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Research Article

# The synergistic interactions of cellulase enzyme activities of *Trichoderma* species in colloidal cellulose bioconversion

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**Abstract:** In this study the cellulytic activity of different species of Iranian *Trichoderma* isolates including *Trichoderma harzianum* (NAS-H101), *T. aureoviride* (NAS-AV106), *T. pleuroticola* (NAS-P109), *T. longibrachiatum* (NAS-L110), *T. ghanens* (NAS-K108), *T. virens* (NAS- Vi114), *T. atroviride* (NAS-A113) and *T. atroviride* (NAS-A112) was studied. The extracellular protein concentration of these isolates was determined by the dye binding method of Bradford. The molecular weight of cellulase enzymes was studied using SDS-PAGE. The lowest extracellular protein production was observed in NAS-K108. The highest Endo and Exo-glucanase activity were observed in NAS-L110 and NAS-A113, respectively. The SDS-PAGE profiles had several enzyme bands such as cellobiohydrolases, endoglucanases and β-glucosidases. The NAS-K108and NAS-P109 had both enzyme bands of CBH I and CBH II, but other isolates had only a sharp enzyme band correlated to CBH I or CBH II. The highest synergy was observed in FPase of NAS-A112, that contained a large amount of Cel 6A (CBH II) and a minor amount of Cel 7B (EG I). The results indicated that NAS-A113 overproduces cellulases, ß-glycosidase, and the extracellular enzymes, which suggest that this species might be utilized as a biological agent and or a source of enzymes for cellulose degradation in colloidal cellulose.

**Keywords:** Cellulase enzyme, *Trichoderma* spp., SDS-PAGE

## Introduction

The demand for cellulases and hemicellulases is growing rapidly because of their numerous current and potential applications. At present, cellulases and hemicellulases are widely used in food, beer and wine, animal feed, textile and laundry, pulp and paper biotechnology, agriculture, and research and development (Bhat, 2000). Cellulases are produced by various organisms, but due to highest extracellular yields, the most important sources for industrial production are filamentous fungi such as *Trichoderma*, *Penicillium*, *Aspergillus* and *Phanerochaete* species (Nieves *et al.*, 1998; Esterbauer *et al.*, 1991; Vinzant *et al.*, 2001). The *Trichoderma* species produce at least two exoglucanases (cellobiohydrolases, CBHs, EC 3.2.1.91) Cel6A (CBHII) and Cel7A (CBHI), five endoglucanases (EGs, EC 3.2.1.4) Cel5A (EGII), Cel7B (EGI), Cel12A (EGIII), Cel45A (EGV) and Cel61A (EGIV), as well as two β-glucosidases (BGLs, EC3.2.1.21) Cel1A (BGLII) and Cel3A (BGLI) for cellulose degradation (Saloheimo *et al.*, 2002; Foreman

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*et al.*, 2003; Grishutin *et al.*, 2004). During an enzymatic hydrolysis process, in which the three enzyme classes (CBH, EG and BG) are used to break down cellulose into sugars, typically, glucose is released quickly in the beginning of the process after which the hydrolysis rate is slowing down due to the high substrate recalcitrance (Lynd *et al.*, 2002); if only one enzyme class were used for hydrolysis, the process would be hampered. Cooperative action, often designated as *synergy*, of the three cellulolytic enzyme classes is essential for efficient enzymatic hydrolysis process. Synergy between cellulolytic enzymes is a term used for the phenomenon that the overall degree of hydrolysis of a mixture of enzyme components is greater than the sum of the degrees of hydrolysis by the enzymes individually. Synergisms between the various classes of the cellulose degrading enzymes have been reported and investigated extensively (Zhang *et al.*, 2004). Most studies reported so far have investigated binary or ternary enzyme mixtures focusing on *endo*(glucanase)-*exo*(glucanase) (e. g. EG-CBH) and/or *exo*(glucanase)-*exo*(glucanase) (e. g. CBH I-CBH II) type of cooperative action with or without the addition of external β-glucosidase activity (Jeoh *et al.*, 2002, 2006; Watson *et al.*, 2002; Zhang *et al.*, 2004).

A major problem today for effective and economical biological use of *Peronosporomycetes*, is the too high production cost of cellulases. Other difficulties include the relatively slow growth rates of cellulase-producing fungi, the long induction period for cellulase expression, the low specific activity of cellulases and the suboptimal levels of β-glucosidases (Kadam, 1996). Another important factor to reduce the costs of enzyme production is the further improvement of cellulase production and effectiveness. Selection of an ideal cellulase producer is of great importance.

Since cellulase activity plays important role in (mycoparasitism) antagonism mechanism of *Trichoderma* species, extracellular enzymatic activity of the strains was assayed. In this report, the impact of complex cellulase mixtures produced from *Trichoderma* species with Phosphoric Acid Swollen cellulose (PASC) as a carbon source on Avicel, carboxy methyl cellulose, cellubiose and filter paper were investigated to screen and find the best Iranian isolate of *Trichoderma* with the maximum enzyme activity for saccharification of cellulose. This study presents a comparison between some Iranian isolates *T. harzianum (NAS-H101), T. aureoviride (NAS-AV106), T. pleuroticola (NAS-P109), T. ghanens (NAS-K108), T. virnse (NAS-Vi114), T. longibrachiatum (NAS-L110), T. atroviridae (NAS-A112), T. atroviridae (NAS-A113)*) cellulase enzyme activity, and the extracellular protein concentration and protein profile of them using SDS-PAGE.

## Materials and Methods

### Phosphoric acid swollen cellulose (PASC) production

Phosphoric acid swollen cellulose (PASC) was used as the main carbon source since it is known to induce the production of cellulase enzyme (Zhang *et al.*, 2006). Colloidal cellulose was produced by pretreatment of microcrystalline cellulose (Avicel) in ortho-phosphoric acid 85% (w/v) for 24h at 4 °C to increase colloidal property in cellulose so as to be easily accessed by the enzymes. After acid pretreatment, the materials were filtered through cheese cloth filter. Then, the solid fraction was thoroughly washed by distilled water to neutralize the pH (~5.0), frozen at -70 °C for 24h, freeze dried for 48h and milled to mesh size 53-125 µm.

### Culture condition of fungi

*Trichoderma aureoviride (NAS-AV106), T. longibrachiatum (NAS-L110), T. ghanens (NAS-K108), T. harzianum (NAS-H101)* and *T. longibrachiatum (NAS-L110),* were isolated from the soil samples collected from Khorasan province (NSTRI-Nuclear Agriculture Research School collection), *T. virnse (NAS-Vi114), T. atroviridae (NAS-A112)* and *T. atroviridae (NAS-A113)* were received from Dr. Rahnama





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at Gorgan University of Agricultural Sciences & Natural Resources and serial diluted on *Trichoderma* selective medium (Papavizas and Lumsden, 1982). To prevent bacterial growth, the culture medium was amended with 50mg.$L^{1}$ ampicillin, 15mg.$L^{-1}$ tetracycline and 30mg.$L^{-1}$ spectinomycin. *NAS-P109* was obtained from the Persian Type Culture Collection (PTCC) strain number 5142. The fungi were subsequently maintained on potato dextrose agar (PDA) prepared according to manufacturer's instructions. The mycelia growth and morphology were observed after 1-3 days incubation at 28 °C.

**Cellulase production**

*Trichoderma* isolates were maintained on agar media (MYG agar medium) containing; malt extract: 5, yeast extract: 2.5; glucose: 10; agar: 20 $gl^{-1}$. Spore suspensions were prepared from seven-day-old slant cultures in sterile saline solution and used as an inoculum of $1 \times 10^7$ spores/ml of medium. The spores were pelleted by centrifugation at 4500 × g for 10min, and washed twice in sterile saline solution. Seed cultures were produced in *Trichoderma* complete medium (TCM) (Shahbazi *et al.*, 2016). The medium was adjusted to pH 4.8 and supplemented with 0.3% w/v of glucose. Cultures were produced in 50ml volumes of TCM in 250ml Erlenmeyer flasks shaken at 180 rpm at 28 °C for 24 h. To induce production of cellulase enzymes, washed mycelium was transferred to 25 ml of *Trichoderma* fermentation medium (TFM) (Shahbazi *et al.*, 2016). This medium was adjusted to pH 4.8 and supplemented with 0.5% w/v of PASC. Growth conditions were as described previously and triplicate flasks were harvested after 72 h. Estimation of protein and extracellular cellulase activity was assayed in *Trichoderma* fermentation medium after centrifugation at 4500 × g for 7 min at 4 °C.

**Estimation of protein and cellulase activity**

The protein content in the TFM supernatant was estimated after 72 h fermentation by the dye binding method of Bradford (Bradford, 1976). The amount of protein was calculated using bovine serum albumin (BSA) as a standard. A standard curve was prepared using 0, 2, 8, 12, 20 and 25 µg protein per ml in supernatant of TFM medium, pH 4.8. The test was carried out using 150 µl of supernatant of TFM or standard and 3ml of Bradford reagent. The experiments were replicated three times. The absorbance was read at 595 nm using spectrophotometer (Jenway, USA). Avicelase, carboxymethyl cellulase (CMCase) and ß- glucosidase activity were determined by measuring the amount of glucose released from substrates by the dinitrosalicylic acid (DNS) method with glucose as the standard. The reaction mixtures contained 0.5 ml of 50g.$l^{-1}$ Avicel, CMC and bacterial cellulase in 0.05M citrate buffer (pH 4.8) and 0.5 ml of each supernatant of TFM medium. The reactions were terminated by adding 3 ml of 3, 5-dinitrosalicylic acid (DNS) after incubation at 50 °C for 60 min. The mixtures were also mixed well, then placed into a boiling-water bath for 5 min, and cooled to room temperature. The absorbance of the reaction solutions was measured at 540 nm. The International unit (IU) of activity is defined as the amount of enzyme that liberates l µmol of glucose per minute in a standard assay. Also, for filter paper assay (FPase), a filter paper strip of Whatman No. 1 (50 mg) was suspended in 1.0 ml of citrate buffer. After addition of the enzyme (0.5 ml), the reaction medium was incubated for 60 min at 50 °C. Finally, the reduced sugars were measured by DNS method (Gama and Mota, 1998).

**Electrophoresis and molecular size determination**

Protein samples (40 ml) from TFM supernatants were precipitated with equal volume of acetone and precipitated proteins were re-suspended in double distilled water in final volume of 1ml, frozen and kept at -70 °C until usage. The molecular weight of the cellulase was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a 4% (stacking) and 12.5% (separating) polyacrylamide gel based on Laemmli (1970). Before electrophoresis, equal volume of sample





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buffer (100 µl) that contained 65mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, and 0.2% (w/v) blue bromophenol was added to the protein sample (100 µl) and boiled for 5min and loaded on the gels. The proteins were separated at constant Ampere of 20 mA using the running buffer that contained 25 mM Tris, 192 mM glycine, and 0.1% (w/v) SDS, pH 8.3. The gels were stained with Coomassie Brilliant Blue R-250 in methanol–acetic acid-water (5: 1: 4, v/v), and decolorized in methanol-acetic acid-water (1: 1:8, v/v) (Laemmli, 1970).

## Results

### Estimation of protein and enzymes' activity

The extracellular protein concentration of different species of *Trichoderma* was determined by Bradford's dye binding method. The amount of protein was calculated using bovine serum albumin (BSA) as a standard. Results are shown in Fig. 1. Protein concentration ranged from 50.06 to 86.75 µg.ml$^{-1}$ in supernatant of TFM medium of the studied fungi. The highest and lowest protein content was 86.75 and 50.06 µg/ml in supernatant of *NAS-Vi114* and *NAS-K108,* respectively. Finally, the results showed that protein content (µg/ml) production in TFM for all studied fungi is different significantly at $p < 0.05$.

The results of enzyme activity of a different strain of *Trichoderma* in TFM supernatant after 72 h incubation at 180 rpm and 28 °C with different substrates (arabinoxylan, pectin, Avicel, carboxy methyl cellulose (CMC), cellubiose and filter paper) are shown in Figs. 2 and 3. These results indicate variations in the enzyme activity values of the different strains of *Trichoderma*. These values are significantly different at $p < 0.05$. The activities of the enzymes are shown as international units (U), in which, one unit of activity is defined as the amount of enzyme required to liberate 1µmol of product per hour. The amount of reducing sugar released was estimated by the dinitrosalicylic acid method (DNS) using glucose as the standard.

Fig. 2a shows the production of Arabinoxylanase (U/ml) by different species of *Trichoderma.* The *NAS-H101*, *NAS-AV106* and *NAS-P109* isolates secreted approximately 2 times more Arabinoxylanase than the other studied species. Fig. 2b shows the production of pectinase by different isolates of *Trichoderma*. The species of *NAS-K108*, *NAS-Vi114*, *NAS-K108, NAS-A112* and *NAS-A113* isolates secreted approximately 2-fold higher pectinase than the other studied species.

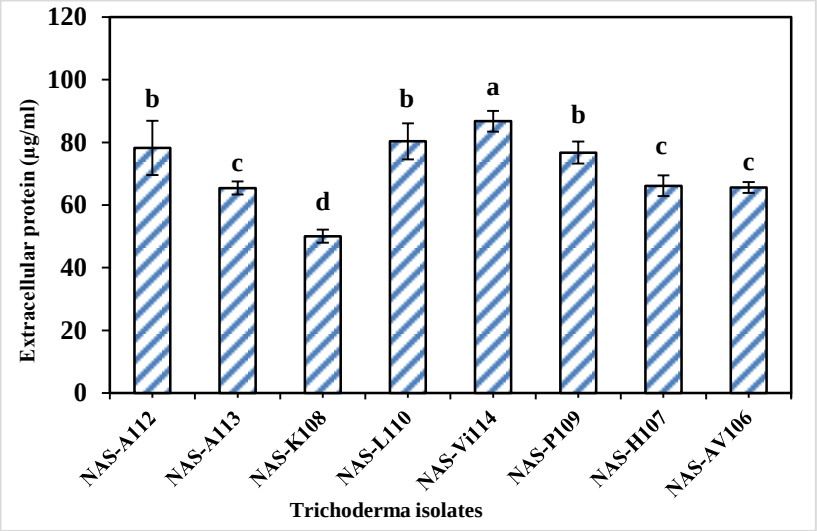


**Figure 1** extracellular protein production (µg/ml) of different *Trichoderma* isolates after 72 h fermentation in TFM supernatant at 180 rpm and 28 °C.





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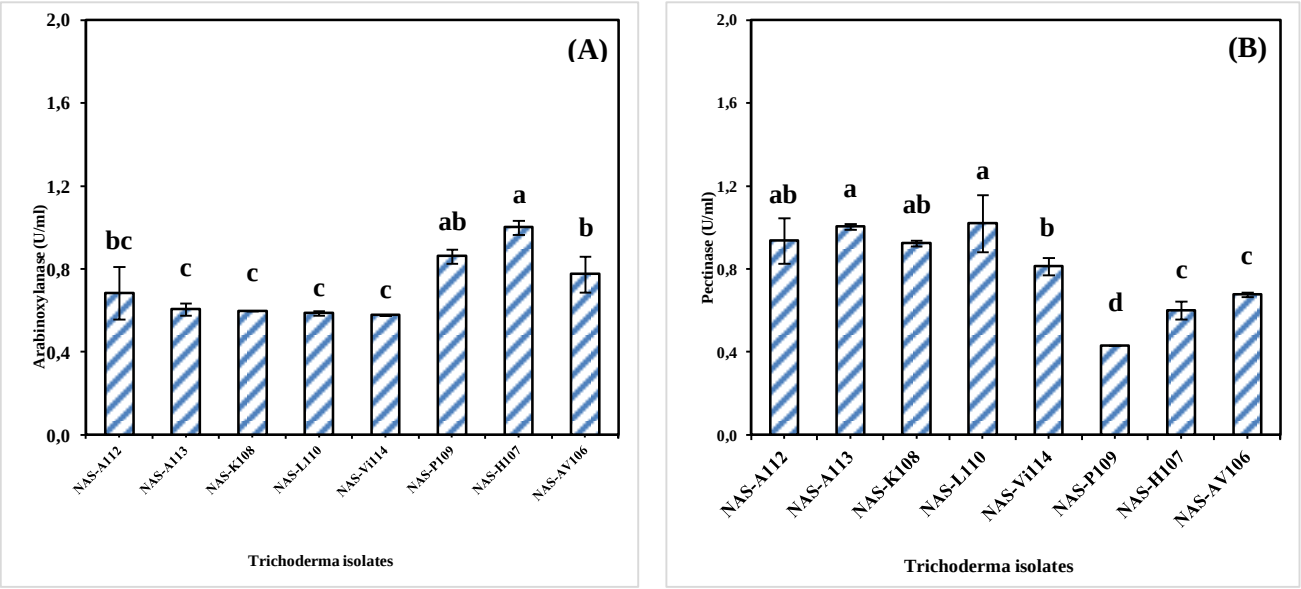


**Figure 2** (A) Arabinoxylanase and (B) Pectinase production (U/ml) of the *Trichoderma* isolates.

To examine the influence of the enzymes expression on colloidal cellulose hydrolysis, the activities of FPA, cellobiohydrolase, endoglucanase and β-Glucosidase were investigated and the results are shown in Fig. 3. Avicelase activities have been analyzed using pure Avicel, and results are given in Fig. 3a. The highest Avicelase activity obtained was 9.93U/ml in supernatant *NAS-A113*. The lowest Avicelase activity which was only 3.27 U/ml was that of the culture supernatant of *NAS-H101*.

CMCase activities have been analyzed using CMC, and results are given in Fig. 3. The highest CMCase activity obtained was 5.18 U/ml in supernatant of *T. reeesei*. The lowest CMCase activity only 3.57 U/ml was measured in the culture supernatant of *T. viride*. Also, the results showed that the highest FPase was about 12mg/ml in supernatant of *NAS-A112*.Significant differences were observed in FPase activity among different studied isolates (Fig. 3).

**Electrophoresis and molecular size determination of proteins**

The electrophoresis patterns obtained by SDS-polyacrylamide gel electrophoresis (PAGE) analysis of the extracellular proteins of TFM supernatants of *NAS-H101* (lane 1), *NAS-AV106* (lane 2), *NAS-P109* (lane 3), *NAS-L110* (lane 4), *NAS-K108* (lane 5), *NAS-Vi114* (lane 6), *NAS-A112* (lane 7) and *NAS-A113* (lane 8) are shown in Fig. 4. There are observable differences in the protein banding pattern ranged from 11 to 245 KDa.

Based on the results, Cel5A and Cel7B were observed in all isolates of *Trichoderma* spp. Cel7A enzyme was observed in molecular weight of 68 KDa for *NAS-AV106*, *NAS-P109* and *NAS-K108* SDS-PAGE profiles that were considered as a Cel7A (CBH I). Also, Cel6A (CBH II) was observed in *NAS-K108*, *NAS-Vi114*, *NAS-A112* and *NAS-A113* SDS-PAGE profiles with molecular weight of 63 KDa (Fig. 4). Cel12A enzyme bond was observed only as a very weak bond in SDS-PAGE profile protein of *T. reesei* and *T. aureoviride (NAS-AV106)* with molecular weight of 25.5 KDa. The sharp bonds were observed in molecular weight of 18 KDa for *T. harzianum* and *NAS-A113* that probably were related to endoglucanase enzymes. In addition, Cel 3A (BGL I) was observed in molecular weight of 75 KDa for *NAS-H101* and *NAS-A112* SDS-PAGE profiles. Cel 1A (BGL II) was observed in molecular weight of 111 KDa for *NAS-P109*, *NAS-H101*, *NAS-A112* and *NAS-A113* SDS-PAGE profiles.





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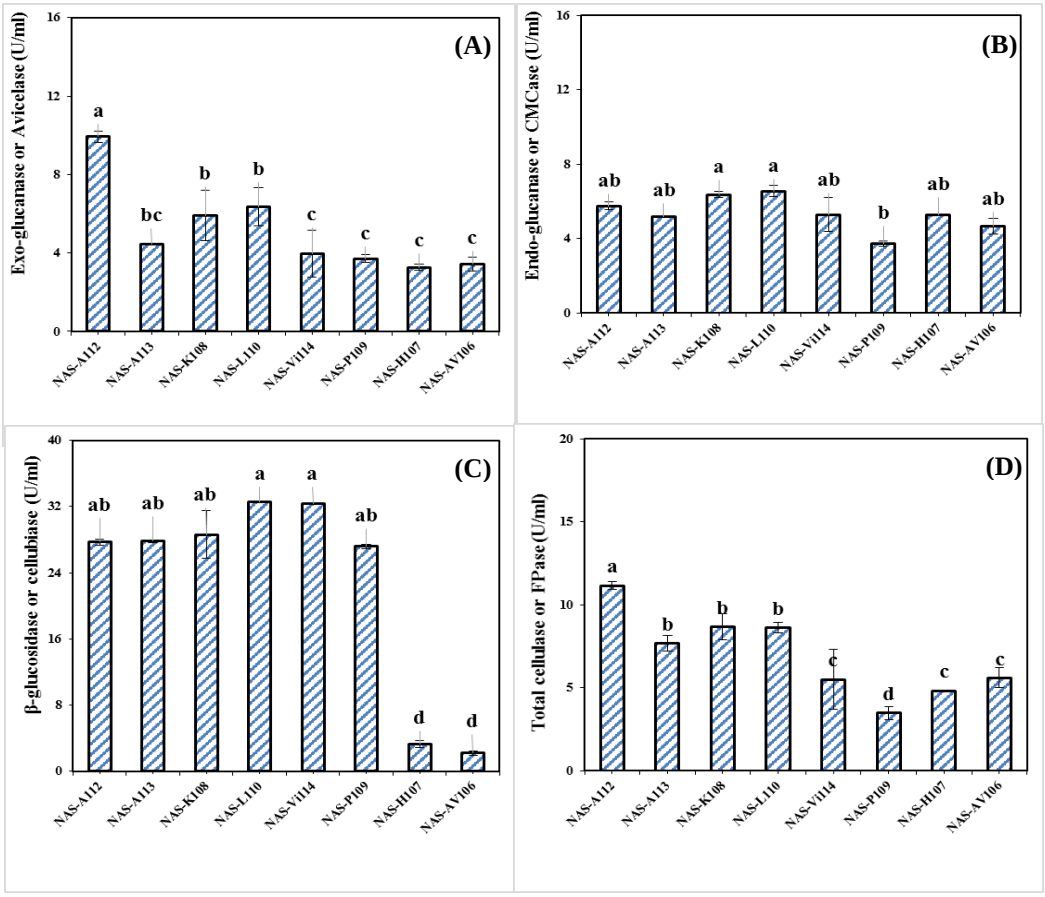


**Figure 3** The enzyme activities of the different isolates of *Trichoderma*: (A) Avicelase or Cellobiohydrolase activity (CBH), (B) Endoglucanase (EG) or CMCase activity and (C) Cellubiase or β-1, 4-glucosidase activity (U/ml) and (D) Total cellulase or FPase activity.

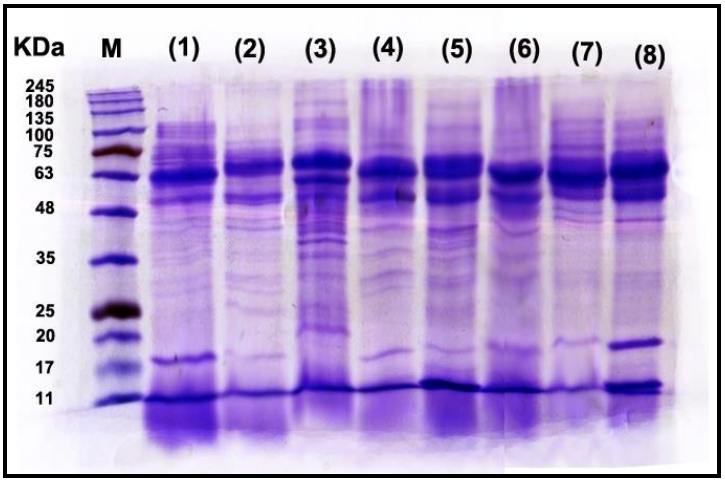


**Figure 4** SDS-PAGE profile of cellulase enzymes: (M) protein marker, (1) *Trichoderma harzianum*, (2) *T. viride*, (3) *T. reesei*, (4) *T. longibrachiatum*, (5) *T. ghanens*, (6) *T. virens*, (7) *T. atroviride* 1-3, (8) *T. atroviride* 60-22.





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## Discussion

Determining the extracellular protein concentration is not always a simple task, since various factors may interfere with the final result (Zaia *et al.*, 1998). There are three main factors that influence these measurements: (a) each protein dosage method is based on a different identification and quantification principle; (b) the presence of non-protein components in the enzymatic solution and/or reaction medium can be a source of error if they interfere with the results of the quantitative method; and (c) other non-cellulase proteins present in the enzyme preparation may compromise the interpretation of the specific activity data. Such differences are also due to the fact that different species of enzymes have different primary structures, in addition to different degrees of glycosylation. Therefore, these factors are reflected in the response of the proteins from different species of *Trichoderma*. In our study *T. ghanens* (NAS-K108) and *T. virnse* (NAS-Vi114) showed the lowest and highest protein content, respectively.

Substrates for cellulase activity assays can be divided into two categories, based on their solubility in water. Ionic substituted carboxymethyl cellulose (CMC, as a soluble substrate) is often used to determine endoglucanase activity, called CMCase, because endoglucanases cleave intramolecular β-1, 4- glucosidic bonds randomly, resulting in a dramatic reduction in the degree of polymerization (DP) (i. e., specific viscosity) of CMC. The structure of the endoglucanase is known, and the active-site residues in the enzymes are situated in a cleft that can accommodate the carboxymethyl groups in such a manner that the individual glucose units can be attacked. Glucanase enzyme production of *Trichoderma* species have been studied by Pandey *et al.* (2014). Based on the results optimum temperature and pH for enzyme production was 50 °C and 5.0, respectively. Also they introduced CMC as the best enzyme introducer compared to wood dust substrate. Also, cellulase activity of *T. reesei* was improved significantly by adding 50 µmol/ml of gibberellins hormone in culture medium (Laamerad and Ansari, 2015).

Among pectinase-producing fungi *Aspergillus niger* certainly is the frequently used one (Ismail, 1996; Castilho *et al.*, 2000), while among *Trichoderma* genus, few species are recorded as good pectinase producers, like *T. lignorum* (Mabrouk *et al.*, 1979) and *T. pleuroticola* (Haltmeier *et al.*, 1983). Jagavati *et al.* (2012) reported the increased level of cellulose production including filter paper activity, CMCase and β-glucosidase activity in co-culture of *Trichoderma* sp. and *Aspergillus* sp. The polygalacturonase assay applied in current study did not discriminate between endo- and exo-polygalacturonase (pectinase) activities. Both an exo- and an endo-polygalacaturonase have been purified from *T. reesei* QM9414 when grown on citrus pectin as carbon source (Markovic *et al.*, 1985). Furthermore, two endo-acting polygalacturonases from *T. reesei* grown on selective alkaline treated sugar beet pectin have been purified and characterized (Mohamed *et al.*, 2003). Consequently, the varying level of polygalacturonase activity (Fig. 2b) measured during growth on colloidal cellulose could indicate that different species of *Trichoderma* produce several polygalacturonases; either different isoforms or with different mode of action.

Evaluation of FPA, cellobiohydrolase, endoglucanase and β-Glucosidase activities showed that *T. atroviridae* (*NAS-A113*) and *T. harzianum* (*NAS-H101*) had the highest and lowest avicellase activity. Exoglucanases cleave the accessible ends of cellulose molecules to liberate glucose and cellobiose. *T. reesei* cellobiohydrolase (CBH) I and II act on the reducing and non-reducing cellulose chain ends, respectively (Zhang and Lynd, 2004). CBHs (exoglucanases) are classified as exo-acting based on the assumption that they all cleave β-1, 4- glycosidic bonds from chain ends releasing cellobiose and some glucose molecules. Commercial Avicel (also called microcrystalline cellulose or hydrocellulose) is





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used for measuring exoglucanase activity because it has a low degree of polymerization of cellulose and it is relatively inaccessible to attack by EGs despite some amorphous regions. Enzymes that show relatively high activity on Avicel and little activity on CMC are identified as exoglucanases (Maki *et al.,* 2009).

Based on the results of CMCase activity, *T. reeesei* had the highest (5.18 U/ml) and *T. viride* had the lowest (3.57 U/ml) enzyme activity. EGs (CMCase) can randomly hydrolyze internal glycosidic bonds in cellulose chains. CBHs (exoglucanases) are classified as exo-acting based on the assumption that they all cleave β-1, 4-glycosidic bonds from chain ends releasing cellobiose and some glucose molecules. Commercial Avicel (also called microcrystalline cellulose or hydrocellulose) is used to measure exoglucanase activity because it has a low degree of polymerization of cellulose and it is relatively inaccessible to be attacked by EGs despite some amorphous regions. Enzymes that show relatively high activity on Avicel and little activity on CMC are identified as exoglucanases (Maki *et al.,* 2009). β-Glucosidase can accelerate cellulose degradation by reducing end product inhibition and thus plays an important role in this synergistic action (Kovács *et al.,* 2008). The reason for the low secretion of this enzyme into the fermentation broth of *NAS-H101* and *NAS-AV106* is that the major part of the β-glucosidase is tightly bound to the cell walls of the fungus during cultivation and some part of the enzyme may be found inside the cells (Kubicek, 1981). It is worth noting that very high β-glucosidase activity does not further increase the hydrolytic capacity and the overall FPase, as reported by Kovács *et al.* (2008). In addition, it also suggests that other accessory enzymes, such as xylanase may compensate the function of β-glucosidase, increase the biomass accessibility, and consequently contribute to the improvement in the sugar yield. Besides measuring the enzyme activities, it was very important also to determine the hydrolytic capacity of the produced enzymes on the lignocellulosic materials. In many cases there is no correlation between the initial hydrolysis of a Whatman No.1 filter paper strip (FPA assay) and the liberation of reducing sugars from the lignocelluloses. It may happen that not the best strain (according to FPA enzyme activity) will be the choice of practical applications. Therefore, the enzyme complex giving the highest glucose yield was not the one having the highest FPase activity. Filter paper activity (FPA) was measured. Data are represented as the mean of three independent experiments; error bars express the standard deviations. Breuil *et al.* (1992) suggested to look at the profile of the individual sugars (especially cellobiose and glucose) released during the filter paper assay in order to be able to better predict the ability of a cellulase mixture to hydrolyze cellulosic materials. The most common total cellulase activity assay is the FPA using Whatman No. 1 filter paper as the substrate, which was established and published by the International Union of Pure and Applied Chemistry (IUPAC) (Ghoseh, 1987).

In this study several molecular bands were observed in the protein profiles, while the TFM supernatant (not inoculated) had no specific protein binding. Cel5A is an EG that belongs to GH family5. The enzyme has an estimated molecular weight of 42 kDa, but has an apparent molecular weight of 48 kDa on SDS-PAGE gel due to glycosylation. It has a pI of 5.5-5.6 (Shoemaker *et al.,* 1983). This enzyme was observed as very weak and faint band in all isolates' SDS-PAGE profiles. Cel5A hydrolyzes the β-1,4-glycosidic bonds in cellulose using the retaining mechanism (Henrissat *et al.,* 1985). The amount of expressed Cel5A has been estimated to be between 5-10% of total expressed cellulase in *T. reesei* (Ståhlberg, 1991; Ilmen *et al.*, 1997).

Cel7A is a GH family 7 CBH and it was the first *T. reesei* GH family 7 cellulase that was discovered (Wey *et al.,* 1994). Cel7A has an estimated molecular weight of 52 kDa, 66 kDa on a SDS-PAGE, and it has a pI of 4.3 (Fägerstam *et al.,* 1977; Shoemaker *et al.,* 1983). Cel7A is the major cellulase produced by *T. reesei*, and it has been estimated that 50-





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60% of total expressed cellulase in the fungus is Cel7A (Ståhlberg, 1991; Ilmen *et al.*, 1997). It is probably the key enzyme needed for hydrolysis of crystalline cellulose by the fungus. Cel7A is a processive enzyme that hydrolyzes the glycosidic bonds in cellulose using the retaining mechanism and it has been shown that the enzyme preferably hydrolyzes the cellulose chain from the reducing end (Barr *et al.*, 1996; Divne *et al.*, 1998). This enzyme was detected in *NAS-AV106, NAS-P109* and *NAS-K108* SDS-PAGE profiles (68 KDA) that were considered as a Cel7A (CBH I). Cel6A is a GH family 6 CBH and has an estimated molecular weight of 47 kDa, 53 kDa on a SDS-PAGE, and it has a pI of 5.9 (Fägerstam and Pettersson, 1980; Bhikhabhai *et al.*, 1984). Cel6A is a processive enzyme that hydrolyzes the glycosidic bonds in cellulose using the inverting mechanism and it has been shown that the enzyme preferably hydrolyzes the cellulose chain from the non-reducing end (Barr *et al.*, 1996; Boisset *et al.*, 2000). There have been reports that Cel6A possesses some endoglucanase activity (Nutt *et al.*, 1998). The amount of expressed Cel6A has been estimated to be between 17-20% of total expressed cellulase in *T. reesei* (Ståhlberg, 1991; Ilmen *et al.*, 1997). Cel6A (CBH II) was observed in *NAS-K108, NAS-Vi114, NAS-A112* and *NAS-A113* SDS-PAGE profiles with molecular weight of 63 KDa (Fig. 4). Cel7B is a GH family 7 EG and has an estimated molecular weight of 48 kDa, 50-55 kDa on a SDS-PAGE, and it has a pI of 4.5 (Shoemaker *et al.*, 1983; Bhikhabhai *et al.*, 1984). Cel7B is homologous to Cel7A, with about 45 % sequence identity. The main difference between the two GH family 7 structures is that the substrate-binding cleft is less covered by extended loops in the endoglucanase (Cel7B) than in the exoglucanase (Cel7A). Cel7B hydrolyzes the glycosidic bonds in cellulose using the retaining mechanism. The amount of expressed Cel7B has been reported to be between 6-10% of total expressed cellulase in *T. reesei* (Ståhlberg, 1991; Ilmen *et al.*, 1997). Cel7B enzyme bonds were observed in SDS-PAGE profile protein of all *Trichoderma* spp. with molecular weight of 54 KDa. Cel12A is a GH family 12 EG and the enzyme has a molecular weight of 25 kDa with a neutral pI of 7.5 (Ülker and Sprey, 1990; Sprey and Bochem, 1992; Hayn *et al.*, 1993). Cel12A hydrolyzes the glycosidic bonds in cellulose using the retaining mechanism. The two catalytic residues in Cel12A are the two carboxylates E116 and E200 (Okada *et al.*, 1998). The amount of expressed Cel12A has been reported to be less than 1% of total expressed cellulase in *T. reesei* (Ülker and Sprey, 1990). The specific function for *T. reesei* Cel12A is not known. Some biochemical data on Cel12A can be found in the literature, including studies of activity on soluble substrates (Hayn *et al.*, 1993), and insoluble cellulase (Sprey and Bochem, 1992). There have been reports that Cel12A, besides cellulose activity, has an activity against β-glucan and xylan (Hayn *et al.*, 1993; Karlsson *et al.*, 2002). It has been shown that Cel12A has an ability to induce extension of type I cell walls from cucumber and wheat (Yuan *et al.*, 2001). Cel12A enzyme bond was observed only as a very weak bond in SDS-PAGE profile protein of *T. reesei* and *T. aureoviride (NAS-AV106)* with molecular weight of 25.5 KDa. The sharp bonds were observed in molecular weight of 18 KDa for *T. harzianum* and *NAS-A113* that probably were related to endoglucanase enzymes. Some biochemical data on EG can be found in the literature, including studies of activity on soluble substrates (Hayn *et al.*, 1993), and insoluble cellulase (Sprey and Bochem, 1992). There have been reports that EG, besides cellulose activity has an activity against β- glucan and xylan (Hayn *et al.*, 1993; Karlsson *et al.*, 2002). Also, Cel 3A (BGL I) was observed in molecular weight of 75 KDa for *NAS-H101* and *NAS-A112* SDS-PAGE profiles. Cel 1A (BGL II) was observed in molecular weight of 111 KDa for *NAS-P109, NAS-H101, NAS-A112* and *NAS-A113* SDS-PAGE profiles. β- Glucosidase hydrolyzes the soluble oligosaccharides, produced by cellulases, to glucose. The addition of β-glucosidases into the *T. reesei* cellulases





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system achieved better saccharification than the system without β-glucosidases (Xin *et al.*, 1993). β- Glucosidases hydrolyze the cellobiose which is an inhibitor of cellulase activity.

## Conclusion

The total cellulase system consists of endoglucanases, exoglucanases, and β-D-glucosidases, all of which hydrolyze crystalline cellulose synergically. Synergism between endoglucanases and exoglucanases is the most widely studied type of synergy and is among the most quantitatively important for hydrolyzing the crystallization of crystalline cellulose. Cellulose crystallinity has long been thought to play an important role in enzymatic hydrolysis. The concept that cellulose structure is divided into two regions, an amorphous region that is easy for enzymes to digest and a crystalline region that is difficult to digest, is extremely appealing. This provides a ready explanation of observed cellulose digestion kinetics, where enzymes more rapidly digest the 'easy and presumed amorphous' material before more slowly digesting the more difficult crystalline cellulose. In addition to substrate properties, experimental conditions also affect the extent of synergy observed. Total cellulase activity assays are always measured using insoluble substrates, including pure cellulosic substrates such as Whatman No. 1 filter paper, cotton linter, microcrystalline cellulose, bacterial cellulose, algal cellulose; and cellulose-containing substrates such as dyed cellulose, α-cellulose, and pretreated lignocellulose. Avicel contains some amorphous cellulose and soluble cellodextrans, which can act as substrates for both exo- and endo-glucanases. There is no highly specific substrate to test exoglucanase activity in cellulase mixtures (Wood and Bhat, 1988). However, this substrate could not be used to determine CBH II activity of *T. reesei*, thus it is not an effective representation of true exoglucanase activity for this strain (van Tilbeurgh *et al.*, 1982, 1985). Avicel has the highest ratio of chain ends to accessible internal β-glucosidic bonds among model cellulosic substrates. The *T. reesei* CBH I and CBH II can cleave several bonds following a single adsorption even before the dissociation of the enzyme substrate complex (Valjamae *et al.*, 1998). Therefore, the actions of CBH I and CBH II result in a gradual decrease in the degree of polymerization (DP) of cellulose (Valjamae *et al.*, 1998).According to the SDS-PAGE profile of proteins (Fig. 4), *NAS-K108* and *NAS-P109* have both enzyme bonds of CBHI and CBH II, but other isolates have only a sharp enzyme bond correlating to CBHI or CBH II. The high Avicelase activity in *NAS-A113* is due to production of high extracellular protein concentration and CBH II. The specific activity of CBH II has been found to be nearly twice that of CBH I in most studies (Medve *et al.*, 1994; Nidetzky and Claeyssens, 1994). The SDS-PAGE profile of *NAS-P109* protein has several enzyme bands such as CBH and BGL, but no significant sharp bands correlating to EG was observed. Therefore, the lowest EG activity was observed in *NAS-P109*. Efficient overall hydrolysis of crystalline cellulose by cellulases requires the synergistic action of both EGs and CBHs, as reviewed by Teeri and Koivula (1995). Maximum synergism is usually obtained with a large amount of exo-enzyme and a minor amount of endo-enzyme (Reinikainen, 1994). It is also known that the degree of synergy is dependent on the substrate used (Nidetzky and Steiner, 1993).This occurrence was observed in FPase of *NAS-A112*, that contains of a large amount of CBH I and CBH II and a minor amount of EG. For the determination of total cellulolytic activities of cellulase mixtures or their complex synergistic action, more heterogeneous substrates such as filter paper can be used. The total cellulase system consists of endoglucanases, exoglucanases, and β-D-glucosidases, all of which hydrolyze crystalline cellulose synergically. The highest FPase activity was observed in *NAS-A112*.A widely accepted theory for the synergistic action between CBH and EG is the so-called 'endo-exo' model. Initially, EG hydrolyses internal β-1, 4-gicosidic bonds randomly in the chains at the surface of the cellulose fibers and thereby producing free





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chain ends. EGs initiate attack (especially in amorphous regions) creating additional sites for hydrolysis by CBH to yield small oligosaccharides (mainly cellobiose). Therefore, the high values of FPase activity in *NAS-A113* are due to presence of CBH and EG enzymes and cooperative behavior or synergism that occurs between the CBH and EG to bring about the complete hydrolysis of cellulose to glucose. Also, the presence of BGL I and II in SDS-PAGE profile of *NAS-H101* protein resulted in high FPase activity compared with *NAS-P109*. β-glucosidase has been shown to greatly increase the rate and extent of hydrolysis by ensuring the efficient hydrolysis of cellobiose reducing the influence of the end product inhibition.

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# اثر متقابل فعالیت آنزیم‌های سلولاز در گونه‌های تریکودرما تحت تأثیر سلولزهای کلوییدی مختلف

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**چکیده:** در این تحقیق فعالیت سلولیتیکی جدایه‌های ایرانی گونه‌های مختلف تریکودرما از جمله *Trichodrma harzianum* (*NAS-H101*)، *T. aureoviride* (*NAS-AV106*)، *T. pleuroticola* (*NAS-P109*)، *T. longibrachiatum* (*NAS-L110*)، *T. ghanens* (*NAS-K108*)، *T. virens* (*NAS-Vi114*)، *T. atroviride* (*NAS-A113*) و *T. atroviride* (*NAS-A112*) مورد بررسی قرار گرفت. میزان پروتئین خارج سلولی این جدایه‌ها با استفاده از روش رنگ‌آمیزی بردفورد اندازه‌گیری شد. وزن مولکولی آنزیم‌های سلولاز با استفاده از الکتروفورز ژل پلی اکریلامید مشخص شد. کم‌ترین میزان پروتئین خارج سلولی در جدایه *NAS-K108* تولید شد. بیش‌ترین میزان فعالیت اندوگلوکانار (CMCase) و اگزوگلوکاناز (آویسلاز) به‌ترتیب در جدایه‌های *NAS-L110* و *NAS-A113* به‌دست آمد. در پروفایل الکتروفورز ژل پلی اکریلامید چندین باند آنزیمی از جمله سلوبیوهیدرولازها، اندوگلوکانازها، بتاگلوکوزیدازها مشاهده شد. دو جدایه *NAS-K108* و *NAS-K109* هر دو باند آنزیمی CBHI و CBHII را داشتند ولی در سایر جدایه‌ها یک باند آنزیمی مشخص مربوط به یکی از آن‌ها دیده شد. بیش‌ترین اثر افزایشی مربوط به FPase در جدایه *NAS-A112* می‌باشد که دارای مقدار زیادی Cel6A (CBHII) و مقدار کمی Cel7B (EGI) است. نتایج نشان می‌دهد که جدایه NAS-A113 مقدار زیادی سلولاز، بتاگلوکوزیداز و آنزیم‌های خارج سلولی تولید می‌کند، از این‌رو می‌توان این جدایه را به‌عنوان یک عامل بیولوژیکی و منبع آنزیمی برای تجزیه سلولز در سلولز کلوئیدی پیشنهاد نمود.

**واژگان کلیدی:** آنزیم سلولاز، تریکودرما، الکتروفورز ژل پلی اکریلامید



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