activities were determined with the CMCase method. Enzyme not subjected to any metal ion treatment was taken as control

Effect of cellobiose

The effect of cellobiose was determined by incubating the partially purified cellulase with different concentration of cellobiose. 0.5 ml of partially purified enzyme and was mixed with cellobiose stock, to achieve a final concentration of 0.0025%, 0.025%, 0.05%, 0.10%, 0.15%, 0.25% & 0.5% . The enzyme activity was determined with the CMCase method. Enzyme not subjected to cellobiose treatment was taken as control

Effect of Glucose

The effect of glucose was determined in the presence of different concentration of glucose. 0.5 ml of partially purified enzyme and glucose stocks were mixed to achieve a final glucose concentration of 0.0025 %, 0.025 %, 0.05 %, 0.10 %, 0.15%, 0.25%. The enzyme activity was determined by CMCase assay. Enzyme not subjected to glucose treatment was taken as control.

Effect of Xylose

The effect of xylose was determined in the presence of different concentration of xylose. 0.5 ml of partially purified enzyme was mixed with glucose stocks to achieve final glucose concentration of 0.025 %, 0.05 %, 0.10 %, 0.15 %, 0.20%, 0.25% & 0.30%. Enzyme activity was determined using CMCase assay and compared against control, not subjected to xylose treatment.

Kinetic characterization of cellulose

Kinetic characterization was carried out in terms of K_m & V_{max} of cellulase. Experiments were carried out at 60 °C in 0. 05 M sodium-citrate buffer pH 4.8 at substrate (CMC) concentrations 5, 10, 15, 20, 25 & 30 and 35 mg/ml. Activity of partially purified cellulase was estimated using different concentrations of substrate solutions.

Chemical modification

Modification of Tryptophan by N-Bromosuccinamide

Partially purified cellulase from *Trichoderma sp.* was treated with varied concentration of NBS solution in a ratio of 9:1 such that in the 5 different reaction sets the final concentrations of NBS should be 0.1µM, 1.0 µM, 3.0 µM, 5.0 µM and 10 µM. All the reaction sets were incubated for at room temperature for 30 minutes. Untreated enzyme was used as positive control. After incubation, activity of the modified enzyme was estimated. Activity of the chemically modified enzyme was estimated by CMCase assay.

Modification of Tyrosine by N-Acetylimidazole

N-Acetylimidazole is a mild and selective protein acetylating reagent. It preferably acetylates exposed tyrosine residues but can also react with thiol groups and Є-amino groups of lysine. Partially purified cellulase (1ml) was treated with 100 µM N-Acetylimidazole in a ratio of 9:1 and was incubated for 1 hour at room temperature. After incubation 250 µl of reaction product was taken out and its activity was measured. The rest of the reaction product was dialyzed using 12 kDa dialysis bag for 12 hours at 4° C against 0.05 M Sodium-citrate buffer of pH 4.8. After dialysis the activity of enzyme was again measured. Chemically non-treated enzyme and 100 µM solution of N-Acetylimidazole were used as positive control. All the solutions were freshly prepared immediately before use, in 0.05 M sodium-citrate buffer of pH 4.8. Chemically modified and unmodified enzymes were assayed by CMCase assay.

Application Of Cellulose

Enzyme treatment of Agro-residues

The agroresidues were oven dried at 70 °C for overnight. 500 mg of each of the agro residue was taken and subjected to different treatments as described below:

1. 5 ml of distilled water was added to the samples and subjected to autoclaving at 121 °C (15 psi) for 15 minutes.
2. 5 ml of 0.5 % H_2SO_4 was added to the samples and subjected to autoclaving at 121 °C (15 psi) for 15 minutes.
   Reducing sugars released after the treatments was analyzed.
3. The samples were further subjected to enzymatic treatment after adjusting the pH to 4.5. Both commercial enzyme (Sigma, USA) and enzyme from *Trichoderma sp.* at an equivalent of 7.5 IU were added Final volume of the solution was made to 9.5 ml using buffer. Samples were incubated at 37 °C for 16 h and reducing sugars released were again measured

RESULTS AND DISCUSSION

Production of Cellulose

The production of cellulase from *Trichoderma sp.* was carried out on basal cellulase production medium. Flasks were inoculated with 1 ml of spore suspension and incubated at 30 °C and 200 rpm. The observation are presented in Table 1 showed that maximum enzyme production of 4.34 IU/ml and specific activity of 13.26 IU/ml was obtained after 144 h of incubation period.

Purification of Cellulose

Attempts were made for the purification of cellulase from *Trichoderma sp.* with a view to achieve an absolute purity of the enzyme produced.

Three phase partitioning

The aqueous phase and interfacial phase were collected and enzyme activity and protein content determined in both the phases. Results presented in Table 2 clearly reveal that the cellulase got bound to the chitosan in the interfacial layer. The specific activity though did not increased to a good extent and a

fold purification of 1.396 was only achieved. In an earlier study[13] it was found that starting with cellulase activity in the range of 9-54 U along with 4 ml of 0.2% (w/v) of chitosan, 97-98% of enzyme activity appeared in the interfacial precipitate during MLFTPP.

Ammonium sulphate precipitation

In order to reduce the protein impurities of the culture supernatant, it was subjected to 0-40 % and then 40- 80% ammonium sulphate saturation. The supernatant and pellet were collected after centrifugation. Enzyme activity and protein content measured and specific activity was calculated. It is very clearly evident from the Table 3 that 0-40 % ammonium sulphate precipitation lead to removal of other proteins, without much loss in the enzyme activity. Also the treatment of supernatant with 80 % ammonium sulphate saturation increased the specific activity, thereby leading to a 7.8 fold purification of cellulase.

Gel filtration chromatography

The partially purified protein sample obtained after ammonium sulphate precipitation was applied on Sephadex G-50. Proteins were eluted using sodium citrate buffer with a flow rate of 1.0 ml /min. 90 fraction of 1.5 ml were collected. Protein content was determined. Cellulase activity was estimated in the eluted fractions showing high protein content. The observations are presented in Table 4. Although, the distribution of enzyme in various fractions was in wide range, results obtained from gel filtration of dialyzed sample of cellulase from *Trichoderma* sp. showed a very high specific activity in fractions 36, 39, 40 and 42. The result needs further confirmation. These fractions were checked for purity of cellulase by SDS PAGE.

Analysis of Purity of cellulase by SDS – PAGE

10% SDS-PAGE followed by Coomassie Brilliant Blue (CBR) staining was performed to examine the protein profiles in various fractions purity and molecular weight of cellulose (Fig. 1). Lane 7 is showing high band intensity due to loading of higher protein concentration (20 μg).

Characterization of Cellolose

All determinations were performed in duplicates and measured against blank samples.

Effect of pH on enzyme activity

The effect of pH on the enzyme activity was investigated by determination of the activity at various pH values. The results were reported in Table 5 and 6 showed that the enzyme was active over a broad pH range from pH 4.0 to 6.0 (Fig. 2 and 3). The highest activity was found at pH 5.0. The results are in accordance with earlier reports, wherein optimum pH for cellulase activity from *Trichoderma sp. reesei*, *Thermoascus aurantiacus* and *Bacillus circulans* were more on the acid side (pH 4.5)[14,15,16]. The endo-ß-glucanase from the psychrotrophic yeast *Rhodotorula glutinis* strain KuJ 2731 was active between pH 2 - 7 with optimal activity at pH 4.5[17]. On the other side, more alkaline cellulases than the studied enzyme were reported such as the cellulases from *Neocallimastix frontalis* and *Bacillus* sp. showed an optimal pH value at 6.0[18,19]. While cellulase from the alkalophilic *Bacillus* sp. No. 1139 was most active at pH 9 and still retained some activity at pH 10.5, it showed no activity at pH 6[20].

Effect of temperature on enzyme activity

The activity of the crude and partially purified cellulase was examined at a temperature ranging from 30-70 °C. The enzyme activity was determined by CMCase assay. The results given in Table 7 and 8 showed that the enzyme was active over a broad range of temperatures (30°C - 70 °C) so far tested (Fig. 4 and 5). The temperature optimum for the enzyme was at 60 °C. Our results correspond with the earlier reports, wherein temperature optimum for endoglucanases from brown-rot fungi is found to be relatively high: 70°C for endoglucanase from *G. trabeum*[21], 60°C, *Phaeolus schweinitzii*[22] and *O. placenta*[23], and 50°C, *C. puteana*[24].

pH stability

The pH stability of cellulase was investigated by measuring the residual activities after 6, 18 and 24 h of incubation in sodium-citrate buffer, pH 4.8 at 25°C. The results obtained in Table 9, Figure 6 indicated that the enzyme retained more than 78.8 % of its normal activity till 6 h of incubation.

Temperature stability

The purified cellulase I solution was incubated in 50 mM sodium citrate buffer at different temperatures. The results presented in Table 7 showed that the enzyme was highly stable at temperatures of 60 °C under the described conditions and measuring the residual activities after 3, 6 and 24 h (Table 10 and Fig. 7).

Effect of various chemicals:

Cellulase activity of the partially purified enzyme from *Trichoderma sp.* was measured using CMCase assay in the presence of various compounds: 2-mercaptoethanol, dithiothreitol, potassium ferrocyanide and urea (0.02 %, final concentration). It is evident from the observations presented in Table 11, Figure 8 that various chemicals do not have any negative effect on cellulase activity.

Effect of divalent cations

The effect of metal ions on the enzyme activity was examined by measuring the activity in the presence of 10 mM of each metal ion. From the data given in Table 12, Figure 9; it could be noticed, that the metal ions had a wide variety of effects on the activity. $Fe3^{+}$ was found to be highly inhibitory for cellulase activity, whereas Cu^{2+} and Co^{2+} were inhibitory to a small extent.

Effect of Cellobiose

The effect of cellobiose on partially purified cellulase from *Trichoderma sp.* was examined by incubating the enzyme with varying concentrations of cellobiose and measuring the activity. The observations are presented below in Table13, Figure 10. Observations clearly reveal that cellobiose concentrations with 0.1% and above were inhibitory for cellulase activity. The results need to be further confirmed.

Effect of Glucose

The effect of glucose on the activity of partially purified cellulase from *Trichoderma sp.* was examined in the presence of different concentrations of glucose. Observations presented in Table 14, Figure11 very clearly reveal that concentrations above 0.025% are inhibitory for cellulase activity from *Trichoderma sp.* The results need to be further confirmed.

Effect of xylose

Effect of xylose on cellulase activity from *Trichoderma sp.* was studied by incubating the enzyme in presence of varying concentrations of xylose. It is clear from the Table 15, Figure 12 that xylose at lower concentrations was found to be stimulatory for cellulase activity, whereas increase in the concentration above 0.2% showed slight negative effect. The results need to be further confirmed.

Enzyme Kinetics

K_m and V_{max} for the enzyme were determined using various concentration of CMC as substrates. The enzyme assay was carried out at pH values (4.8) and at 60°C. The formation of reducing sugars in the reaction mixtures was estimated by CMCase assay. Observations are presented in table 16, Figure13. The K_m and V_{max} values of 7 mg/ml and 0.456 μmoles/ml/min. was obtained for partially purified cellulase from *Trichoderma* sp. (Table 17)

Chemical modification

The use of chemical modification to study the role of tryptophans in cellulose binding involves endoglucanase II from the fungus *Trichoderma reesei*[25]. EG II comprises a catalytic domain connected to a CBD from family I by a linker. The protein contains 11 tryptophans, 9 in the catalytic domain, and 2 in the CBD. CBDs in family I are about 35 amino acids long, folded into wedge-shaped, three-stranded P-barrels. Four aromatic residues, either tyrosine or tryptophan, are strictly conserved in these CBDs. Three of the aromatic residues are exposed on a hydrophobic face of the wedge. In $CBD_{EG\ II}$, one of these exposed aromatic amino acids is a tyrosine, two are tryptophans[26].

It is thought that the aromatic amino acid residues such as tryptophan, tyrosine packs sugar rings giving extra stability and specificity to enzyme substrate complex[27]. Keeping the above studies in mind, cellulase from *Trichoderma sp.* was modified with various chemical probes and efforts were made to conclude the role of probable amino acid residues like tryptophan and tyrosine present in various sites of cellulase.

Modification of Tryptophan by NBS

Modification of tryptophan residues present in the catalytic domain (SBD) and other sites using oxidation by N- Bromosuccinimide was carried out by the protocol described in Materials and Methods. Cellulase activity was further assayed.

The observations presented in Table 18 clearly reveal that N- Bromosuccinamide shows strong inhibition of cellulase as it showed complete inactivation of cellulase from *Trichoderma sp.* at a low concentration of 5 µM. The results are in accordance[28] wherein they have shown that after 10 min of incubation, the purified EG-III was strongly inhibited and lost up to 90% of the initial activity when incubated with 5 µM NBS, .

In another study, the modification of the equivalent of one and two tryptophans by NBS reduces binding of CBDcex to cellulose by 70% and 90%, respectively. This confirms the direct role of the exposed aromatic residues in the binding of CBDcex to cellulose[29]. Although adsorption to cellulose does afford some protection against NBS, as evidenced by the increased quantity of NBS required to oxidize all of the tryptophan residues, the polypeptide can still be oxidized completely when adsorbed. This suggests that, whereas the binding appears to be irreversible overall[30], *Bio/Technology* 76044071, each of the exposed tryptophans interacts reversibly with cellulose.

Effect of N-acetylimidazole on cellulase activity

The effect of N-acetylimidazole, a mild acetylating reagent on the catalytic activity of cellulase was studied. The cellulase activity was further estimated. Observations are presented in Table 19. The results show that the enzyme retained 60.2 % of the enzyme activity after chemical treatment. This result indicates that acetylation of tyrosyl groups changes the enzymatic activity of cellulase. Further experimentation using enzyme inhibitor is required to confirm the prediction. Later, when the chemically modified enzyme was subjected to dialysis, no activity was retained back. This indicates that the acetylation of tyrosine by N-Acetylimidazole looks like a non-reversible reaction. Further experimentation are needed for the confirmation. In an earlier report, modification of endoglucanase from a mutant strain of *Trichoderma sp.* with N-acetylimidazole (50 mM) over an extended period of 3 h did not result in any loss of their activity indicating that tyrosine residues are not involved in the catalysis[28].

Enzymztic treatment of Agro-residue

The agroresidues were subjected to different treatments as described below:

1. **Physical treatment :** autoclaving at121 ℃ (15 psi) for 15 minutes.
2. **Chemical treatment followed by physical treatment:** 0.5 % H_2SO_4 was added to the samples and subjected to autoclaving at 121 °C (15 psi) for 15 minutes.
3. The samples were further subjected to enzymatic treatment after adjusting the pH to 4.5. Both commercial enzyme (Sigma, USA) and enzyme from *Trichoderma sp.* at an equivalent of 7.5 IU were added. The results presented in table 20 and 21 clearly show that chemical treatment followed by physical treatment is found to support high sugar release as compared to just physical treatment.

Maximum conversion of 22.76 % and 22.14% was obtained from Agro-waste1 and Agro-waste 2 using *Trichoderma* sp. cellulase which was high as compared to commercial cellulase, thus proving the hydrolytic potential of the selected cellulase.

Table 1: Cellulase production from *Trichoderma sp.*

Incubation period (hr)	CMCase activity(IU/ml)	FPase activity (IU/ml)	Specific activity IU/mg protein for CMCase
144	4.34	0.80	13.26

Table 2: Purification of cellulase from *Trichoderma sp.* using Three phase partitioning

Serial No.	Enzyme activity IU/ml	Protein content mg/ml	Specific activity IU/mg protein
Culture filtrate	4.55	0.315	13.26
Inter phase	1.13	0.150	18.52
Aqueous phase	---	0.066	0.0

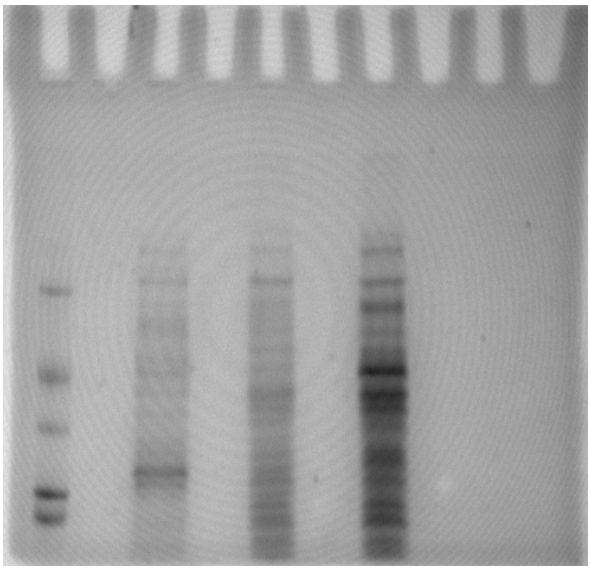
Table 3: Partial purification of cellulase from *Trichoderma sp.* using ammonium sulphate precipitation

saturation		Enzyme activity IU/ml	Protein content mg/ml	Specific activity IU/mg protein
Culture filtrate-	-	308.0	22.26	13.83
0 - 40	Pellet	0.521	0.82	0.635
	Supernatant	291.0	20.34	14.30
40 - 80	Pellet	121.46	1.128	107.67
	Supernatant	0.00	0.90	0.00

Table 4: Analysis of fractions obtained after gel filtration

Fractions	Enzyme activity (IU /ml)	Protein content (mg/ml)	Specific activity (IU/mg protein)
24	0.058	0.0097	6.03
25	0.337	0.0210	16.0
26	0.459	0.0192	23.9
27	0.574	0.0176	32.61
28	0.452	0.0115	39.30
29	0.682	0.0342	19.94
30	0.686	0.0188	36.48
31	0.485	0.0084	57.73
32	0.295	0.0076	38.36
33	0.330	0.0130	25.38
34	0.330	0.0111	29.72
35	0.344	0.0103	33.39
36	0.372	0.0030	121.17
37	0.544	0.0315	17.26
38	0.311	0.0115	27.04
39	0.290	0.0011	263.23
40	0.243	0.00028	846.6
41	0.224	0.0046	48.69
42	0.147	0.0007	210.0

Lane 1 2 3 4 5 6 7 8



Lane 1: Molecular weight marker
Lane 3: Cellulase culture filtrate from *Trichoderma sp.*
Lane 5 & 7: Partially purified cellulase from *Trichoderma sp.*

Table 5- Activity of cellulase culture filtrate of Trichoderma sp. at different pH

pH	Enzyme activity (IU/ml)	Relative activity (%)
4.0	1.33	39.46
4.5	2.80	83.08
5.0	**3.37**	**100**
5.5	3.32	98.5
6.0	2.57	76.26

Figure 2: Activity of cellulase culture filtrate of Trichoderma sp. at different pH

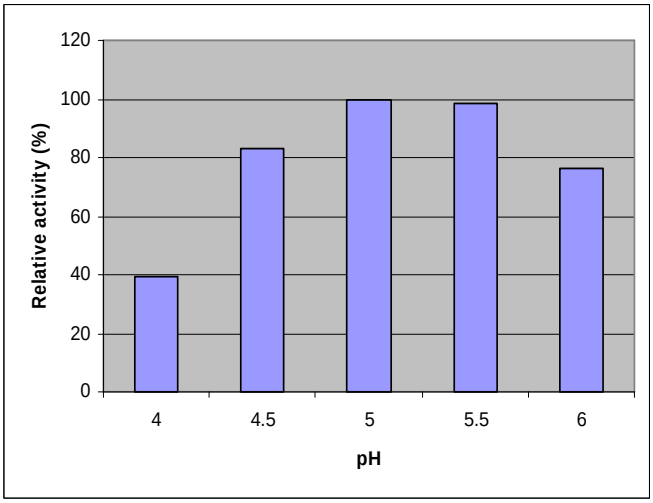


Table 6: Activity of cellulase Partially purified (after gel filtrations)of Trichoderma sp. at different pH

pH	Enzyme activity (IU/ml)	Relative activity (%)
4.0	0.360	52.0
4.5	0.508	73.5
5.0	**0.691**	**100**
5.5	0.597	86.3
6.0	0.574	83.0

Figure 3: Activity of cellulase Partially purified (after gel filtrations)of Trichoderma sp. at different pH

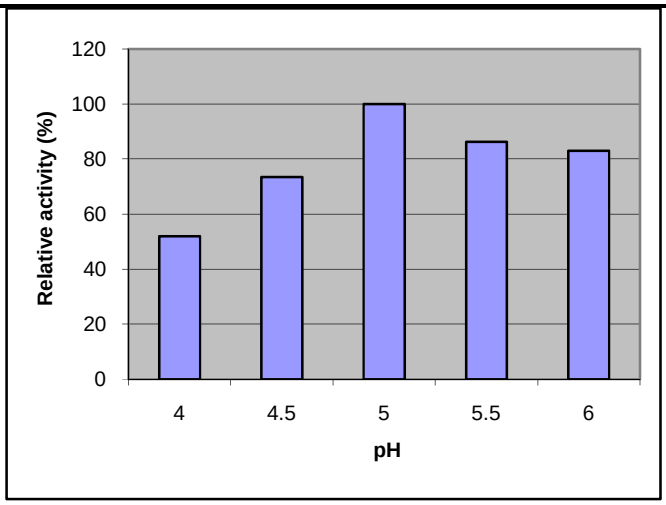


Table 7: Activity of cellulase culture filtrate of Trichoderma sp. at different temperature

Temperature (°C)	Enzyme activity (IU/ml)	Relative activity (%)
30	1.86	54.86
40	1.96	57.81
50	2.75	81.12
60	**3.39**	**100**
70	2.39	70.50

Figure 4: Activity of cellulase culture filtrate of Trichoderma sp. at different temperature

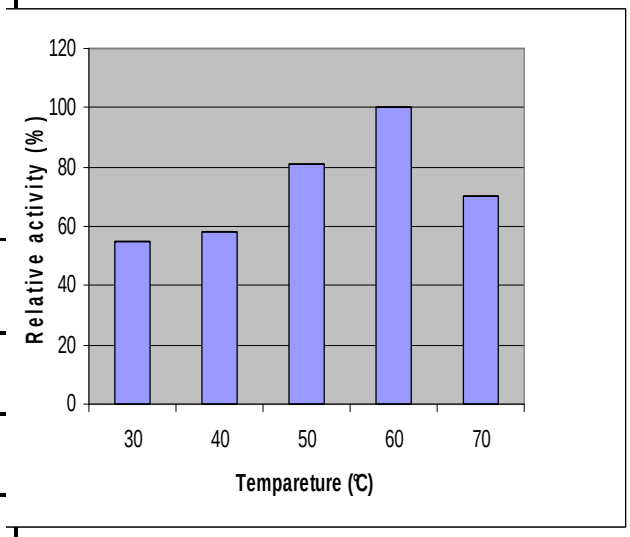


Table 8: Activity of Partially purified cellulase of Trichoderma sp. at different temperature

Temperature (°C)	Enzyme activity (IU/ml)	Relative activity (%)
30	0.393	64.5
40	0.506	83.0
50	0.574	94.0
60	**0.609**	**100**
70	0.473	77.60

Figure 5: Activity of Partially purified cellulase of Trichoderma sp. at different temperature

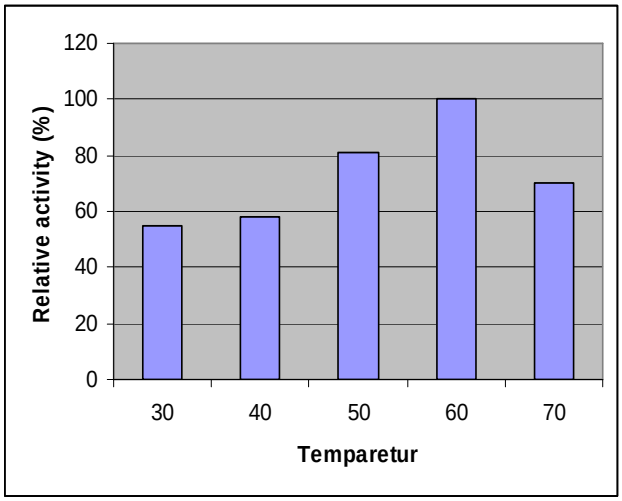


Table 9: Residual activity of cellulase (partially purified)of Trichoderma sp.. at 4.8 pH

Incubation time (h)	Enzyme activity (IU/ml)	Residual activity (%)
control	0.585	100
6	0.47	78.8
18	0.35	59.82
24	0.335	57.26

Figure 6: Residual activity of cellulase (partially purified)of Trichoderma sp.. at pH 4.8

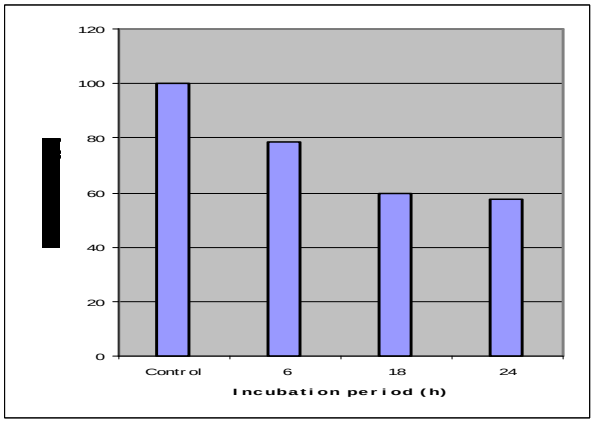


Table 10: Residual activity of cellulase (partially purified) of Trichoderma sp. at 60 in different Time interval

Time interval	Enzyme activity (IU/ml)	Residual activity (%)
control	0.70	100
3 hr	0.43	61.42
6 hr	0.35	50.0
24 hr	0.25	36.78

Figure 7: Residual activity of cellulase (partially purified of Trichoderma sp. at 60 in different Time interval

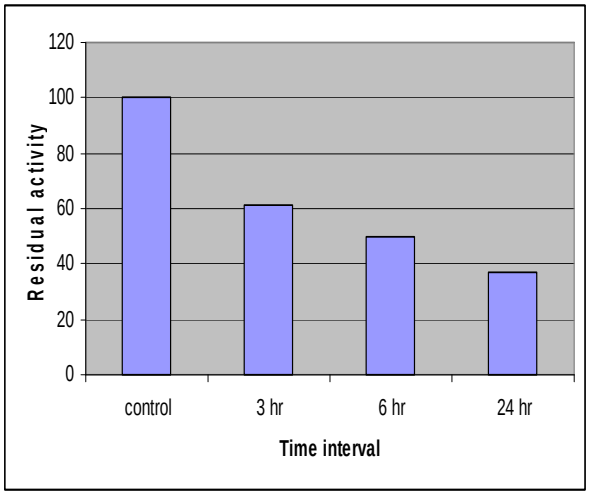


Table 11: ***Effect of various chemicals on partially purified cellulase of Trichoderma sp.***

S .No.	Enzyme activity (IU/ml)	Relative activity (%)
1 Control	0.52	100
2 β-mercaptoethanol	0.51	98.0
3. Dithiothreitol	0.75	144
4. Urea	0.54	103

Figure ***8: Effect of various chemicals on partially purified cellulase of Trichoderma sp.***

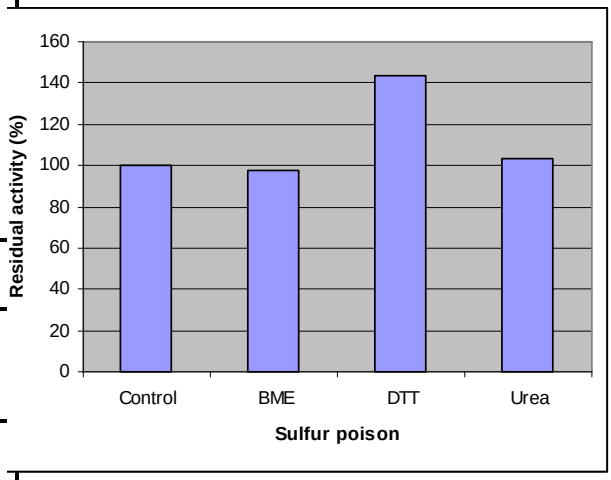


Table 12: ***Effect of*** **divalent cations** ***on partially purified cellulase of Trichoderma sp.***

Divalent cations	Enzyme activity (IU/ml)	lative activity (%)
1.Control	0.105	100
2.$CaCl_2.2H_2O$	0.077	73.16
3.$CuCl_2.2H_2O$	0.041	39.02
4. $CoCl_2.6H_2O$	0.039	36.99
6.$FeCl_3$	0.013	12.19
8.$MgCl_2.6H_2O$	0.086	81.30

Figure ***9: Effect of*** **divalent cations** ***on partially purified cellulase of Trichoderma sp.***

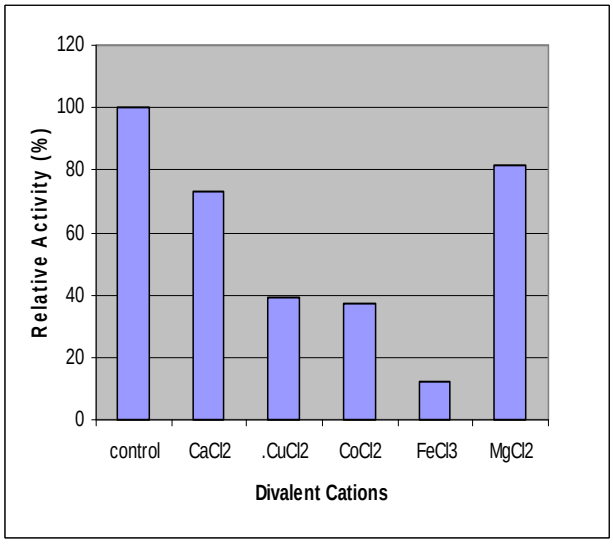


Table 13: ***Effect of Cellobiose on partially purified cellulase of Trichoderma sp.***

S .N	Enzyme activity (IU/ml)	Relative activity (%)
1 Control	0.40	100
2 0.0025%	0.40	100
3 0.025 %	0.39	97.5
4 0.050 %	0.40	100
5 0.100 %	0.38	95
6 0.15 %	0.36	90
7 0.25 %	0.12	30
8 0.5 %	0.0.	0

Figure ***10: Effect of Cellobiose on partially purified cellulase of Trichoderma sp.***

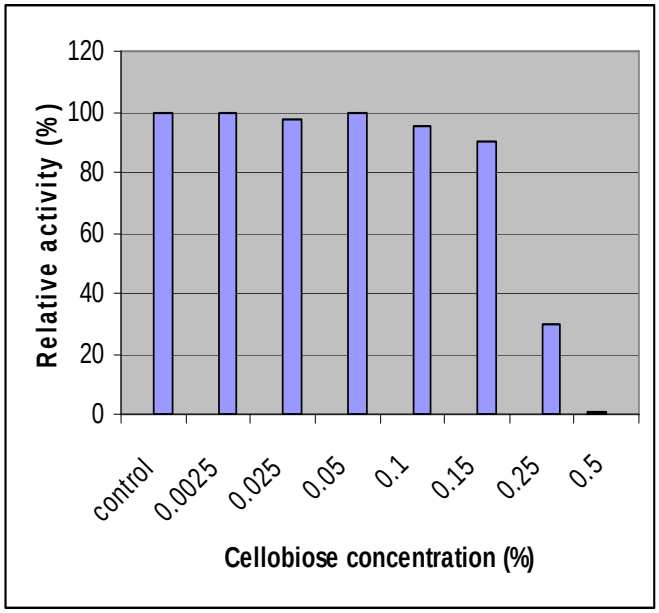


Table 14: ***Effect of Glucose on partially purified cellulase of Trichoderma sp.***

S .N	Enzyme activity (IU/ml)	Relative activity (%)
1 Control	0.58	100
2 0.0025%	0.68	117
3 0.025 %	0.46	79.31
4 0.050 %	0.42	72.41
5 0.100 %	0.38	65.56
6 0.15 %	0.33	56.89
7 0.25 %	0.0	0

Figure ***11: Effect of Glucose on partially purified cellulase of Trichoderma sp.***

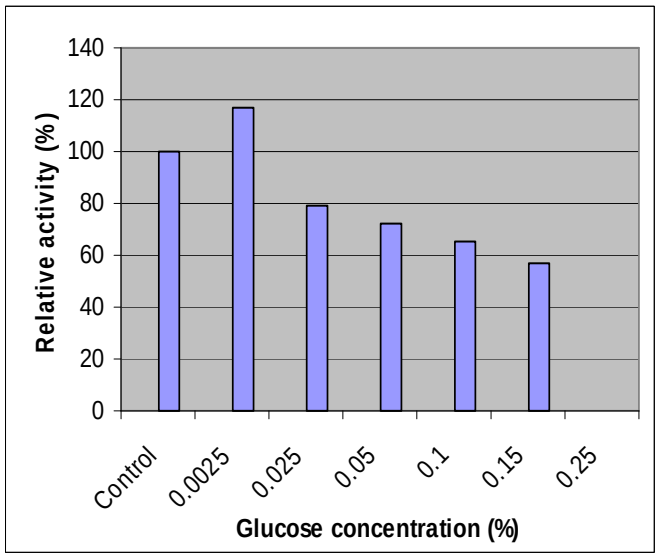


Table 15: ***Effect of xylose on partially purified cellulase of Trichoderma sp.***

S .N	Enzyme activity (IU/ml)	Relative activity (%)
1 Control	0.41	100
2 0.025%	0.55	132
3 0.05 %	0.56	136
4 0.10 %	0.61	147
5 0.15 %	0.54	130
6 0.20 %	0.41	100
7 0.25 %	0.37	89
8 0.3 %	0.31	76

Figure ***12: Effect of xylose on partially purified cellulase of*** *Trichoderma sp.*

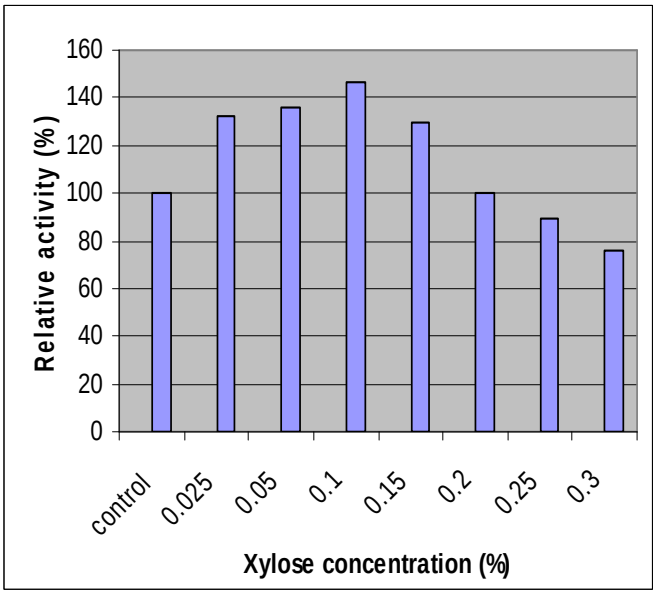


Table 16: Effect of substrate concentration on the initial velocity of partially purified cellulase from Trichoderma sp.

Substrate conc. In % [S]	Enzyme activity IU/ml
0.5	0.126
1 .0	0.269
1.5	0.332
2.0	0.456
2.5	0.412
3.0	0.449
3.5	0.449

Figure 13: Effect of substrate concentration on the initial velocity of partially purified cellulase from Trichoderma sp.

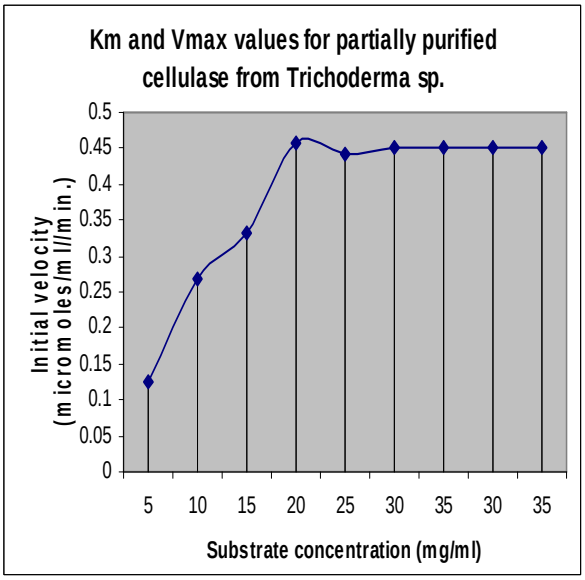


Table 17: *K_m and V_{max} of cellulase*

Enzyme source	Enzyme	K_m with CMC (mg/ml)	V_{max} (µmoles/ml/min)
	Partially Purified	7	0.456

Table 18: *Activity of Partially purified Cellulase before and after treatment with NBS*

Enzyme	Conc. of NBS (µM)	Enzyme activity (IU / ml)	Residual activity (%)
Untreated enzyme	---	0.107	100
NBS-treated enzyme	0.10	0.107	100
	1.0	0.093	86.9
	3.0	0.089	83.17
	5.0	0	0
	10	0	0

Table 19: *Activity of cellulase before and after treatment with 10 µM N-acetylimidazole*

Reaction step	Sample	Enzyme activity (IU / ml)	Relative activity (%)
Before dialysis	Native enzyme	0.73	100
	Chemically treated enzyme	0.44	60.2
After dialysis	Native enzyme	0.72	100
	Chemically treated enzyme	0.37	51.0

Table 20: % Conversion of Agro-residues into reducing sugars

	Physical treatment	Enzymatic treatment
Agrowaste 1	1.49	10.86
Agrowaste 2	2.63	7.03
Agrowaste 3	1.59	11.29

Table 21: % Conversion of Agro-residues into reducing sugars

	Chemical treatment	Enzymatic treatment	
		Trichoderma sp. cellulase	Commercial cellulase (Sigma)
Agrowaste 1	5.6	22.76	21.02
Agrowaste 2	9.50	17.47	15.63
Agrowaste 3	5.84	22.14	19.46

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