



CELLULASES PRODUCTION UNDER SOLID STATE FERMENTATION USING AGRO WASTE AS A SUBSTRATE AND ITS APPLICATION IN SACCHARIFICATION BY *TRAMETES HIRSUTA* NCIM

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ABSTRACT

Food and energy crisis are the biggest constraint all over the world which has focused lights on need of utilizing renewable resources to meet the future demand. A promising strategy is efficient utilization of lignocellulosic waste and fermentation of the resulting sugars for production of desired metabolites or biofuel. Production of all the cellulase enzymes on wheat bran and different parameters regulating it like pH, moisture ratio (substrate: liquid), temperature and inoculum size has been optimized which found to be 4.5, 1:3, 30°C and 10⁸ spores respectively. Salient feature of partially purified enzyme with stability in the range of 30-50°C under acidic pH range was found to be prominent for industrial applications, moreover in this study, *Trametes hirsuta*, an efficient cellulase producer, was observed to be an effective species for saccharification of wheat straw to enhance the sugar yield. Enzymatic hydrolysis of wheat straw with 15 FPU of cellulase from the species showed 73% yield in 20 hrs. It may prove to be a suitable choice for the industrial saccharification of lignocellulosic biomasses.

Keywords: Agro waste, cellulases, solid state fermentation (SSF), saccharification

INTRODUCTION

The major constituents of the terrestrial plants cell wall is Cellulose which originates from micro-fibrils (2-20nm in diameter and 100 – 40,000nm long). It make up the mesh network of the cell wall. It is a foremost component of natural and manking produced wastes.

The requirement for expenses of renewable resources to cope up the food and fuel demand has focused the attention towards cellulose use which is the sustainable sources for fuel (Lynd *et al.*, 2002). The major obstacle to the widespread utilization of this important resource is the absence of economically feasible technologies for overcoming the recalcitrant of cellulosic biomass. Hence, there is a considerable economic interest in the development of processes for effective utilization of cellulosic waste as inexpensive carbohydrate sources. A promising strategy for efficient utilization of lignocellulosic waste and fermentation of the resulting sugars for production of desired metabolites or biofuel. The growing concerns about shortage of fossil fuels, the emission of greenhouse gases and air pollution by incomplete combustion of fossil fuel has also resulted in an increased focus on production of bioethanol from lingo cellulose (Sheehan and Himmel, 1999; Zaldivar *et al.*, 2001).

Currently, there are two major ways of converting cellulose to glucose: chemical versus enzymatic. Enzymatic hydrolysis of cellulose is an important reaction in nature for it marks the initial step in cellulose deterioration. One promising approach is to hydrolyze the cellulose to glucose with fungal enzymes and then to ferment the glucose to ethanol which could be used as a liquid fuel (Mandels *et al.*, 1974). Chemical hydrolysis of celluloses are of less concern due to the environmental hazards generated with the involvements of acids and alkaline reagents. The research on both methods has for decades occupied the attention of many investigators world wide. Cellulase is a synergistic enzyme that is used to break up cellulose into glucose or other sugar moieties. The fungal cellulase observed to comprise three hydrolytic enzymes: endo-(1,4)- β -D-glucanase (endoglucanase, endocellulase, CMCase [EC 3.2.1.4]), which cleaves- linkages at any point, mainly in the amorphous region of cellulose, exo-(1,4)- β -D-glucanase (exocellulase, cellobiohydrolase, cellulase (microcrystalline), avicelase [EC 3.2.1.91]), which releases cellobiose from both reducing or non-reducing end, commonly crystalline parts of cellulose and β -glucosidase (cellobiase [EC 3.2.1.21]), which releases glucose from cellobiose and short-chain cellooligosaccharides (Bhat and Bhat, 1997).

The production of cellulase and hemicellulases carried out with either solid or liquid state fermentation processes. But Solid state fermentation (SSF) is advantageous over submerged fermentation because of superior enzymes yield,

better oxygen circulation and less efforts in downstream procession. The low estimated costs of SSF are due to the rather traditional preferential claim of SSF, viz. SSF can be applied with use of complex agricultural waste substrate and encompassing low-cost technology regarding sterility and regulation demands. However, attempts to reduce costs by using cheap substrates have hampered biotechnological progress in SSF because of the strongly increased diversity in SSF research. There is no consensus on either the methods, the microorganisms or the substrate applied rather it will help to access the system. The broad range of substrates application represents an especially critical problem. One of the key merit of SSF has always been the possibility of using substrates that are abundant, cheap, and not applicable to SmF. It resembles the natural habitat for the filamentous fungi to grow and produce the fermented products. Surprisingly, biological parameters, like enzyme stability of produced enzyme at high temperature or extreme pH, is also shown best in SSF (Deschamps and Huet, 1985; Acuna-Arguelles *et al.*, 1995). Catabolite repression or protein degradation by proteases were severe problems in SmF often reduced or absent in SSF (Solis-Pereira *et al.*, 1993; Aguilar *et al.*, 2011).

For cellulase production, SSF is most preferred, due to its lower capital investment and lower operating cost (Rodriguez and Anroman, 2005). Additionally, SSF finds greater use in solid waste management, energy conservation and synthesis of secondary metabolites. SSF also allows the use of low-cost agricultural substrates for enzyme production, in turn reduces the production cost. Previously, agricultural residues like wheat bran, wheat straw, rice straw, bagasse, corn stover etc can be applied in production of cellulase. In some cases physical and chemical pretreatments are necessary before the agricultural residues are used for cellulase production, due to their recalcitrant nature.

The report on the production of cellulases by *Trametes hirsuta* are majority with liquid state fermentation, which needs optimized conditions and sophisticated instruments for the production of cellulases which ultimately adds to the cost of the enzyme production. We report here the production of FPA, CMCase, β -glucosidase by *Trametes hirsuta* NCIM. in solid state fermentation using cheap lignocellulosic material, partial purification and characterization of Endoglucanase. The study also focuses on sachharification of wheat straw with crude cellulases.

MATERIAL AND METHODS

Fungus

The *Trametes hirsuta* strain used in the present study was provided by NCIM, National chemical laboratory, Pune, India. It was maintained at 4°C on PDA (Potato dextrose agarose) slants.

Cellulase production under solid state fermentation

Cellulase production was studied in flasks containing wheat bran as a substrate. The basal medium used for moistening agent was (g L⁻¹), Peptone 1, Urea 0.3, Ammonium sulphate 1.4, Calcium chloride 0.3, Magnesium sulphate 0.3, dipotassium hydrogen phosphate (KH₂PO₄) 2, (mg L⁻¹), Zinc sulphate 1.4, Cobalt chloride 1, Manganese sulphate 1.6, Ferrous sulphate 5. The basal medium was sterilized at 121°C for 15 min at 15 lbs pressure.

Inoculum

Inoculum was prepared from 7 days old culture that had been grown on PDA slants at 30°C. Spore suspension was prepared by adding sterile distilled water containing 0.1% Tween 80 to each slant and was brushed lightly with a sterile wire loop. Spore count was done using Neubauer's chamber.

Enzyme extraction

The content of each flask was extracted using 30 ml of 50mM sodium acetate buffer (pH 4.6) and filtered through wet muslin cloth by thorough squeezing. The extract was centrifuged at 8000 rpm for 20 min, clear supernatant was collected and used as crude enzyme for further analysis.

Enzyme assay

Filter paper assay was used to estimate total cellulase activity in the crude enzyme preparation and endo-β-1,4-glucanase activity was determined using carboxymethylcellulose (CMC) as the substrate as per the method of Mandels *et al.* (1975) in which one unit was defined as the amount of enzyme required to liberate one mole of glucose per minute. Reducing sugars in the enzymatic hydrolysate of biomass was estimated by 3,4 dinitrosalicylic acid (DNS) method (Miller, 1959). β-glucosidase activity was measured as reported by Kubicek (1983) using p-nitrophenyl β-D-glucopyranoside (PNPG) as substrate and one unit of β-glucosidase is defined as the amount of enzyme liberating one mole of p-nitrophenol per minute under standard assay conditions. Protein estimation was done by Lowry's (1951).

Effect of pH on production of cellulases

The effect of pH was studied by using different initial pH 3.5, 4.0, 4.5, 5.0 of the fermentation medium. The pH was adjusted by 1N HCl. The flasks were inoculated with solution containing 1x10⁸ spores. All flasks were incubated at 30°C with intermittent shaking.

Effect of moisture ratio on production of cellulases

Different moisture ratio (1: 2, 1: 3, 1: 4, 1: 5 as Substrate: Liquid) were used for the production of cellulases. The flasks were inoculated with solution containing 1x10⁸ spores. All flasks were incubated at 30°C with intermittent shaking and the effect of moisture ratio on enzyme production was studied.

Effect of Temperature on production of cellulases

To study the effect of different temperature on cellulase production. The flasks were inoculated with solution containing 1x10⁸ spores and incubated at different temperatures 20 °C, 30°C, 40°C, 50°C with intermittent mixing.

Effect of Inoculum size on production of cellulases

Different inoculum size of fungal culture (1 x 10⁵- 1 x 10⁸) were studied for the production of cellulases. The flasks were inoculated with solution containing 1x10⁸ spores. All the flasks were incubated at 30°C with intermittent shaking.

Partial purification of endoglucanase

Partial purification of the enzyme was performed by ammonium sulfate fractionation method. A calculated amount of ammonium sulphate powder was added to the enzyme extract with constant stirring at 4°C to achieve 40% saturation. After incubation for 2 hour, the content was centrifuged at 12,000 g at 4°C for 30 min. The precipitate was dissolved in minimum volume of Acetate buffer (0.05 M, pH 4.6). Dissolved enzyme was further purified with Sephadex

G-75 column chromatography. The enzyme activity and protein estimation were carried out from the desalted sample.

Gel permeation chromatography

5.0 gm of Sephadex G-75 matrix was soaked in five times the volume in double distilled water for overnight swelling. The autoclaved gel matrix was added to the glass column with the dimensions (1 x 30 cm). After equilibration of column the sample was loaded and purified fraction of 1 ml volume were collected and used for further study.

Characterization of partially purified Endoglucanase

Effect of temperature on activity and stability of Endoglucanase

The optimum temperature for partially purified enzyme was obtained by assaying the relative activity of enzyme at different temperatures 30°C, 40°C, 50°C, 60°C, 70°C, 80°C. The thermostability of Endoglucanase was monitored by incubating the enzyme solutions at a fixed temperature, in the range of 30°C to 70°C for 60 minutes and the activity was deduced after incubation.

Effect of pH on activity and stability of Endoglucanase

The relative activity of Endoglucanase was determined at different pHs. The optimum pH of Endoglucanase was determined by preparing the substrate in respective buffers of different pH ranging from 3 to 8. Citrate buffer (0.05 M) was used for pH 3, Acetate buffer (0.05 M) was used for pH 4 & 5, Phosphate buffer (0.05 M) was used for 6-8. To test the pH stability, the partially purified enzyme was incubated for 120 minutes at room temperature using respective buffers having pH range 3 to 8 as described above. The residual activity was estimated by following the standard procedure.

Effect of Metal Ions on Activity of Endoglucanase

The effect of different metal ions on enzyme activity was determined by incorporating 5.0 mM of KCl, CuSO₄, MgCl₂, CoCl₂, HgCl₂, CaCl₂, AgNO₃, FeSO₄, MnSO₄ and NaCl in to the reaction.

Saccharification studies

0.5 gm of delignified substrates such as wheat straw, rice straw and sugarcane bagasse were prepared in Sodium acetate buffer (0.05 M, pH 4.6) with 15 FPU of enzyme extract and incubated at 50°C for 24 h. Sampling was done at every 4 h intervals and amount of reducing sugar liberated was calculated.

Delignification of solid substrates

Twenty gram of chopped rice straw was suspended in 80 ml of 2% aqueous solution of NaOH at 85°C for 1 h, with solid to liquid ratio as 1: 4. The solid residue was collected by filtration and washed extensively with distilled water until it reached to neutral pH. The pre treated rice straw was dried in the oven at 70°C to maintain constant weight and used as the substrate for saccharification experiments (Jeya *et al.*, 2009).

RESULTS AND DISCUSSION

Optimization of fermentation parameters

Regardless of the fermentation process that is used to grow cells, it is necessary to monitor and control parameters starting from the selection of optimum inoculum volume, moisture content, pH, temperature etc. Changes in one of these parameters can have a dramatic effect on the yield of cells and the stability of protein product. The high rate of metabolism supports the critical period of metabolite production. The meaning of optimization in this context needs careful consideration of the environmental and nutritional parameters for growth and production. Solid state fermentation has numerous advantages over submerged fermentation (SmF), including superior productivity, simple technique, less investment, less energy requirement and low waste water generation and better recovery of product (Asgher *et al.*, 2006).

Two major impediments to exploit the commercial potential of Cellulases are yield stability and cost of cellulase production. Therefore, research should be also focus at use of the commercial potential of existing and new cellulase producers (Coral *et al.*, 2002). Generally the substrate exploit in SSF are generally insoluble in water. In practice, water is absorbed onto the substrate particles, which can then be used by microorganisms for growth and metabolic activities. Bacteria and yeast grows on the surface of the substrate while fungal mycelium penetrates into the particles of the substrates (Pandey, 1992). Natural waste materials such as wheat straw, wheat bran and sugarcane bagasse have been effectively utilized as a major carbon source for the production of cellulase enzyme by fungal strains, though the yield is variable, due to effect of substrate

(carbon source) on the growth of cellulolytic organisms (Mandels, 1985; Zhu et al., 1988; Lakshmikanth and Mathur, 1990).

Effect of pH on cellulase production

The influence of pH was studied by adjusting the pH of the medium which is used to wet the substrate. Higher enzyme activities were observed at pH 4.5. Maximum activities for Endoglucanase, β -glucosidase (BGL) and Exoglucanase (Filter paper activity) were found to be 38.3 U \cdot gm⁻¹, 30.86 U \cdot gm⁻¹ and 13.97 U \cdot gm⁻¹ respectively (Figure 1). It was observed that an initial pH of 4.5 was ideal for cellulase production (Xia and Cen, 1999).

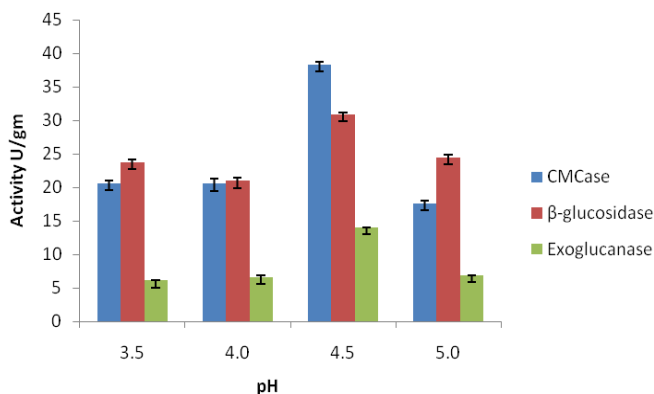


Figure 1 Effect of pH on production of cellulases by *Trametes hirsuta*. Enzyme activities were measured according to standard assay conditions and were expressed as Units per gram dry substrate. (X axis = pH and Y axis = Cellulase activity in terms of Units/gm)

Production of most of the fungal cellulases was reported in the pH range of 4.5-5.0 (Latifian et al., 2007; Wen et al., 2005). In SSF process, most precaution was taken for optimizing the initial pH of the medium because of the fact that extracellular enzymes are stable only at a particular pH and there may be rapid denaturation at lower or higher values (Kalra and Sandhu, 1986). Optimal pH is very important for the growth of microorganism and its metabolic activities. As the metabolic activities of the microorganism are very sensitive to changes in pH, change in pH affects cellulase production by fungi. On the basis of the data obtained pH 4.5 considered to be suitable for cellulase production by *Trametes hirsuta*.

Effect of Moisture ratio on cellulase production

Highest activities of Endoglucanase, β -glucosidase were observed as 48.49 U \cdot gm⁻¹, 26.28 U \cdot gm⁻¹ with substrate to moisture ratio 1: 3 and Exoglucanase activity 22.81 U \cdot gm⁻¹ of substrate with substrate to moisture ratio 1 : 2 (Figure 2).

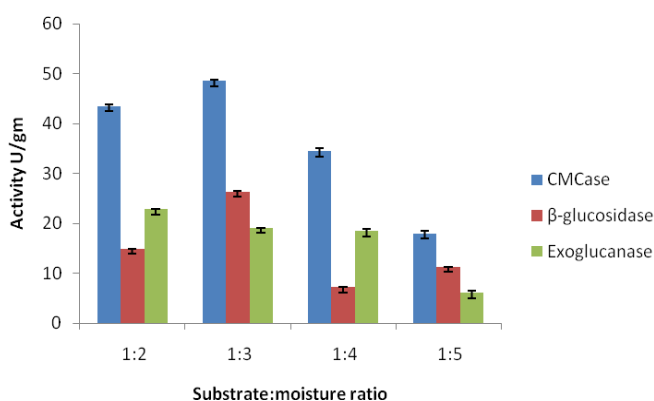


Figure 2 Effect of moisture ratio on production of Cellulases by *Trametes hirsuta*. Enzyme activities were measured according to standard assay conditions and were expressed as Units per gram dry substrate.

It was observed that further increase in moisture affects the enzyme production negatively. Ahmed (2008) and Sodhi et al. (2005) reported that moisture content of the substrate is one of the critical factors influencing the outcome of SSF, lower moisture content causes a reduction in solubility of nutrients provided to organism by SSF, a lower degree of swelling and higher water tension. On the other side, reduction in enzyme production at high moisture may be due to the reduction in substrate porosity, changes in the structure of substrate particles,

reduction of gas volume and decreasing in microbial growth (Baysal et al., 2003).

Moisture level as an optimizing factor is often neglected in submerged fermentations. In case of SSF it is a critical parameter as the microorganism grows near the surface of the solid substrate particles associated with low moisture content (Raimbault and Alazard, 1980). Transfer of oxygen affects the growth and metabolism, thus, substrate should contain suitable amount of water to enhance mass transfer.

Pandey, (2000) concluded that the degree of hydrate of one of the substrates play an important role on the growth of the fungus and subsequently the enzyme production. Water causes the swelling of the substrate and facilitates good utilization of substrates by the microorganisms. Low moisture may reduce the solubility of lignin and swelling capacity of substrate causing high water stress, and consequently decrease in growth and enzyme production (Raimbault and Alazard, 1980). Increasing moisture level is believed to have reduced the porosity of substrate, thus limiting the oxygen transfer into the substrate (Raimbault and Alazard, 1980). It is also known that the fungus grows and produces maximum enzyme activity at lower water additions.

Effect of Temperature on cellulase production

Optimum temperature for cellulase production was observed to be 30°C. As higher Endoglucanase, Exoglucanase and β -glucosidase activities were found (80.43 U \cdot gm⁻¹ and 28.09 U \cdot gm⁻¹, 33.5 U \cdot gm⁻¹) respectively at 30°C. Further increase or decrease in the incubation temperature did not result in a corresponding increase in the yield. Thus, high level of cellulase production was noticed at 30°C (Figure 3).

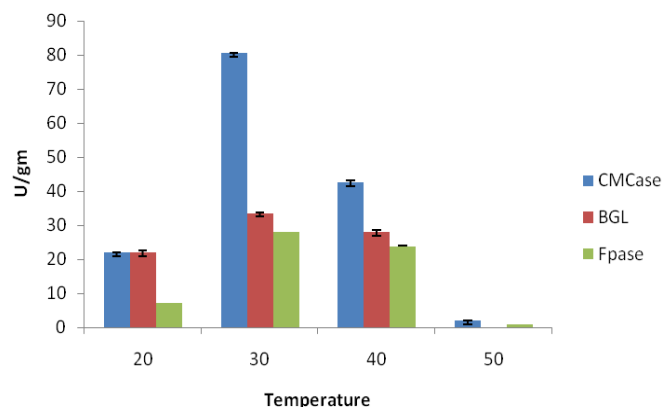


Figure 3: Effect of Temperature on cellulase production by *Trametes hirsuta*. Enzyme activities were measured according to standard assay conditions and were expressed as Units per gram dry substrate. (X axis = Temperature in °C and Y axis = Cellulase activity in terms of Units/gm)

Incubation temperature influences the transport of several species of enzyme across the membrane (Poorna and Prema, 2007; Mohana et al., 2008). Maximum enzyme production was recorded at 30°C and production was reduced at temperatures higher than 30°C (Yang et al., 2006).

Effect of Inoculum size on cellulase production

It is important to provide an optimal size of inoculum in fermentation cycle, as the lesser inoculum level can result in insufficient biomass and permit the growth of undesirable organism, whereas high inoculum size may produce too much biomass and deplete the nutrients necessary for the production (Pandey et al., 2003). Irrespective of type of fermentation, whether SSF or submerged fermentation, inoculum size influence the yield of final product greatly (Prakasham et al., 2006).

Maximum activities were observed with inoculum size i.e 1 x 10⁸ spores (Figure 4). With increase in inoculum size, a corresponding increase in enzyme activity was observed. Maximum Endoglucanase, β -glucosidase and Exoglucanase activities recorded were 90.75 U \cdot gm⁻¹, 35.7 U \cdot gm⁻¹ and 29.02 U \cdot gm⁻¹ respectively (Figure 4). The inoculum size can influence the growth of the organism and hence the efficiency of enzyme production and high inoculum size supports adequate growth and eventually efficient enzyme production.

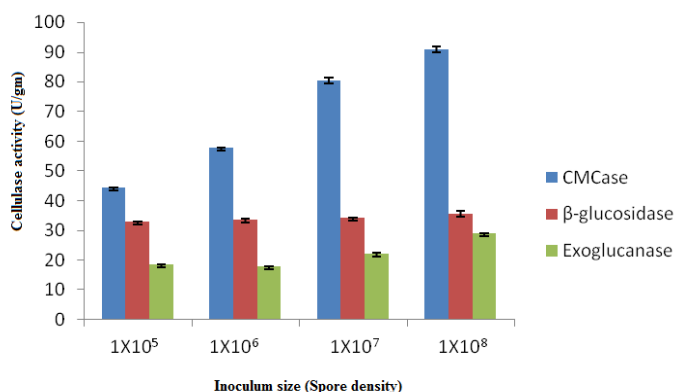


Figure 4: Effect of Inoculum size on production of cellulases by *Trametes hirsuta*. Enzyme activities were measured according to standard assay conditions and were expressed as Units per gram dry substrate. (X axis = Inoculum size (Spore density) and Y axis = Cellulase activity in terms of Units/gm)

Partial purification of Endoglucanase

A state of high purity is generally not required in processes such as saccharification of lignocellulosic waste. However, it is advisable to remove several unwanted compounds from cell free fraction of culture filtrate. One of the methods used for this purpose is ammonium sulphate precipitation of the selective protein from a mixture of proteins. The ammonium sulphate precipitated fractionation (40% saturation) followed by gel permeation chromatography of crude cellulases lead to a final recovery of the enzyme, endo- β -1,4-glucanase with increased purity by 5.2 fold. The partially purified enzymes thus obtained were further subjected for characterization studies (Table 1).

Table 1 Partial purification of endo- β -1,4-glucanase

Extract	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Fold Purification
Crude	249.2	286.8	0.86	1
40 % ammonium sulphate	82.5	54	1.52	1.8
Sephadex G-75 Purified	22.5	5	4.5	5.2

Effect of Temperature on Activity and Stability of Endoglucanase

The effect of temperature on Endoglucanase activity was examined over a temperature range of 30°C – 80°C. Figure 5 shows the enzyme activity which increased gradually with rise in the temperature up to the 60°C and decline sharply thereafter. The relative enzyme activities were low at 30°C, raised to a maximum at 60°C (100%) and then dropped at 80°C to (61%). The result shows that the optimum temperature for endo- β -1,4-glucanase was 60°C.

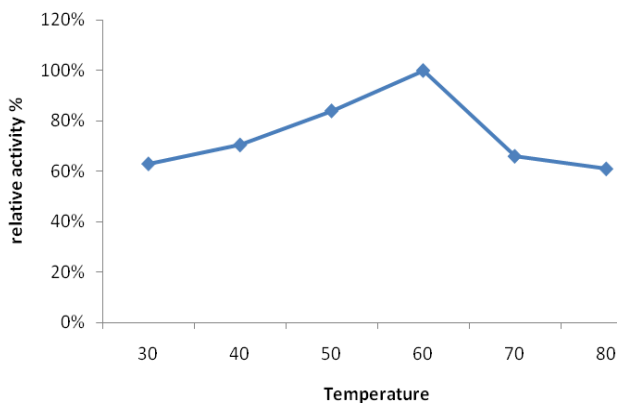


Figure 5 Effect of temperature on activity of partially purified endo- β -1,4-glucanase of *T. hirsuta*. Enzyme activities measured under standard assay conditions and expressed as relative activity of the maximum. (X axis = Temperature in °C and Y axis = Relative activity in %)

Effect of Temperature on Stability of Endoglucanase

Stability of enzyme in the temperature range of 30°C to 70°C revealed the following observations which are elucidated in Figure 6. Endo- β -1,4-glucanase was stable up to 50°C as the enzyme exhibited nearly 100% relative activity. These results are in agreement with other reports. However, further increase in the temperature resulted in sharp decrease in the enzyme activity which may be attributed to thermal inactivation of the enzymes.

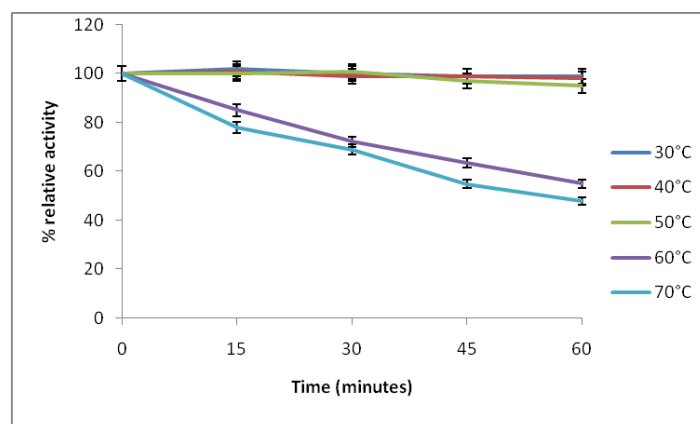


Figure 6 Effect of temperature on stability of partially purified endo- β -1,4-glucanase of *T. hirsuta*. Enzyme activities measured under standard assay conditions and expressed as residual activity. (X axis = Time in minutes and Y axis = % relative activity)

Effect of pH on Activity of Endoglucanase

The effect of pH on Endoglucanase activity was examined by using different pH, ranging from 3-8. The buffers used were 0.05M Citrate buffer (pH-3), 0.05 M Acetate buffer (pH 4-5) and 0.05 M Phosphate buffer (pH 6-8) and the enzyme was stable at pH 4.5-5.0 (30 min at 50 °C). This means that the Endoglucanase from *Trametes hirsuta* is fairly stable and active at a pH range of 4.5 -5.0. Figure 7 shows the optimum pH range for the activity of Endoglucanase is between 4.5-5.0.

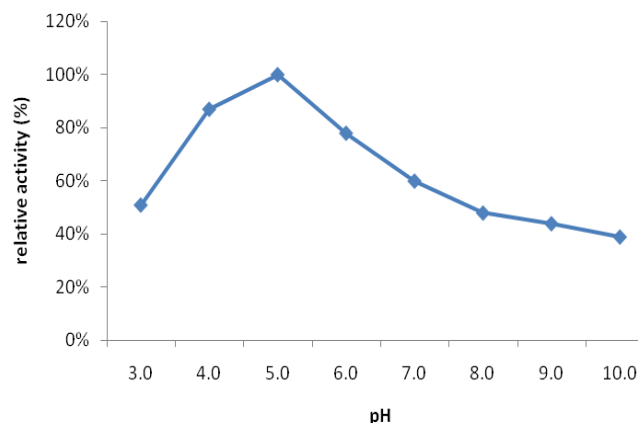


Figure 7 Effect of pH on activity of partially purified endo- β -1,4-glucanase of *T. hirsuta*. Enzyme activities measured under standard assay conditions and expressed as relative activity of the maximum. (X axis = pH and Y axis = Relative activity in terms of %)

Effect of pH on Stability of Endoglucanase

Figure 8 depicts the pH stability studies in the range of pH 3.0 to 8.0. It shows that endo- β -1,4-glucanase was highly stable in the acidic range between pH 4.0 and 5.0 (relative activities >90%). Stability of enzyme decreased with further increase in pH, which was unexpectedly low even at neutral pH. However, changes in pH resulted in a rapid decline in the enzyme activity, it retained activity at pH 8.0 after 2 h of incubation, narrating that the enzyme is not stable in the alkaline region. *Zhu et al. (1988)* found that Endoglucanase was more stable in the range of pH 2-5.

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