*Pak. J. Bot.*, 41(3): 1411-1419, 2009.

# PRODUCTION AND PURIFICATION OF CELLULOSE-DEGRADING ENZYMES FROM A FILAMENTOUS FUNGUS *TRICHODERMA HARZIANUM*

**SIBTAIN AHMED, AMMARA BASHIR, HUMA SALEEM, MUBSHARA SAADIA AND AMER JAMIL***

*Molecular Biochemistry Lab., Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad-38040, Pakistan.*

**Abstract**

The major components of plant cell walls are cellulose, hemicellulose and lignin, with cellulose being the most abundant component. Cellulose degrading-enzymes provide a key opportunity for achieving tremendous benefits of biomass utilization. *Trichoderma harzianum*, a filamentous fungus, produces cellulose degrading enzymes and has been exploited by the industry. In this study, three cellulases, exoglucanase (EXG), endoglucanase (EG) and β-glucosidase (BGL) were partially purified from *T. harzianum*. The optimal pH, temperature and incubation time for cellulases production was found to be 5.5, 28ºC and 120 h respectively. To promote cellulases production, we evaluated the effects of glucose, carboxymethylcellulose (CMC), corn cobs, birchwood xylan and wheat bran as carbon sources for cellulase production. The results showed that CMC induced cellulases production whereas glucose repressed the synthesis of cellulases. The fungus was cultivated with 1% CMC for 120 h at 28ºC, pH 5.5 and the resulting culture filtrate was used for cellulase purification. EXG, EG and BGL were partially purified from culture filtrate of fungus by Ammonium sulfate precipitation followed by gel filtration chromatography on Sephadex G200 and on Sephadex G50. After final purification step specific activities (IU/mg$^{-1}$) of the enzymes were; EXG: 49.22, EG: 0.63 and BGL: 0.35 with 21.87-, 7.15- and 1.74- fold purification, respectively.

## Introduction

Plant cell walls are the most abundant renewable source of fermentable sugars on earth (Himmel *et al*., 1999; Saleem *et al*., 2008) and are the major reservoir of fixed carbon in nature (Yang *et al*., 2007). The major components of plant cell walls are cellulose, hemicellulose and lignin, with cellulose being the most abundant component (Han *et al*., 2003). Plant biomass comprises on average 23% lignin, 40% cellulose and 33% hemicellulose by dry weight (Sa-Pereira *et al*., 2003). Annually, 830 Gt of renewable plant biomass is formed consisting mainly of cellulose and hemicelluloses (Rauscher *et al*., 2006). Plant biomass is an alternative natural source for chemical and feedstocks with a replacement cycle short enough to meet the demand of the world fuel market (Kulkarni *et al*., 1999).

Cellulose consists mainly of long polymers of β 1-4, linked glucose units and forms a crystalline structure (Shallom & Shoham, 2003). 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). Cellulases are important industrial enzymes and find applications in several industrial processes (Hanif *et al*., 2004; Jamil *et al*., 2005). Researchers have strong interests in cellulases because of their applications in industries of starch processing, grain alcohol fermentation, malting and brewing, extraction of fruit and vegetable juices, pulp and paper industry, and textile industry (Gao *et al*., 2008; Zhou *et al*., 2008). One of the potential applications of

*Corresponding author: amerjamil@yahoo.com

cellulases is the production of fuel ethanol from lignocellulosic biomass (Duff & Murray, 1996), which is a good substitute for gasoline in internal combustion engines. The most promising technology for the conversion of the lignocellulosic biomass to fuel ethanol is based on the enzymatic breakdown of cellulose using cellulase enzymes (Holker *et al*., 2004; Ahamed & Vermette, 2008).

Many fungal strains secrete higher amounts of cellulases than bacterial ones, with *Trichoderma* as the leading one (Amouri & Gargouri, 2006). Most commercial cellulases are mesophilic enzymes produced by the filamentous fungus *Trichoderma reesei* and *Aspergillus niger*. This process reflects well the fact that filamentous fungi are naturally excellent protein secretors and can produce industrial enzymes in feasible amounts (Bergquist *et al*., 2002). Cellulases produced by *T. harzianum*, is the most efficient enzyme system for the complete hydrolysis of cellulosic substrates into its monomeric glucose, which is a fermentable sugar.

In this paper we report production and partial purification of cellulases (EXG, EG and BGL) from *Trichoderma harzianum*.

## Materials and Methods

**Chemicals:** Carboxymethylcellulose (CMC), glucose and birchwood xylan were from Sigma Chemical Co., USA. Corn cobs and wheat bran were purchased from local market of Faisalabad, Pakistan. All the other chemicals used were of analytical grade unless otherwise stated.

**Fungal strain:** *Trichoderma harzianum* was used in this study and was maintained at 4$^{o}$C after growing for 7 days in MYG medium (0.2% malt extract, 0.2 % yeast extract, 2% glucose and 2% agar) at 28 $^{o}$C (Saadia *et al*., 2008).

**Culture conditions:** For the production of cellulases (EXG, EG and BGL) in liquid state fermentation, the fungus was grown in 500 mL Erlenmeyer flask containing 100 mL of the Vogel's medium (Ahmed *et al*., 2007). Concentrations of the nutrients were 5 g/L Trisodium citrate, 5 g/L $KH_2PO_4$, 2 g/L $NH_4NO_3$, 4 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4$, 1 g/L peptone and 2 g/L yeast extract. Glucose (1%) was used as carbon source for inoculum preparations whereas 1% CMC was used as carbon source in cultivation medium. pH of the medium was adjusted to 5.5. The inoculum preparations were completed by 24 h of cultivation at 28$^{o}$C in an orbitaly shaker (150 rpm). A 10 mL of the liquid culture from the inoculum was transferred to 1000 mL Erlenmeyer flasks containing 250 mL of Vogels medium for 5 days at 28$^{o}$C with shaking at 150 rpm (Ahmed *et al*., 2005). Liquid state cultures were harvested by centrifugation at 10, 000 rpm, for 20 min., at 4$^{o}$C (Ali *et al*., 2009). The resulting supernatant was called as crude enzyme preparation.

**Determination of optimal pH, temperature and incubation time for cellulases production from *T. harzianum*:** The optimum pH for cellulases production was estimated at various pH values between 4.0 to 7.0 with appropriate buffer at 28$^{o}$C. For determination of optimum temperature for cellulases production, the reactions were carried out at 22$^{o}$C to 34$^{o}$C with 2$^{o}$C interval at pH 5.5. The mycelia were grown at the test pH or temperature under the conditions mentioned above. Cellulases activities were determined from the culture filtrate. Effect of various time periods on cellulases production was investigated from *T. harzianum* at pH 5.5 and 28$^{o}$C.

**Effect of various carbon sources on cellulases production:** *T. harzianum* was grown in Vogel's medium with different carbon sources viz., glucose, CMC, corncobs, birchwood xylan and wheat bran to check their effect on the expression of cellulases. All samples were analyzed in triplicate and the mean values calculated.

**Purification of cellulases:** All the purification steps were performed at 4°C unless otherwise stated. EXG, EG and BGL were purified by ammonium sulfate precipitation followed by gel filtration chromatography on Sephadex G200 and Sephadex G50 coulmn. For the purification of cellulases the crude extract of 5 days culture grown on 1% CMC was subjected to ammonium sulfate precipitation at different concentrations of $(NH_4)_2 SO_4$ saturation (0 to 80%). After precipitation the pellet was redissolved in sodium acetate buffer and dialyzed overnight against the same buffer. The dialyzed samples were oaded on to Sephadex G200 column (30 cm) pre-equilibrated with 50 m*M* sodium acetate buffer, pH 5.0. The cellulases were eluted at a linear flow rate of 30 cm/h. Different fractions of the enzymes were pooled, concentrated and applied to Sephadex G50 column. Elutions were performed at a flow rate of 30 cm/h. The concentrated EXG, EG and BGL fractions were subjected to enzyme activity. Protein concentration was determined by Bradford method (Bradford, 1976) using bovine serum albumin as standard. Protiens in the coloumn effluents were monitored by measuring $A_{280}$.

**Cellulases assay:** Cellulases activities were assayed in reaction mixture (I mL) containing 1% substrate avicel (for EXG) or CMC (for EG) or salicin (for BGL) in 0.05 *M* acetate buffer, pH 5.0 and appropriately diluted enzyme solution. After incubation at 60°C for 30 min., the reaction was stopped by adding 3mL Dinitorsalycilic acid solution (Shamala & Sereekanth 1985). One unit (IU) of enzyme activity was defined as the amount of enzyme required to liberate I μmole of glucose or p-nitrophenol from the appropriate substrates under the standard conditions.

## Results and Discussions

**Effect of pH, temperature and incubation time on cellulase production:** Studies were performed in shake flasks to optimize different fermentation conditions for cellulases production from *T. harzianum*. The optimum pH for the fungal growth for maximal EXG, EG and BGL production was found to be 5.5 at 28°C. (Fig. 1). The temperature for cellulases production of *T. harzianum* was optimized. The optimum temperature for maximal EXG, EG and BGL production was found to be 28°C at pH 5.5 (Fig. 2). Further increase in temperature resulted in decrease in cellulases production. Time course for cellulases production by *T. harzianum* was also investigated. Optimum EXG, EG and BGL production was achieved at 120 h of incubation at pH 5.5 and 28°C (Fig. 3). Further increase in incubation time resulted in decrease in EXG, EG and BGL activities.

Our results of optimal pH 5.5, temperature 28°C and incubation time of 120 h for cellulases production from *T. harzianum* are in accordance with earlier reports. The optimal pH for fungal cellulases varies from species to species, though in most cases the optimum pH ranges from 3.0 to 6.0 (Garg & Neelakantan, 1981; Niranjane *et al*., 2007). Rodriguez *et al*., (2005) used pH of 5.5 for the production of celluloytic enzymes from *Colletotrichum lindemuthianum*. Similarly it was found earlier that maximum induction of endoglucanase was achieved at pH 5.5 (Ikram-ul-Haq *et al*., 2001). Maximum production of β-glucosidase by *Aspergillus terreus* was achieved with the pH in the range of 4.0-5.5 (Pushalkar *et al*., 1995).

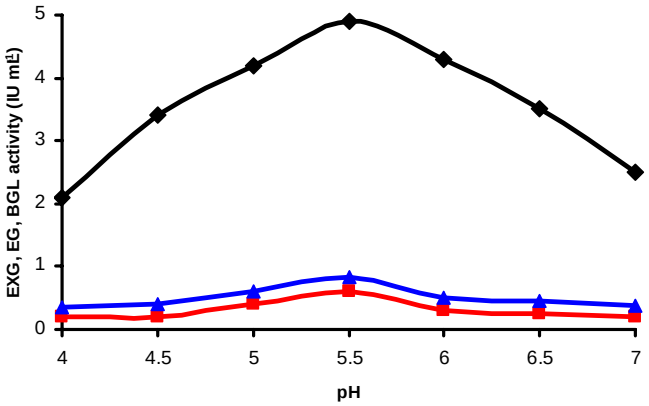


Fig. 1. Influence of pH on cellulase production by *T. harzianum*: (♦) EXG; (■) EG; (▲) BGL.

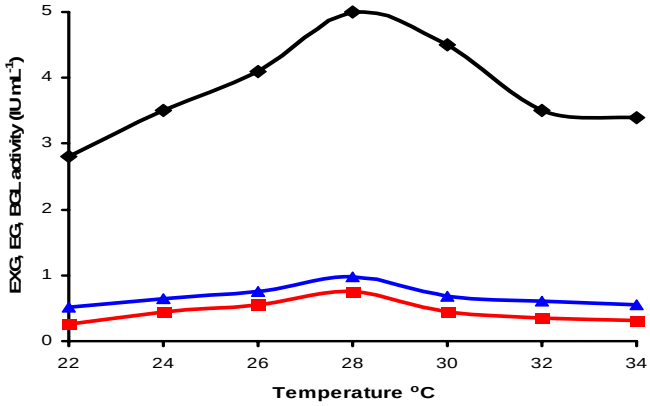


Fig. 2. Effect of temperature on cellulase production by *T. harzianum*: (♦) EXG; (■) EG; (▲) BGL.

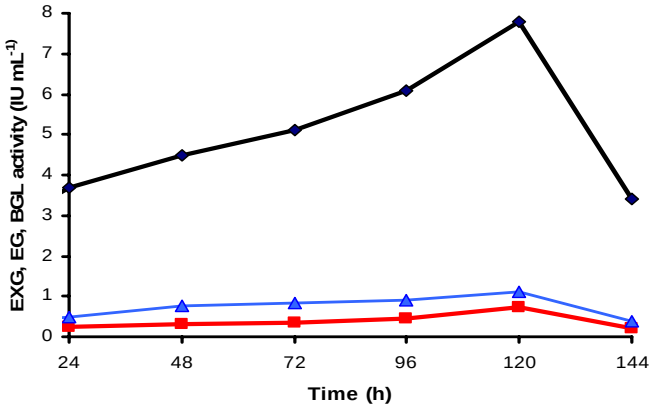


Fig. 3. Time course of cellulase production by *T. harzianum*: (♦) EXG; (■) EG; (▲) BGL.

The temperature of the fermentation medium is one of critical factor that has profound influence on the production of end product. The optimum temperature of 28°C for cellulases production by *T. harzianum* is in accordance with earlier research. Zhou *et al*., (2008) grew *Trichoderma viride* at 28°C for optimal cellulases production. BGL was produced and purified from *Trichoderma harzianum* type C-4 grown at 28°C (Yun *et al*., 2001). Exo β-1, 3 glucanse was produced from *Trichoderma asperullum* at 28°C (Bara *et al*., 2003). Similarly cellulases were produced from *Aspergillus niger* KK2 at 28°C (Kang *et al*., 2004). Maximum cellulase production in *Aspergillus terreus* was obtained at a temperature of 28°C (Sing *et al*., 1996).

Time course of 120 h for optimum cellulases production by *T. harzianum* is in accordance with earlier reports. Cellulase were produced from *Aspergillus niger* KK2 at 120 h incubation (Kang *et al*., 2004). Similarly cellulolytic enzymes were produced by *Aspergillus phoenix* at 120 h incubation (Dedavid *et al*., 2008). Similarly *Aureobasidum pullulans* showed maximum β-glucosidase production at 120 h of cultivation. Likewise, Kirchner e*t al.,* (2005) produced maximum β-glucosidase activity from *Aspergillus niger* C-6 after 96 to 120 h. Time course required to reach maximum level of cellulase activity may be affected by several factors, including the presence of different ratios of amorphous to crystalline cellulose (Ogel *et al*., 2001).

Hence optimum pH 5.5, optimum temperature 28°C and 120 h incubation time were used in all the subsequent experiments.

**Cellulases production with various carbon sources:** *T. harzianum* was grown in Vogel's medium with various carbon sources such as 1% glucose, carboxymethylcellulose (CMC), corn cobs, birch wood xylan and wheat bran at 28°C for 120 h days with shaking at 150 rpm for optimum cellulases production. In this study we found that when glucose was used as a carbon source, very little EXG, EG and BGL activities were detected whereas higher amounts were produced when 1% CMC was used as a carbon source (Table 1).

The production of cellulase is a key factor in the hydrolysis of cellulosic material and it is essential to make the process economically viable. Since the cost of the substrate plays a crucial role in the economics of an enzyme production, therefore different substrates utilized by *T. harzianum* for cellulases production were compared. The choice of an appropriate substrate is of great importance for the successful production of cellulases. The substrate not only serves as a carbon source but also produces the necessary inducing compounds for the organism (Haltrich *et al*., 1996). Reduction in the cost of cellulase production can be achieved by the use of cheap and easily available substrates. In fungi, the production of cellulolytic enzymes is subject to transcriptional regulation by available carbon sources. The cellulase genes are repressed in the presence of glucose. Earlier it has been reported that endoglucanase was induced by CMC but repressed by glucose (Ahmed *et al*., 2005). It was also found that CMC was preferred substrate for EG production (Lucas *et al*., 2001). Similarly, Malik *et al*., (1986) reported that negligible cellulases were produced with glucose as carbon source from *T. harzianum*. Niranjane *et al*., (2007) observed highest yields of cellulases on CMC. In this study, we recorded the similar results with very less cellulases activities in the presence of glucose, while CMC proved to be a strong inducer of cellulase enzymes.

**Purification of cellulases:** Summary of purification procedures of the cellulases is presented in Tables 2-4. Cellulases purification was preformed at 4°C by ammonium sulfate precipitation, gel filtration on Sephadex G-200 coloumn followed by gel filtration on Spehadex G-50 coloumn. The crude culture filtrate was subjected to partial purification, using 25% ammonium sulfate. EXG, EG and BGL fractions were pooled and applied on Sephadex G-200 coloumn. After Sephadex G-200 chromatography,

specific activities of EXG, EG and BGL were increased about 4.79-fold, 2.60-fold and 1.24-fold respectively compared with the crude preparations.

Elutions showing maximum EXG, EG and BGL activities were further purified by Sephadex G-50 chromatography. The purification scheme employed resulted after final purification step 21.87-fold, 7.15-fold and 1.74-fold purification of EXG, EG and BGL respectively. About 10.26%, 90% and 73.75% of the initial activities of EXG, EG and BGL were recovered and the partially purified EXG, EG and BGL have specific activities of 49.22 U $mg^{-1}$, 0.63 U $mg^{-1}$ and 0.35 U $mg^{-1}$, respectively. The data obtained from this study will help in future studies for the economic production of cellulases. Cellulolyitc enzymes produced by *T. harzianum* in this study can be used for biotechnology purposes.

**Table 1. Cellulases (EXG, EG and BGL) activities produced from *T. harzianum* grown on different carbon sources.**

| S. No. | Substrate | EXG activity (IU $mL^{-1}$) Mean ± S. D | EG activity (IU $mL^{-1}$) Mean ± S. D | BGL activity (IU $mL^{-1}$) Mean ± S. D |
|---|---|---|---|---|
| 1. | Glucose | 0.03 ± 0.01 | 0.023 ± 0.02 | 0.05 ± 0.01 |
| 2. | CMC | 7.8 ± 0.15 | 0.79 ± 0.020 | 0.92 ± 0.015 |
| 3. | Corn cobs | 1.84 ± 0.09 | 0.38 ± 0.012 | 0.18 ± 0.01 |
| 4. | Birch wood xylan | 0.64 ± 0.08 | 0.58 ± 0.105 | 0.54 ± 0.25 |
| 5. | Wheat bran | 0.58 ± 0.06 | 0.28 ± 0.05 | 0.37 ± 0.04 |

**Table 2. A summary of purification of EXG from *T. harzianum*.**

| Purification step | EXG activity (IU $mL^{-1}$) | Specific activity (U $mg^{-1}$) | Yield (%) | Purification (fold) |
|---|---|---|---|---|
| Culture supernatant | 7.87 | 2.25 | 100 | 1 |
| $(NH_4)_2SO_4$ precipitation | 6.02 | 3.04 | 5.09 | 1.35 |
| Gel filtration on Sephadex G-200 | 23.18 | 10.78 | 19.6 | 4.79 |
| Gel filtration on Sephadex G-50 | 40.41 | 49.22 | 10.26 | 21.87 |

**Table 3. A summary of purification of Eg from *T. harzianum*.**

| Purification step | EXG activity (IU $mL^{-1}$) | Specific activity (U $mg^{-1}$) | Yield (%) | Purification (fold) |
|---|---|---|---|---|
| Culture supernatant | 0.79 | 0.087 | 100 | 1 |
| $(NH_4)_2SO_4$ precipitation | 1.06 | 0.37 | 13.3 | 4.22 |
| Gel filtration on Sephadex G-200 | 0.45 | 0.23 | 56 | 2.60 |
| Gel filtration on Sephadex G-50 | 0.72 | 0.63 | 90 | 7.15 |

**Table 4. A summary of purification of BGL from *T. harzianum*.**

| Purification step | EXG activity (IU $mL^{-1}$) | Specific activity (U $mg^{-1}$) | Yield (%) | Purification (fold) |
|---|---|---|---|---|
| Culture supernatant | 0.92 | 0.20 | 100 | 1 |
| $(NH_4)_2SO_4$ precipitation | 0.40 | 0.06 | 12.58 | 0.29 |
| Gel filtration on Sephadex G-200 | 0.35 | 0.25 | 40.56 | 1.24 |
| Gel filtration on Sephadex G-50 | 0.68 | 0.35 | 73.75 | 1.74 |

Several workers have purified cellulases previously. Bara *et al*., (2003) purified EXG by gel filtration and ion exchange chromatography and observed 35.7 fold purification with

9.5% yield. Similarly Noronha *et al.,* (2000) has employed gel filtration chromatography for purification of EXG. He further purified EXG by ion exchange chromatography and got 69-fold purification with 0.32% yield. Endoglucanse was purified from *Gymnoascella citrina* by gel filtration chromatography with 27.3 fold purification and final recovery of 25.5% (Jabbar *et al*., 2008).

Piston *et al.,* (1997) purified β- glucosidase by ammonium sulfate precipitation and gel filtration. Similarly Wei *et al*., (1996) purified β-glucosidase by $(NH_4)_2SO_4$ precipitation, ion exchange and gel filtration chromatography. β-glucosidase was purified from *Monascus purpureus* by gel filtration chromatography with 13-fold purification. (Dariot *et al*., 2008). β-glucosidase was purified from *Paecilomyces thermophila* by gel filtration chromatography with 105-fold purification and final recovery of 21.7% (Yang *et al*., 2008).

## Conclusion

The successful use of cellulosic material as carbon source is dependent on the development of economically feasible process for cellulases production. The production of cellulases by *T. harzianum* in liquid state fermentation was investigated. CMC served as a powerful inducer that enhanced the cellulases yields where glucose repressed cellulases activities. The purification scheme employed resulted in 21.87-, 7.15- and 1.74-fold with a yield of 10.26%, 90% and 73.75% of EXG, EG and BGL respectively. Different approaches such as mutagenesis; cloning and overexpression of cellulase genes can be followed in order to increase the cellulases yields.

## Acknowledgement

The work was supported by grant from Pakistan Science Foundation and Higher Education Commission, Government of Pakistan, which is gratefully acknowledgement.

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(Received for publication 12 February 2009)