View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by AJOL - African Journals Online

academicJournals

Vol. 12(28), pp. 4512-4518, 10 July, 2013
DOI: 10.5897/AJB2013.12346
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Isolation, Characterization and Molecular weight determination of Cellulase from *Trichoderma viride*

Salwee Yasmin[1]*, R. L. Mattoo[2] and F. A. Nehvi[1]

[1]Faculty of Agriculture, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir, India.
[2]School of Biotechnology,University of Jammu, India.

Accepted 5 July, 2013

Cellulose hydrolyzing enzyme from fungus *Trichoderma viride* was purified and characterized. The cellulase production was variable depending upon the type of cellulose the fungus grew on; it was higher when grown on cellulose or whatmann filter paper than on other carbon source viz carboxy methyl cellulose. Enzyme purification to homogeneity was carried out by anion exchange chromatography on DEAE-Sepharose. SDS-PAGE revealed molecular mass of 87 kDa. Maximal activity of the enzymes was observed at 50°C at pH 4 and was stimulated by Ca^{2+}, Co^{2+}, Mg^{2+} (test at 10 Mm each) and inhibited by Fe^{2+}. Ethanol at an optimum concentration of 2% stimulated the initial enzyme activity. The end product of cellulase action was glucose and cellobiose. The enzyme therefore qualifies as an exo-β 1, 4-glucanase. Thermostability, pH and stability in the presence of surface active agents make this enzyme potentially useful in industry particularly for ethanol production.

Key words: Enzyme, cellulose, cellulase.

INTRODUCTION

Cellulose is a linear polymer of up to 15,000 D-glucose residues linked by β (1-4) glycosidic bonds and is an enzyme that hydrolyzes the β-1, 4-glycosidic bonds in cellulose to release glucose units with distinction of being the most abundant component of plant biomass, found exclusively in plant cells and is produced in some bacteria and animals (Nishida et al., 2007). A multienzyme complex, components of the cellulose enzymes were first classified on the mode of catalytic action and have also been categorized based on the structural properties. Three major types of enzyme activities are found: 1) Endoglucanase; 2) Exoglucanase and 3) β-Glucosidase. These enzymes carry out hydrolysis by the synergistic action (Liming and Xueliang, 2004; Vinha, 2011).

Cellulose utilization proceeds via organisms that are either aerobic or anaerobic but not both. Cellulases have been widely studied in the case of cellulolytic microorganisms from the soil and resident bacteria ruminants. The cellulolytic system of filamentous fungus Trichoderma reesei has been studied because of its efficiency in degrading native cellulose substrate (Teeri et al.,1992). The present study aimed the isolation and biochemical characterization of cellulose from *Trichoderma* for biotechnological application because any process which could efficiently and economically convert celluloytic material to glucose would be of immense industrial significance (Walsh, 2002).

MATERIALS AND METHODS

Fungal strain

Scrapped Whatmann filter paper 12.5 cm diameter was sandwiched between two thin agar layers (2%) in sterile Petri plates and pre-

*Corresponding author. E-mail: Salwees@yahoo.com.

incubated at room temperature for three days. When no growth occurred they were inoculated. When fungal growth appeared, pure culture was isolated by repeated sub-culturing maintained on 2% potato dextrose agar until a pure fungus was isolated and identified as *Trichoderma viridae*, which was screened for cellulolytic activity using congo red staining technique (Beguin, 1982).

Isolation of Cellulase

Sterile M-9 medium was used for inoculation. Two different carbon source were used namely scrapped Whatmann filter paper and carboxy methyl cellulose (HIMEDIA). The broth with Whatmann filter paper was sonicated for 4 min prior to inoculation. The 250 ml Erlenmeyer flask containing 50 ml media were inoculated by adding a scoop of culture from fresh PDA plate, incubated on gyratory shaker incubator for 9 days at 28.5°C and 120 rpm.The broth was regularly monitored for growth and cellulolytic activity and all the assays were carried out in triplicates and the mean values were subjected to analysis of variance for estimation of standard error of mean and standard error of differences (Johnson et al., 1955).

Protein estimation

Protein exuded in the medium was monitored regularly checking absorbance at 280/260 nm (Warburg and Christian, 1942) using corresponding controls as blank Protein (µg/ml)=1.55A_{280}-0.76A_{260} Assay. The assay was performed using:

Cellulose powder as a substrate

The assay recipe had 5 mg of cellulose and 500 µl of enzyme supernatant in 0.1 M Tris-HCl pH 5.3. After 3 h of incubation at 40°C in a hybridization oven/shaker, the contents were centrifuged and reducing sugars released from cellulose were quantified in the supernatant.

Partially hydrolysed cellulose as a substrate

Whatmann filter paper was scapped and 300 mg of this were mixed with 1 ml of 1N HCl and boiled at 100°C till slurry was formed. The slurry was treated with 1N NaOH and pH 5.3 after 3 h in a hybridization oven/shaker. At the end of the incubation period, the samples were cooled, centrifuged at 4°C at 10,000 *g* for 10 min and an aliquot of the supernatant employed for quantifying by measuring the released reducing sugar (total) as a Schiff base derivative with o-toludine (Cooper et al., 1970). 100 µl aliquot of the broth was added to 3 ml 0-toluidine reagent; suitable blank and glucose as standard were similarly and simultaneously employed. All the tubes were vortexed and placed in a boiling water bath for 12 min. After cooling, the absorbance was read at 630 nm against reagent blank which contained distilled water instead of aliquot.

Purification of cellulase

Whole broth was centrifuged at 10, 000 *g* for 20 min and pellet washed twice with M9 medium. The two supernatents obtained were combined and an aliquot set aside for determining cellulose activity protein profile on the SDS-PAGE prior to anion exchange chromatography. The anion exchange coloumn was packed with DEAE-Sepharose (1 × 20 cm) thoroughly equilibrated with 50 Mm Tris-HCl pH 7.8. The elution was effected with 40 ml linear gradient of NaCl in the range of 0 to 0.5 M in 50 Mm Tris HCl, buffer pH 8.5 and 1.5 ml fractions collected at a flow rate of 1 ml per min. 16 fractions of 1.5ml each were collected.

Molecular weight determination

Samples showing a peak at A_{280} were selected and SDS-PAGE was run .The molecular weight of the cellulase was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and compared with molecular marker - Sigma (Sambrook and Russel, 2001).

Thin layer chromatography

At the end of the assay of finally purified preparation, an aliquot was subjected to TLC, employing HPTLC plate of silica gel and a mobile phase of chloroform:methanol:water (90:65:15). The plates were air dried, sprayed with phenol sulphuric acid mixture (phenol 3 g, sulphuric acid 5 ml,ethanol 92 ml) and spots detected by charring the plate in on hot air oven at 90°C or on a hot plate.

Kinetic properties

Effect of incubation time on enzyme activity

Half a milliliter of enzyme in 0.1 M Tris-HCl was incubated with 0.5% cellulose solution and 50 µl aliquot withdrawn after every hour to determine the amount of reducing sugars released.

Optimum temperature and thermal stability

An aliquot of 500 µl of enzyme in 0.1 M Tris-HCl,pH 5.3 were incubated with cellulose (0.5% final concentration) at different temperature range from 30 to 60°C for determining the optimum concentration. At the end of assay, the amount of reducing sugars was measured using o-toluidine coupled reaction.

Thermal studies were carried out by pre-incubating the enzyme for 3 h at different temperature; room temperature, 0-4°C, (-) 20°C and (-) 80°C followed by enzyme assay.

Optimum pH

Optimum ph was determined by incubating 500 µl of enzyme with 0.5% cellulose solutions at pH ranging from 3 to 10; at the end of the reaction, the supernatant was taken and the amount of reducing sugar determined.

Effect of divalent ions

Enzyme assay was carried out using 0.1 M citrate-phosphate buffer at pH 4 and 50°C in the presence and absence of 10 mM Fe^{2+}($FeCl_2$), Co^{2+}($CoCl_2$), Zn ($ZnSO_4$), Ca^{2+}($CaCl_2$), $Mg^{2+(}$$MgCl_2$), respectively and amount of total reducing sugar was determined after 3 h.

Effect of surface active agents

Cellulase assay was done at pH 4 employing 0.1 M Citrate phosphate buffer and temperature of 50°C in the presence and absence of non-ionic detergent, Triton-X100, at a final concentration of 0.1% and also 0.2 and 5% ethanol and after 3 h of incubation, the amount of reducing sugar was determined and compared.

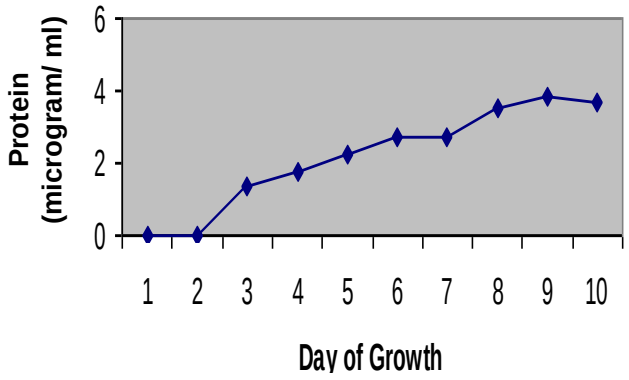


Figure 1. Pattern of growth on whatman filterpaper.

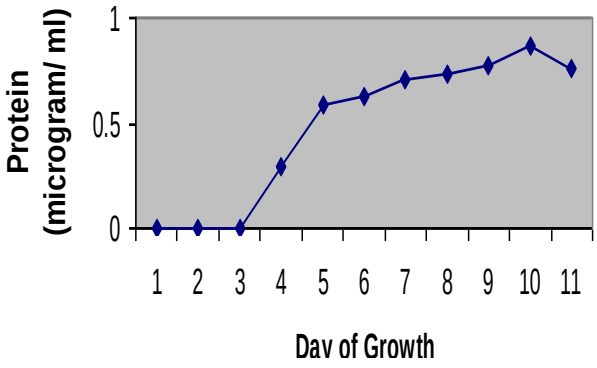


Figure 2. Pattern of growth on carboxymethyl cellulose.

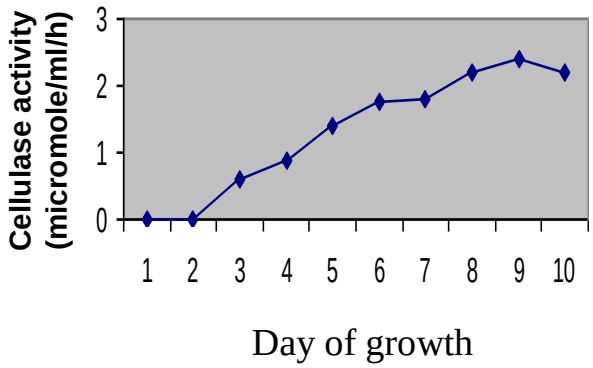


Figure 3. Cellulase activity with Whatman filterpaper.

Comparative efficacy of cellulase action on cellulose and derived cellulose

Cellulase action was compared towards different types of substrates by determining the enzyme activity, employing commercially available cellulose powder (HIMEDIA) and carboxymethyl cellulose.

RESULTS AND DISCUSSION

The present study is related to biochemical characterization of cellulase from Trichoderma strain identified as *T.*

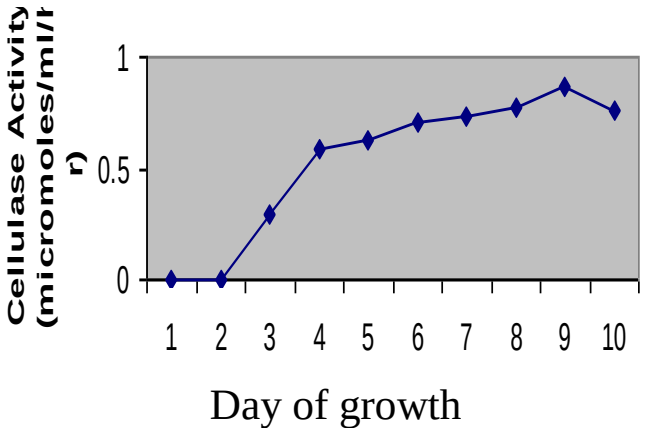


Figure 4. Cellulase activity with carboxymethyl cellulose.

viridae (MTCC). Agneiszka et al. (2002) reported that selection of suitable carbon and energy source has a particular importance in the process of extracellular production of hydrolases by filamentous fungi. The study reveals that pattern of growth on two types of cellulose was significantly different as also reported by Geimba (1999) with maximal growth and cellulose activity with whatman filterpaper on 9th day (2.40 cellulase units) and on the 10th day with carboxymethyl cellulose (0.099 cellulase units) as carbon source where after, it registered a decline (Figure 1 to 4 and Table 1). Similar observation has also been reported by Targonski and Wojcik (1993) in the case of *Trichoderma reesei* M-7. The enzyme being an exoglucanase shows very little activity with carboxymethyl cellulose something that has been observed with glucanase (Takashima et al., 1998).

The purified cellulase shares many enzymatic properties with known glucanases from plants and micro-organisms. By analyzing the products of enzymatic reaction and by measuring the liberated sugars, it is revealed that the purified enzyme is an exo-glucanase. Elution at 0.43 M showed highest cellulase activity of 2.5 units per 1.5 ml for fraction 14 and SDS-PAGE of the fraction revealed a sub-unit molecular weight of 87 kDa (Plate 1). On silica-gel 60 plates, there appeared two products of reaction mixture, namely glucose and cellobiose apparently in almost equal proportion (Plate 2). The two spots correspond with monosaccharide and disaccharide. Since the SDS-PAGE revealed that the enzyme preparation was apparently homogenous with (sub) molecular weight of protein as 87 kDa, it appears that this enzyme is an exo-cellulase that splits disaccharide units and also possesses disaccharidase activity such that monosaccharide are also generated.

Alternatively, this enzyme may have a preference for a trisaccharide such that, a maono and a di-saccharide are generated. The multiplicity of the individual cellulases might be the result of post-translational and/or post secretional modifications of a gene product or might be

Table 1. Pattern of growth and Cellulase activity.

Day	Whatmann filter paper		Carboxymethyl cellulose	
	Protein (µg/ml)	Cellulase unit (µmoles/ml/h)	Protein (µg/ml)	Cellulase units (µmoles/ml/h)
3	1.33	0.6	0.30	0.031
4	1.796	0.9	0.585	0.042
5	2.214	1.40	0.630	0.061
6	2.682	1.76	0.713	0.070
7	2.76	1.80	0.738	0.072
8	3.49	2.20	0.769	0.081
9	3.88	2.4	0.861	0.098
10	3.71	2.21	0.7577	0.099
11			0.721	0.096
SE(m)	0.046			
SE(d)	0.070			
CD ($p < 0.01$)	1.145			

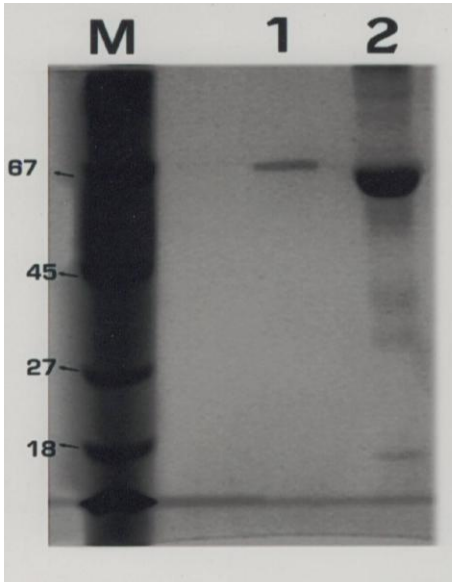


Plate 1. SDS-PAGE M, maker (in kDa); lane 1, purified enzyme; lane 2, crude enzyme.

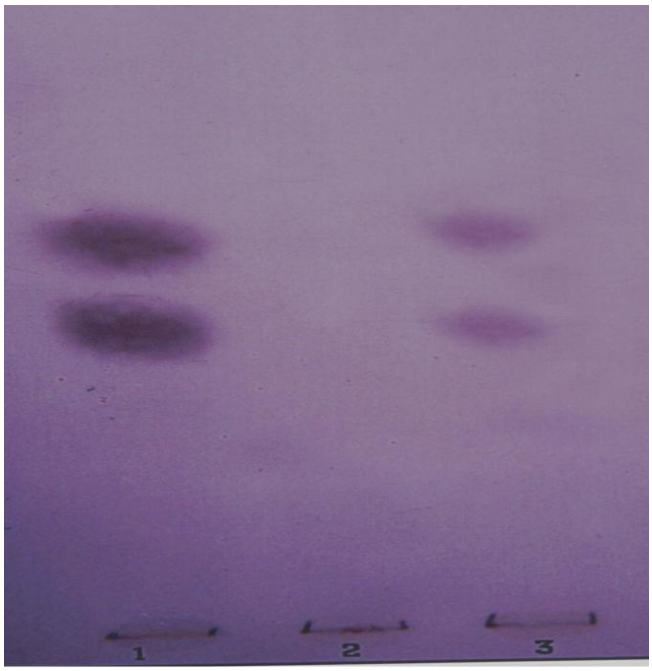
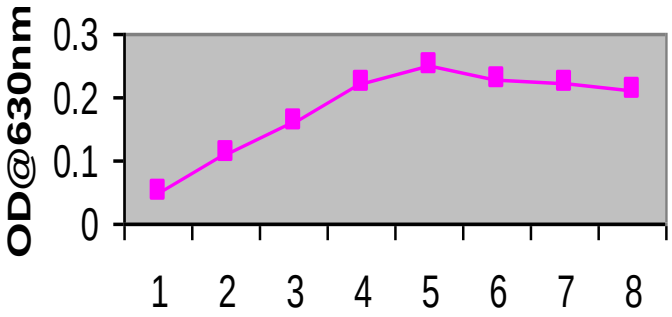


Plate 2. Thin layer chromatography; lane 1, standard (clucose cellobiose); lane 2, (substrate blank); lane 3, experimental.

due to multiple genes as has been reported by Maheshwari et al (2000).

Cellulase enzyme activity was characterized for incubation time, thermal stability, assay temperature, optimum pH, divalent ions and surface active agents. Perusal of Figures 5,6,7,8,9, 10 reveals that with increase in the incubation time, there was corresponding increase in the cellulase until 5 h (0.24) where after, it showed no increase and reached a plateau (Figure 5). Exposing cellulose to temperatures of (-) 80°C, (-) 20°C, 0-4°C and room temperature (30°C), it was observed that the enzyme showed stability at 4°C to 30°C. However freezing or ultra-freezing resulted in 75 and 80% loss in activity, respectively (Figure 6). The maximal cellulose activity was observed on incubation with 0.5% cellulose at 50°C, although considerable activity was also seen at 40°C (71%) and 60°C (79%). At 30°C, it retained about 54% of activity as compared with that observed at 40°C. Therefore the enzyme appears to be active over a wide range of temperature (30 to 60°C) (Figure 7) The maximal cellulose activity was observed at pH 4.0. However, cellulose exhibited nearly 40% of the maximal activity in the entire alkaline pH range (7 to 9) and as much as 70% at pH 3.0 (Figure 8). Several cellulases from plants (Bryne, 1975), fungi (Macarron et al., 1993) and bacteria



SE(m)+/-	0.009
SE(d) +/-	0.0136
CD (p < 0.01)	0.0385

Figure 5. Effect of incubation time on enzyme activity.

SE(m)	1.06
SE(d)	1.56
CD (p < 0.01)	5.32

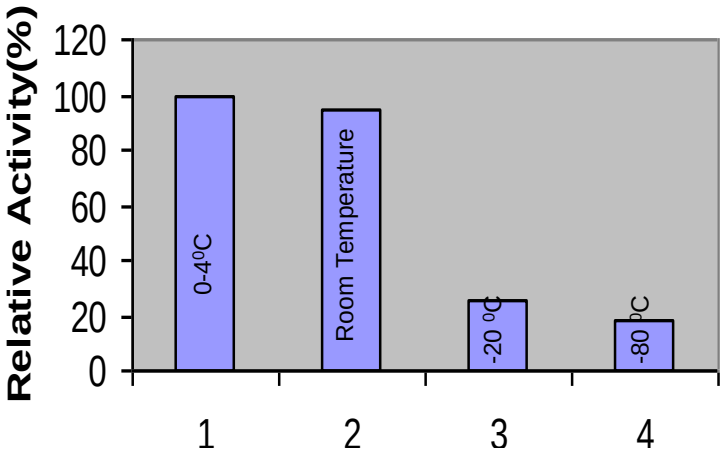
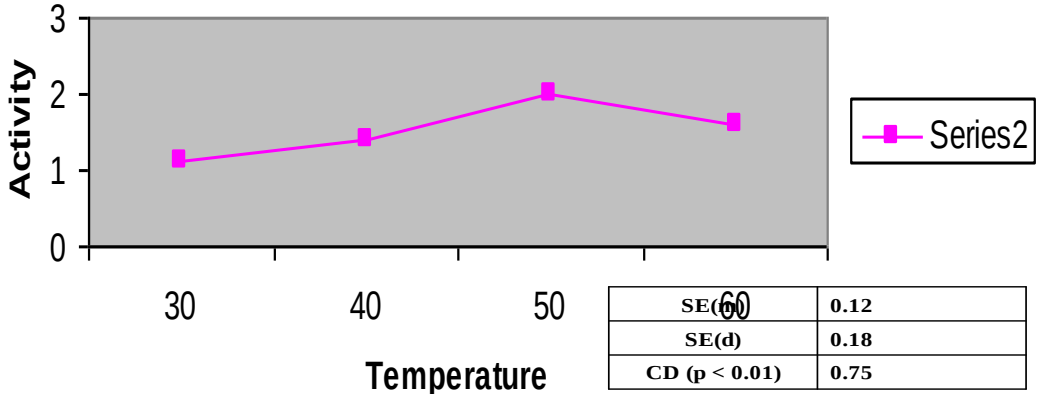


Figure 6. Thermal stability.



SE(m)	0.12
SE(d)	0.18
CD (p < 0.01)	0.75

Figure 7. Assay temperature versus Enzyme activity.

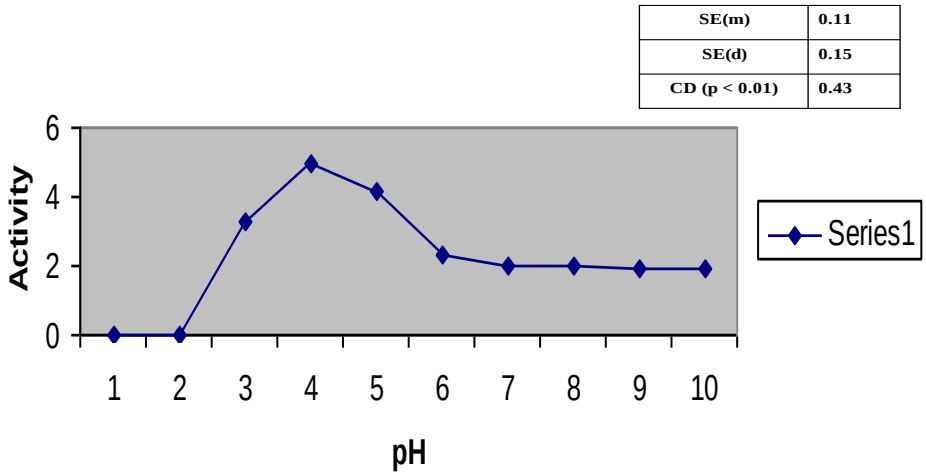


Figure 8. Optimum pH.

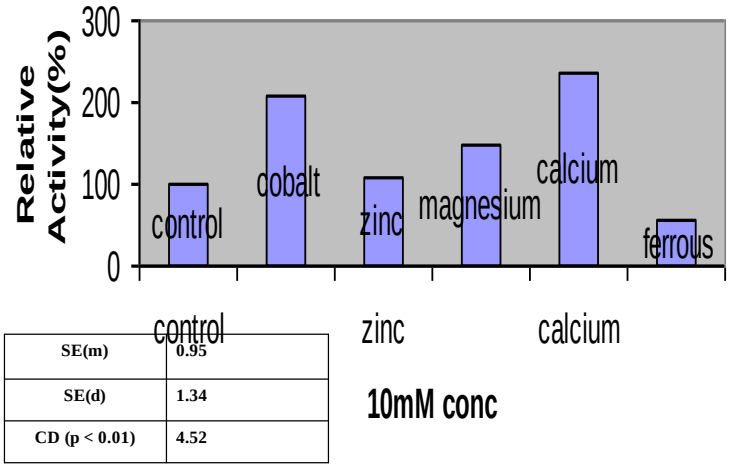


Figure 9. Effect of divalent ions.

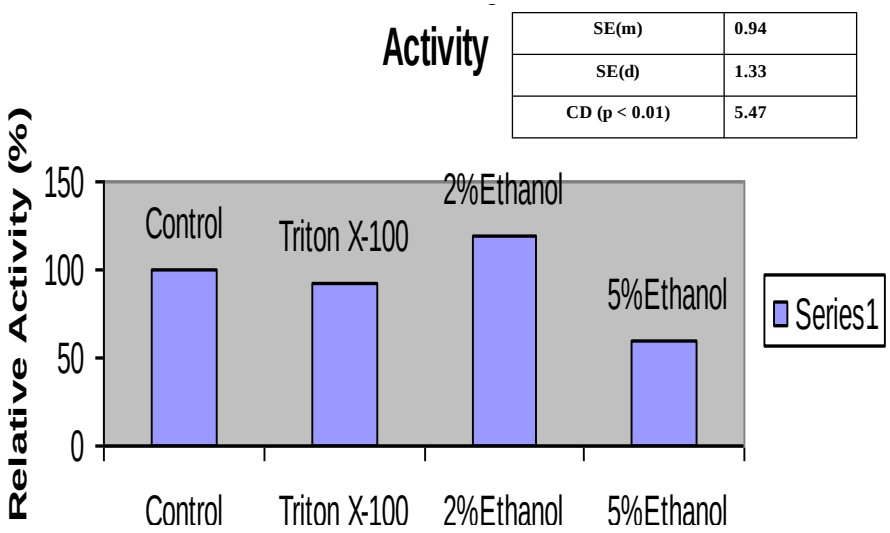


Figure 10. Effect of surface active agents on cellulose.

(Sheweita et al., 1996; Cavicchioli et al., 1991) are active between pH 4 and pH 6.0. Many reports on cellulases show a wide range for optimum pH and temperature. In Kitamoto's studies (1987) on the properties of exo-β-1,4 glucanases from *T. harzianum,* the optimum activity of cellulases was found to be at pH 4.6 and at a temperature of 45°C. A wide range of optimum temperature and pH could be related to the fact that these *Trichoderma* are widespread in soil that may have varied composition and characteristics. The ability of cellulases to be active at diverse pH and temperature conditions could reflect an ability of the enzyme to degrade cellulosic material in a large variety of environmental conditions and thereby allow *Trichoderma* to grow widely in diverse conditions

The biochemical properties, that is, inhibitors and activators, are also important in the characterization of the enzyme. At 10 Mm concentration, maximal stimulition (2 to 2.3 fold) in the enzyme activity occurred with Ca^{2+} and Co^{2+} with Mg^{2+} causing only 50% increase in activity while ferrous resulted in 50% inhibition and Zn^{2+} was found to be ineffective (Figure 9). This is in accordance with the past reports on *Trichoderma hamatum* , wherein there was an increase in activity by about 20% in presence of Ca^{2+} and Mg^{2+} and an insignificant increase in presence of Zn^{2+} ions. Results are in analogy with the earlier reports wherein Ca^{2+} stimulated the activity in *Bacillus pumilus* BpCRI 6, (Kotchoni et al., 2003). Stimulatory effect of Co^{2+} has also been reported in the case of alkaline cellulases in *Bacillus sphaericus* JS 1(Singh, et al., 2004)

The non-ionic detergent TritonX-100 showed no inhibitory effect on the cellulose purified under study. The finding is in accordance with that observed in *B. sphaericus* (Singh et al., 2004). It was observed that ethanol at an optimum concentration (2%) stimulated this activity; similar findings have also been reported for *Candida peltata* (Saha and Bothast, 1996) (Figure 10).

Conclusion

The study has helped in making available cellulose for biotechnological application. The enzyme has wide industrial application in paper and pulp industry, textiles, wastewater treatment, laundry, and for bioengineering for bioethanol production due to its enzymatic capability to degrade cellulose to glucose.

REFERENCES

Agneiszka M, Witkouska D, Robak M (2002). Biosynthesis and properties of beta-1,3-glucanases of *Trichoderma hamatum* Electronic journal of Polish Agricultural University 5(2) Series Biotech

Beguin P (1982). Detection of cellulose activity using congo red stained agar replicas. Appl. Exo-Microbiol. 43(4):777-780

Bryne H, Christou NV, Verma DP, Maclachlan GA (1975).Purification and Characterization of two cellulases from auxin treated Pea epicotyls. Biol. Chem. 250:1012-1018

Johnson, HW, Robinson HF, Comstock RE (1955). Estimation of genetic and environmental variability in soyabean. Agron. J. 47:314-318.

Kitamoto Y, Kono R, Shimotoria MN, Ichi KY (1987). Purification and some properties of exo-beta-1,3-Glucanase from *Trichoderma harzianum*. Agri L. Biol. Chem. 51(12):3385-3386

Kotchoni OS, Shonukan OO, Gachomo WE (2003). *Bacillus pumilus* BpCRI 6, a promising candidate for cellulase production under conditions of catabolite repression. Afr. J. Biotechnol. 2(6):140-146

Maheshwari R, Bharadwaj G, Bhat KM (2000). Thermophilic fungi;Their physiology and Enzymes. Microbiol. Mol. Biol. Rev. 64(3):461-488.

Macarron R, Acebal C, Castillon MP, Domisnguez JM, de la Mata I, Pettersson G, Tomme P, Claeyssens M (1993).Mode of action of endoglucanase III from *Trichoderma reesei*. Biochem. J. 289:867-873

Nishida Y, Suzuki KI, Kumagai Y, Tanaka H, Inoue A, Ojima T (2007). Isolation and primary structure of a cellulase from the Japanese sea urchin *Strongylocentrotus nudus.* Biochimie 89:1002-1011.

Saha BC, Bothast RJ (1996). Production, purification, and characterization of a highly glucose-tolerant novel beta-glucosidase from *Candida peltata* Appl Environ Microbiol. 62(9):3165-3170.

Sambrook J, Russell DW (2001). Molecular Cloning: A Laboratory Manual, Cold Spring Habor Lab Press, pp. A8:40-51.

Singh J, Batra N, Sobti RC (2004). Purification and characterization of alkaline cellulose produced by a novel isolate *Bacillus sphaericus* JS1. J. Ind. Microbiol. Biotechnol. 31:51-56.

Takashima T, Tikura A, Nakamula M, Hidaka H, Uozumi T (1998). Isolation of gene and Characterization of the enzymatic properties of a major exoglucanase of *Humicola grisea* without CBD. J. Biochem 124(4):717-725

Targonski Z, Wojcik W (1993). Biosynthesis of extracellular enzymes by isolates, mutants and recombinant strains of *Trichoderma spp.* Biotechnologia 1(20):14-19.

Teeri TT, Penttila M, Keranen S, Nevalainen H, Knowles JK (1992). Structure, function, and genetics of cellulases. Biotechnology 21:417-445.

Walsh G (2002).Industrial enzymes: proteases and carbohydrases. In: Proteins; Biochemistry and Biotechnology. John Wiley and Sons. Ltd.

Vinha Da FMN, Gravina-Oliveira MP, Franco MN (2011). Cellulase production by *Streptomyces viridobrunneus* SCPE-09 using lignocellulosic biomass as inducer substrate. Appl. Biochem. Biotechnol. 164 (3): 256–267, 2011.

Warburg O, Christian W (1942). Isolation and crystallization of enolase. Biochemistry Z. 310:384-421.