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# EXPRESSION PATTERN OF *TRICHODERMA* CELLULASES UNDER DIFFERENT CARBON SOURCES

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**Abstract**

Production of easily fermentable low-cost sugars demands for an economic and less expensive method of enzymatic hydrolysis of cellulosic biomass. It is highly desirable to compare various fermentation media with different carbon sources for cellulase production. *Trichoderma harzianum* was grown on different carbon sources and monitored for cellulase production. Glucose-grown cultures of *T. harzianum* showed high amount of mycelial growth but no yield of cellulase enzyme. Cellulase expression was also studied herein by comparing the cellulase activities using soluble and insoluble cellulosic carbon sources in the growth media in order to obtain less expensive fermentation media. Outcome of the research will be helpful in the development of low cost system for production of cellulose.

## Introduction

Fungi like *Trichoderma* secrete a large number and a variety of enzymes that can act on the polycassharides found in plant cell walls. These enzymes include cellulases, hemicellulases, pectinases, esterases, oxidoreductases and proteases (Chandra *et al*., 2010).

The polysaccharides especially celluloses and hemicelluloses are very cheap and easily available as wastes from industries like paper and pulp, agriculture, food and feed and municipal. In developing countries these wastes are not been discarded or treated properly and become the major cause of environmental pollution (Dashtban *et al*., 2009). Cellulose is a linear biopolymer of glucopyranose-molecules, connected by β-1,4-glycosidic bonds. Cellulase enzymes, which can hydrolyze cellulose forming glucose and other commodity chemicals, can be divided into three types: endoglucanase (endo-1,4-β-D-glucanase, EG, EC 3.2.1.4); cellobiohydrolase or exoglucanase (exo-1,4-β-D-glucanase, CBH, EC 3.2.1.91) and β-glucosidase (1, 4-β- D-glucosidase, BG, EC 3.2.1.21) (Li *et al*., 2006; Gao *et al*., 2008; Ahmed *et al*., 2009b).

Some species of filamentous fungi secrete more than two hundred glycosyl hydrolases alone (Nagendran *et al*., 2009). *Trichoderma* species has been used for a long time for industrial enzyme production and as an important model system for studying cellulosic and lignocellulosic degradation (Nevalainen *et al*., 1994). Its enzymes have important applications in starch processing, pulp and paper industry, extraction of fruit and vegetable juices, malting and brewing, animal feed production and alcohol fermentation. Many of the extracellular enzymes of *T. reesei* have been biochemically characterized (Stricker *et al*., 2008; Nagendran *et al*., 2009; Sun *et al*., 2010).

In one of previous study at our lab, three cellulases, exoglucanase (EXG), endoglucanase (EG) and β-glucosidase (BGL) were partially purified from *T. harzianum* (Ahmed *et al*., 2009a). The optimal pH, temperature and incubation time for cellulases production were determined. In this study we report the time course effect of different carbon sources like CMC (carboxymethylcellulose), lactose, sigma cellulose, birchwood xylan, wheat bran and alkali treated corn cobs and glucose on the production of cellulases by *Trichoderma harzianum*.

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## Materials and Methods

**Chemicals:** Carboxymethylcellulose (CMC), glucose, Sigma cell and lactose were from Sigma Chemical Co., USA. Corn cobs and wheat bran were gift from Fermentation Lab University of Agriculture, Faisalabad, Pakistan. All the other chemicals used were of analytical grade unless otherwise stated.

**Fungal strain and culture conditions:** *Trichoderma harzianum* (E-58) was maintained on agar slants containing (g/L) Trisodium citrate, 0.5; $KH_2PO_4$, 0.5; $NH_4NO_3$, 0.2; $(NH_4)_2SO_4$, 0.4; $MgSO_4$, 0.02, peptone, 0.1; yeast extract 0.2; glucose 0.2; agar 2.5. Inoculated slants were incubated for 5 days and spores were transferred to inoculum containing Vogel's medium (Vogel, 1956) containing glass beads for uniform suspension. The fungal growth for enzyme production was carried out in the Vogel's medium containing 1% any of the carbon source. Trace element solution (Citric acid. $H_2O$, 5g; $ZnSO_4$ $7H_2O$, 5 g; $Fe(NH_4)_2(SO_4)_2$ 6 $H_2O$, 1 g; $CuSO_4$ $5H_2O$, 250 mg; $MnSO_4$ $H_2O$, 50 mg; $H_3BO_3$, 50mg; $Na_2MoO_4 2H_2O$, 50 mg and $H_2O$ up to 100 mL) was added for optimal growth and enzyme production. The flasks were incubated for 5 days in a shaking incubator at 180 rpm at 28 °C (Aslam *et al*., 2004).

**Enzyme assays:** After each 24 hours crude cultures from different flasks containing different carbon sources were withdrawn and checked for the activity of cellulases. The carbon sources used were lactose, CMC, cellulose (sigma cell), birchwood xylan, wheat bran and alkali treated corn cobs. Three fundamental cellulase enzymes Endoglucanase, Exoglucanase and β-glucosidase were checked for their activity in the crude culture filtrate of all above mentioned carbon sources. In every assay DNS (Dinitorsalycilic acid) method (Shamala & Sereekanth, 1985) was employed to check the activity of enzymes. Enzyme (0.2 mL crude culture filterate) was incubated with 1.8 mL of 1% substrate in 0.2 M acetate buffer at 50°C for 30 minutes. The DNS (3 mL) was added to stop the reaction and boiled for 10 minutes in boiling water bath. After cooling the reaction, OD was noted at 575 nm. The enzyme activities were determined by comparing the amount of product formed.

**Protein estimation:** Protein was estimated using Bradford's reagent (Bradford, 1976). To 1.5 ml Bradford reagent 50 µL protein (crude enzyme) was added and OD was checked at 595 nm after 10 minutes.

## Results and Discussion

*Trichoderma harzianum* was grown in Vogel's media having trace element solution and Tween 80. It was observed that using trace element solution the mycelial growth was very high and enzyme production reaches its maximum within 48 hours. Tween 80 was used to inhibit the pellet formation so as to increase protein concentration and cellulase activity (Domingues *et al*., 2000). Furthermore it also helps in increasing the production of cellulases (Reese & Maguire, 1969). Regulation of expression of three cellulases was monitored in the fungal species. Carbon sources like Carboxymethylcellulose (CMC), Sigma cellulose, lactose, alkali treated corn cobs, wheat bran and glucose were used to investigate their influence on the cellulases production. Vogel's media (Vogel, 1956; Montenecourt & Eveleigh, 1977) was used for the present study. Repression of the enzymes was noticed during growth on glucose. When the glucose was added after 24 or 48 hours the cellulase level remains constant. Literature survey also shows that expression of a large majority of the cellulase genes that have been studied in *H. jecorina* and other filamentous fungi is believed to be induced during growth on cellulosic substrates and does not occur during growth on glucose (Messner & Kubicek, 1991;

Cullen & Kerston, 1992; Kubicek *et al*., 2009). Protein was determined using Bradford method (Bradford, 1976). Figure 1 shows the enzyme activities of exoglucanase, endoglucanase and β-glucosidase using CMC as a substrate. Endoglucanase showed the higher activity on CMC as compared to exoglucanase and β-glucosidase. This explains that CMC is a specific substrate of endoglucanase (Xiao *et al*., 2005). The enzyme activities of exoglucanase, endoglucanase and β-glucosidase using sigma cell as a substrate were very low during first three days having lowest activity of endoglucanase, but with time the activity of endoglucanase increased (Fig. 2). Since sigma cell (cellulose) is not easy to be broken down, its activity was less as compared to CMC. Figure 3 shows the enzyme activity using lactose as a carbon source in the growth media. Lactose is a disaccharide and is readily used by fungus to induce cellulases. Kubicek (2009) studied the induction of cellulases using lactose and revealed that the induction of cellulase transcription by this disaccharide required simultaneous degradation of the D-galactose moiety of lactose and the alternative reductive D -galactose catabolic pathway. However, as the experiments were performed on the crude cultures and cellulose needs synergistic action of different enzymes so other enzymes may be involved in giving the cellulase activity. Addition of glucose to the growth media containing cellulosic meterials results in negligible increase in the formation of cellulases (Figs. 6 and 7). Cellulase formation was also monitered in the culture filtrate which had only glucose as a carbon sources in the medium, which shows almost zero activity but gave large amount of mycelial mass. As glucose is the end product of cellulose degradation by cellulases, so the presence of glucose in the medium shows the end product inhibition (Muthuvelayudham & Viruthagiri, 2006). Szijarto *et al*., (2004) also found similar results while monitoring the cellulase production in the glucose-grown cultures of *T. reesei* Rut-C30 and under pulse additions of Solka-floc. Cullen & Kerston (1992) explained in the same way that enzyme synthesis is inhibited by end-product like glucose. However, Messner & Kubicek (1991) detected low levels of *T. reesei* exoglucanase (CBHII) in glucose medium. Rapid growth in the mycelia was noticed with glucose in the media but with lactose, CMC, sigma cell, wheat bran and corn cobs, there was slow mycelial growth. Induction in *Trichoderma* has been shown to occur at the transcriptional level. Cellulases are induced in most of fungi only when cellulose or an inducer exists (Suto & Tomita, 2001). It has also been suggested that low level of expression of cellulases mediate the induction by producing soluble compounds that enter the cell and cause induction (Teeri *et al*., 1987; Kubicek *et al*., 2009). *Trichoderma* needs low level (basal cellulases) for the induction of cellulases. These cellulases (basal cellulases) would digest cellulose and release oligosaccharides that enter into the cell and trigger expression of cellulases. Carle-Urioste *et al*., (1997) revealed that the basal expression of the cellulase was required for induction of its own transcripts by cellulose. Figure 4 and 5 show the enzyme activities using insoluble and relatively complex cellulosic substrate i.e., wheat bran and alkali treated corn cobs in the growth media. As shown in the figures the enzyme activities were low as compared to the above discussed media, and increase in activities with time was also low. This explains that as wheat bran and alkali treated corn cobs are complex substrates so the enzymes found difficult to penetrate into these substrates. Liming & Xueliang (2004) used corn cob residues for the production of cellulases using *Trichoderma reesei* ZU-02. They found that the cellulase production was the same with corn cobs and commercially available purified cellulose. In our case, corn cobs were not finely ground which results in lower enzyme activity. As purified celluloses are very expensive therefore by using the finely ground corn cobs, cellulase yield can be increased.

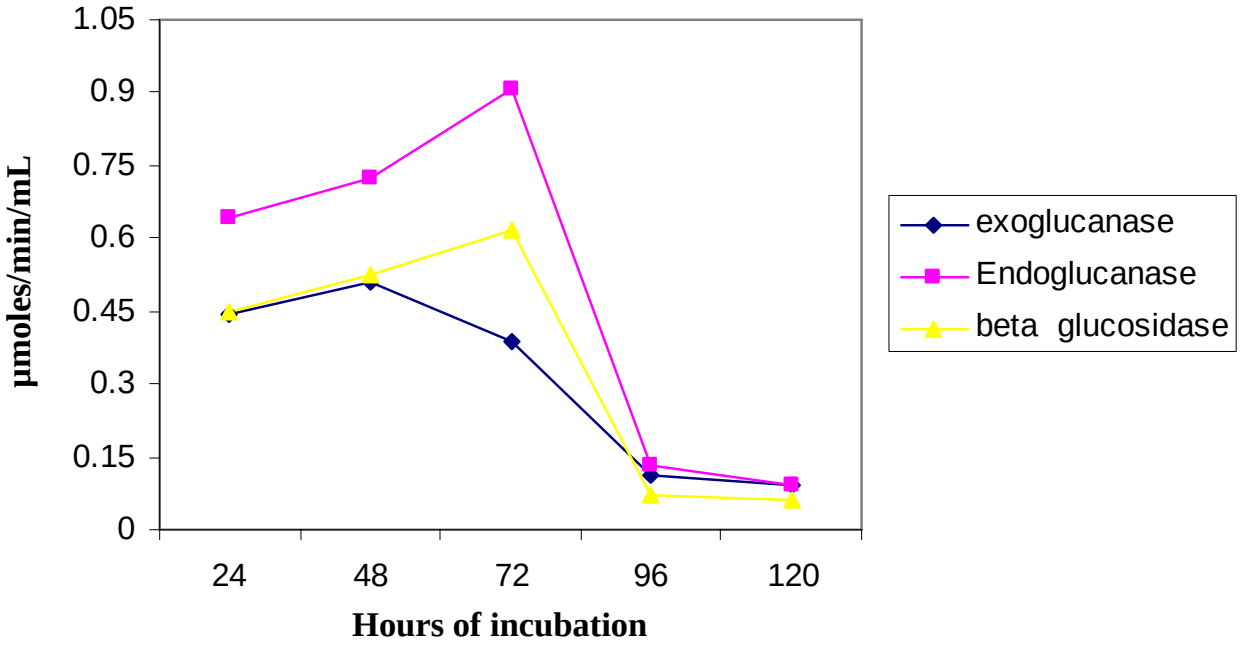


Fig. 1. Cellulase activity in the culture filtrate of *T. harzianum* with CMC as a carbon source. DNS assay was used to test the reducing sugars formed during hydrolysis. Avicel, CMC and salicin were used as a substrate in the assays of exoglucanase, endoglucanase and β-glucosidase, respectively. No enzyme control was used as a blank.

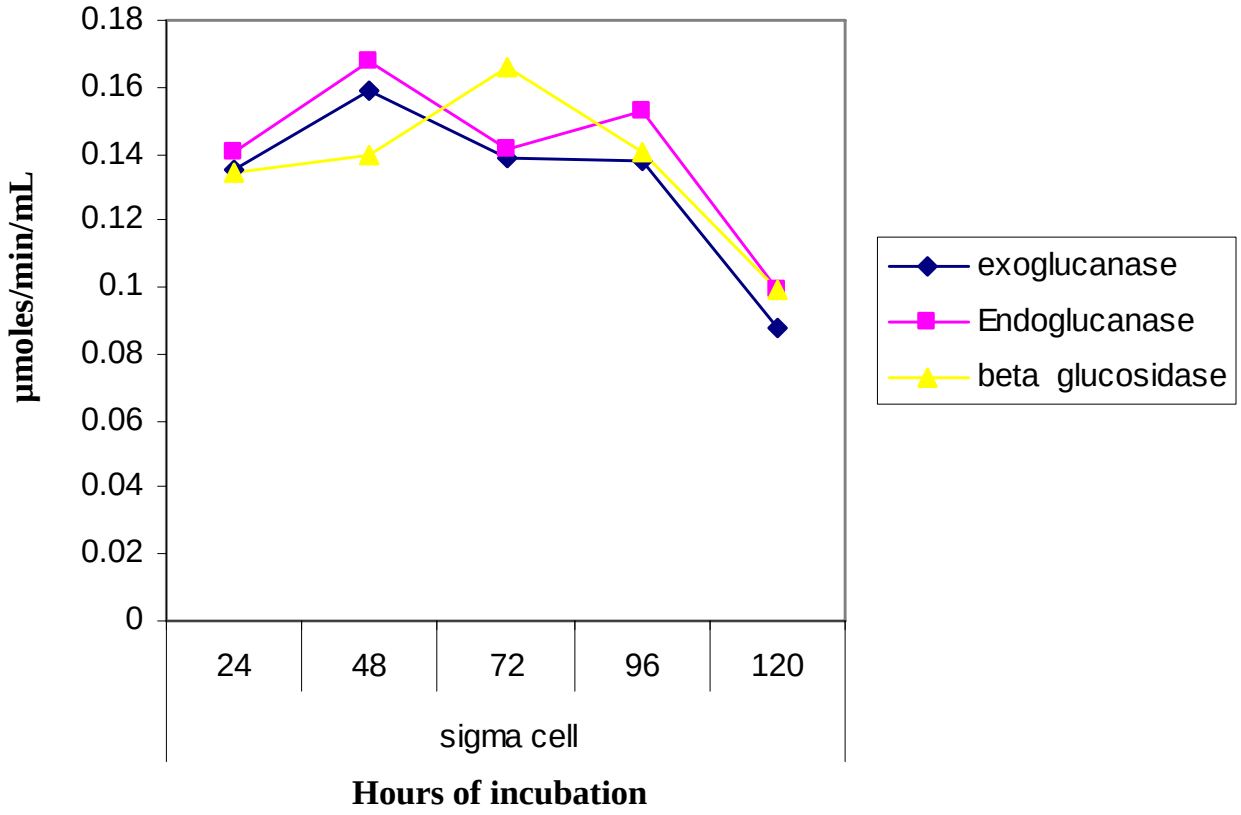


Fig. 2. Cellulase activity in the culture filtrate of *T. harzianum* with sigma cell as a carbon source. DNS assay was used to test the reducing sugars formed during hydrolysis Avicel, CMC and salicin were used as a substrate in the assays of exoglucanase, endoglucanase and β-glucosidase respectively. No enzyme control was used as a blank.

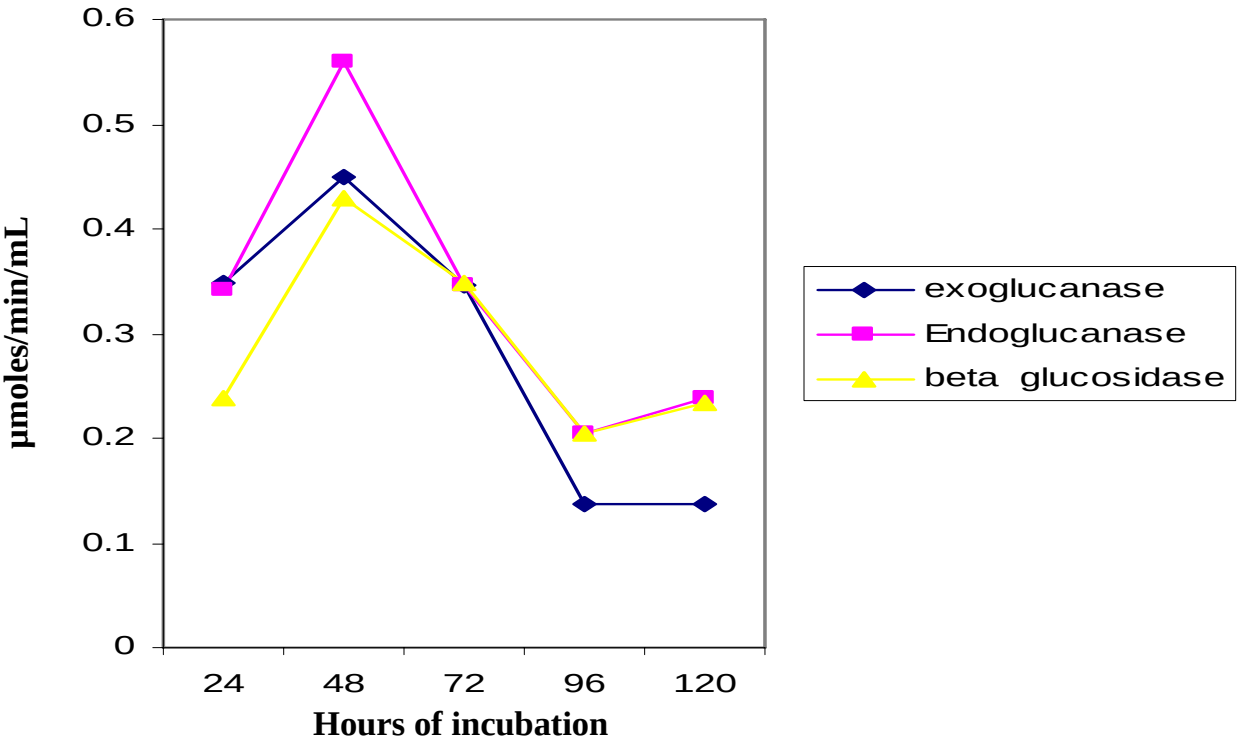


Fig. 3. Cellulase activity in the culture filtrate of *T. harzianum* with lactose as a carbon source DNS assay was used to test the reducing sugars formed during hydrolysis. Avicel, CMC and salicin were used as a substrate in the assays of exoglucanase, endoglucanase and β-glucosidase respectively. No enzyme control was used as a blank.

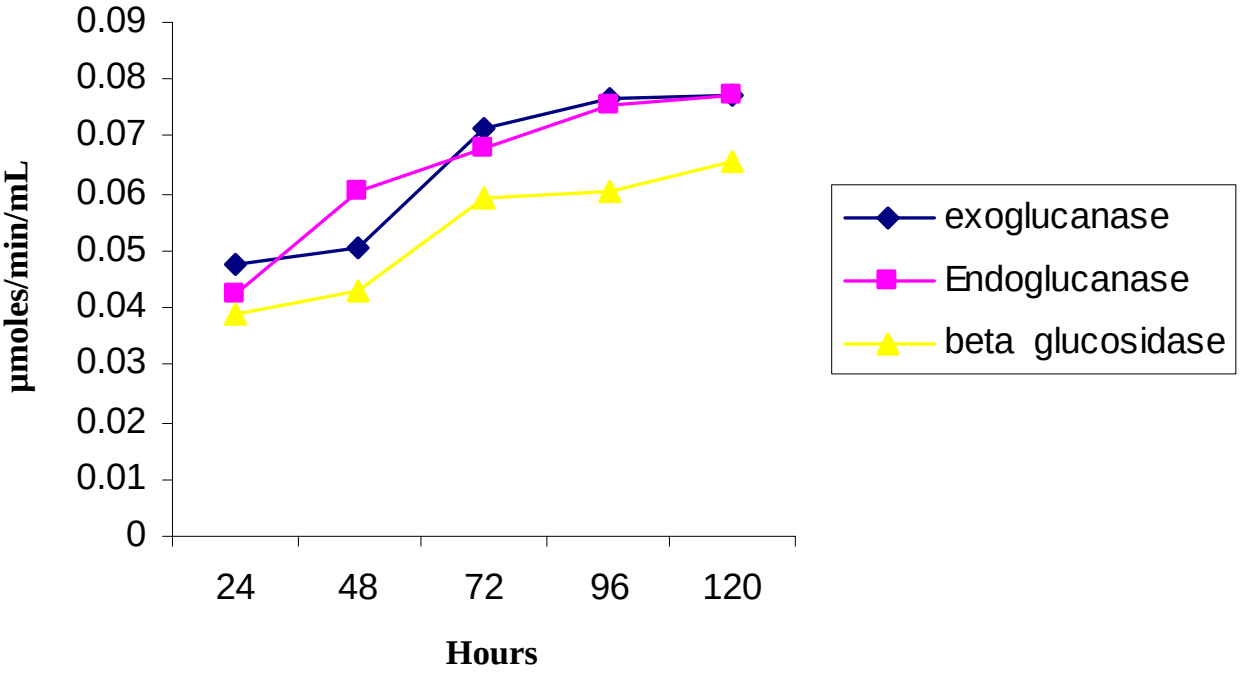


Fig. 4. Cellulase activities in the culture filtrate of *T. harzianum* with wheat bran as a carbon source. DNS assay was used to test the reducing sugars formed during hydrolysis. Avicel, CMC and salicin were used as a substrate in the assays of exoglucanase, endoglucanase and β-glucosidase respectively. No enzyme control was used as a blank

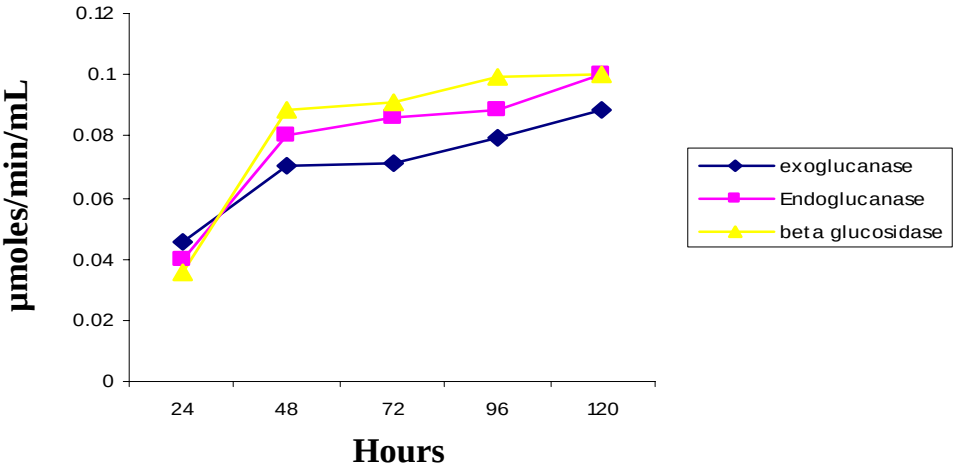


Fig. 5. Cellulase activities in the culture filtrate of *T. harzianum* with corn cobs as a carbon source. DNS assay was used to test the reducing sugars formed during hydrolysis. Avicel, CMC and salicin were used as a substrate in the assays of exoglucanase, endoglucanase and β-glucosidase respectively. No enzyme control was used as a blank.

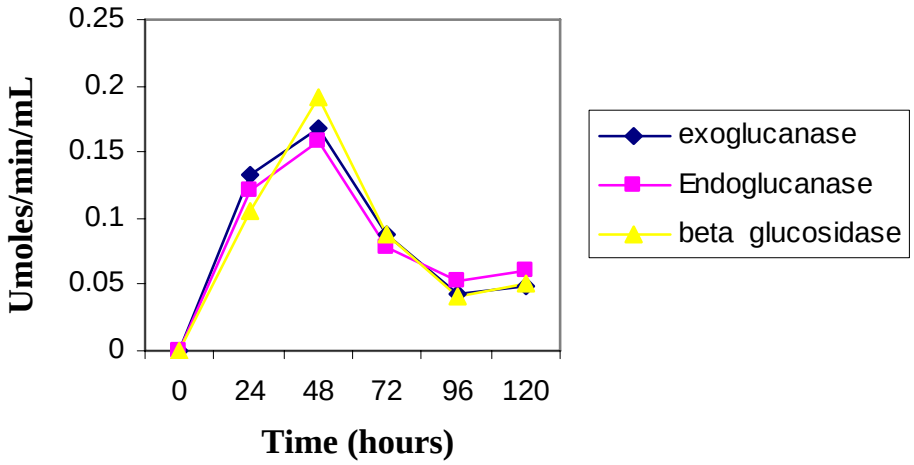


Fig. 6. Cellulase assay on the media containing sigma cell with addition of glucose. Glucose (1%) was added after 48 hours of incubation in the media containing sigma cellulose as carbon source. Cellulase assay was performed after every 24 hours to monitor the presence of cellulase enzyme.

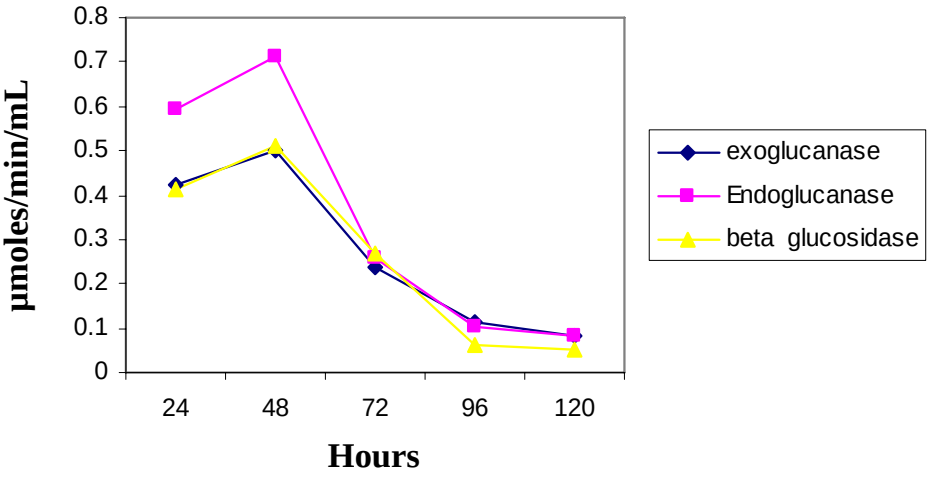


Fig. 7. Cellulase assay on the media containing CMC with addition of glucose. Glucose (1%) was added after 48 hours of incubation in the media containing CMC as carbon source. Cellulase assay was performed after every 24 hours to monitor the presence of cellulase enzyme.

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