J. Crop Prot. 2016, 5 (3): 397-412
doi: 10.18869/modares.jcp.5.3.397

Research Article

The impact of different physicochemical parameters of fermentation on extracellular cellulolytic enzyme production by *Trichoderma harzianum*

Samira Shahbazi[1*], Hamed Askari[1] and Shideh Mojerlou[2]

1. Nuclear Science and Technology Research Institute (NSTRI), Atomic Energy Organization of IRAN (AEOI), Karaj, Iran.
2. Department of Horticulture and Plant Protection, Shahrood University of Technology, Shahrood, Iran.

Abstract: This study was undertaken to find out the optimum physicochemical parameters of fermentation, i.e. pH, incubation temperature and incubation time for the cellulase enzyme production of *Trichoderma harzianum*. The extracellular protein content was estimated by the dye binding method of Bradford. Endo-glucanase (EG), exoglucanase (or Cellobiohydrolase; CBH), β-glucosidase and total cellulase activity were investigated. The molecular weight of cellulase enzymes was studied using SDS-PAGE. To identify the predominant catalytic components in optimum conditions of enzyme production, cellulases were separated by an adapted two-dimensional electrophoresis technique. Estimated optimum conditions for cellulase enzyme were found as: pH 6.5, incubation temperature 28°C and incubation time 72 h. The SDS-PAGE profiles showed several enzyme bonds such as CBHs, EGs and BGLs. The *T. harzianum* had both enzyme bonds of Cel7A (CBHI) and Cel7B (EG). Finally, the results of the 2D PAGE analysis showed that the profile of protein in optimium conditions of enzyme production had several enzymes such as CBHs, EGs and the high values of cellulose activity due to synergism that occurred between the CBH and EG.

Keywords: *Trichoderma harzianum*, Cellulase enzyme, SDS-PAGE, 2D-PAGE

Introduction

Cellulose forms the basic structural ingredient of the cell wall of eukaryotic plants and algae and is also found as a major constituent of the cell wall of true fungi (Cannon and Anderson, 1991). It is therefore, the most abundant bio-polymer on the earth, of which 180 billion tons is produced per year (Zhao *et al*., 2007). It is a simple straight chain polymer of glucose molecules that are linked at the β, 1-4 position. Cellulose is most commonly obtained from wooden parts of trees and cotton plant but is also derived from flax, jute and hemp (Cannon and Anderson, 1991; Zhao *et al*., 2007). The enzyme system for the conversion of cellulose to glucose involves at least three types of cellulases including endoglucanase (EG, E.C.3.2.1.4), cellobiohydrolase (CBH, E.C.3.2.1.91) and β-glucosidase (BGL, 3.2.1.21). EGs act randomly along the cellulose chains to produce cellulose fragments. CBH acts as exoglucanase to release the disaccharide cellobiose. The BGLs then hydrolyze cellobiose to yield glucose (de Palma-Fernandez *et al*., 2002). Biodegradation of lignin and xylan requires laccase (EC.1.10.3.2), lignin

Handling Editor: Naser Safaie

* **Corresponding author**, e-mail: sshahbazi@nrcam.org
Received: 12 April 2016, Accepted: 28 May 2016
Published online: 22 June 2016

[DOR: 20.1001.1.22519041.2016.5.3.2.5]

peroxidase (EC. 1.11.1.14), endo-1,4-β-D-xylanxylano hydrolase (EC.3.2.1.8), β-xylosidase (EC.3.2.1.37) and several other accessory enzymes (Baldrian, 2006). All these enzymes work synergistically to hydrolyse cellulose by creating new accessible sites for each other, removing obstacles and relieving product inhibition (Eriksson *et al*., 2002; Valjamae *et al*., 2003). Consequently, these reactions can be affected by different obstacles such as the use of high substrate concentrations, the presence of lignin, which shields the cellulose chain and adsorbed enzymes, crystallinity of cellulose, surface area, pore size, degree of polymerization (DP) and hemicellulose content (Chang and Holtzapple, 2000; Arantes and Saddler, 2010). Enzymatic saccharification of cellulosic biomass has been considered as an ecofriendly method that replaces conventional chemical treatments. A wide variety of microorganisms are involved in cellulase production including bacteria and fungi (Maki *et al*., 2009; Singhania *et al*., 2010). Most of the cellulase exploited for industrial applications are from filamentous fungi such as *Trichoderma* spp., *Penicillium* spp., *Fusarium* spp., *Humicola* sp., *Phanerochaete* spp. etc., where a large number of cellulases are generated (Singhania *et al*., 2010). Cellulase enzymes are mainly used in food, pulp and paper and textile industries. Environmental and nutritional factors are known to have marked effects on enzyme production by microorganisms. There are, therefore, variations in optimum conditions for cellulase production. In this study, the optimization of cellulase enzyme production by *Trichoderma harzianum* as functions of incubation time, incubation temperature and pH of fermentation was investigated. Also, protein profiles of the produced enzymes were examined.

Materials and Methods

Culture condition of fungi

Trichoderma harzianum was isolated from the soil samples collected from different agricultural fields (Khorasan province, Iran) by serial dilution on *Trichoderma* selective medium (Papavizas and Lumsden, 1982). To prevent bacterial growth, the culture medium was amended with 50 $mg.l^{-1}$ ampicillin, 15 $mg.l^{-1}$ tetracycline and 30 $mg.l^{-1}$spectinomycin. 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 harzianum was maintained on agar media (MYG agar medium) containing ($g.l^{-1}$): malt extract, 5; yeast extract, 2.5; glucose, 10; agar, 20. Washed spore suspension was prepared from seven-day-old slant cultures in sterile saline solution and used as an inoculum of 1×10^7 spores/ml of medium. The spores were pelleted by centrifugation at 4500 × g for 10 min, and washed twice in sterile saline solution. Seed culture was produced in *Trichoderma* complete medium (TCM) which contained ($g.l^{-1}$): bactopeptone, 1.0; urea, 0.3; KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 1.4; $MgSO_4.7H_2O$, 0.3; $CaCl_2.6H_2O$, 0.3; $FeSO_4.7H_2O$, 0.005; $MnSO_4$, 0.002; $ZnSO_4$, 0.002; $CoSO_4.7H_2O$, 0.002 and 2 $ml.l^{-1}$, Tween 80. The medium was adjusted to pH 4.8 and supplemented with 0.3% w/v of glucose. Cultures were produced in 50 ml volumes of TCM in 250ml Erlenmeyer flasks shaken at 180 rpm at 28˚C for 24 h. To induce production of cellulase enzymes washed mycelia were transferred to 25ml of *Trichoderma* fermentation medium (TFM) which contained ($g.l^{-1}$): urea, 0.3; KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 1.4; $MgSO_4.7H_2O$, 0.3; $CaCl_2.6H_2O$, 0.3; $FeSO_4.7H_2O$, 0.005; $MnSO_4$, 0.002; $ZnSO_4$, 0.002; $CoSO_4.7H_2O$, 0.002 and 2 $ml.l^{-1}$, Tween 80. This medium was adjusted to different pH (4, 4.8, 5.5 and 6.5) and supplemented with 0.5% w/v of Phosphoric Acid Swollen cellulose (PASC). Growth conditions were as described previously and triplicate flasks were harvested after different incubation times (48,

[DOR: 20.1001.1.22519041.2016.5.3.2.5]

72, 96 and 120 h) at different incubation temperatures (25, 28 and 34 °C). Estimation of protein and extracellular cellulase activity was assayed in *Trichoderma* fermentation medium (TFM) after centrifugation at 4500 × g for 7 min at 4 °C.

Estimation of protein and cellulase activity

The protein content in the TFM supernatants was estimated in different conditions of fermentation by the dye binding method of 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 3 ml of Bradford reagent. The standards and tests were replicated three times. The absorbance was read at 595 nm on a spectrophotometer (Jenway, USA). Avicelase, Carboxymethylcellulase (CMCase) and bacterial cellulase 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 50 $g.l^{-1}$Avicel, CMC and bacterial cellulase in 0.05M citrate buffer (pH 4.8) and 0.5ml of each supernatant of TFM medium. After incubation at 50 ˚C for 60min, the controls and samples were taken out of the 50˚C bath. The reactions were terminated by adding 3 ml of 3, 5- dinitrosalicylic acid (DNS). The mixtures were also mixed well, then placed into a boiling-water bath for 5min, 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 µmole of glucose per minute in a standard assay. Also, for FPase assay, 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 (Bradford, 1976; Gama and Mota, 1998).

Electrophoresis and molecular size determination

Protein samples (5 ml) from TFM supernatants were precipitated with equal volume of acetone and precipitated proteins were resuspended in double distilled water in final volume of 100 µl, frozen and kept at -70 ˚C until they were used. The molecular weight of the cellulase was determined by sodium dodecyl sulfate–poly-acryamide gel electrophoresis (SDS–PAGE) with a 4% (stacking) and 12.5% (separating) polyacrylamide gel based on Laemmli (1970). Before electrophoresis, equal volume of sample buffer (100 µl) that contained 65 mM Tris–HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 0.2% (w/v) bromophenol blue was added to the protein sample (100 µl) and boiled for 5 min and applied to loading on the gels. The proteins were separated at constant amperage of 25 mA using the running buffer containing 25 mMTris, 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).

Isoelectric focusing and 2D SDS–PAGE

Regardless of the initial protein concentration in the TFM supernatant of optimum fermentation condition, the same amount of protein (1200 µg) was used for 2-D gel. Proteins were precipitated by incubating the supernatant with 13.3% (w/v) trichloroacetic acid (TCA) in cold acetone containing 0.2% (w/v) dithiothreitol (DTT) for 1 h at 4 °C. Following centrifugation at 6000 g for 15 min at 4 °C, the pellet was resuspended in 20 ml cold acetone and incubated on ice for 30 min. Centrifugation was then repeated at 6000 × g for 15 min at 4 °C; the supernatant was removed and the pellet was air dried prior to resuspension in 360 µL of a sample solution

[DOR: 20.1001.1.22519041.2016.5.3.2.5]

consisting of 8 M urea, 2% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 0.002% (w/v) bromophenol blue, 40 mM DTT, 0.5% Bio-Lyte® 3/10 ampholyte in double distilled water. The sample was used directly on 17 cm immobilized strips (3–10, pH gradient (IPG), (Bio-Rad, CA, USA) and rehydrated by applying 360 µL of sample. IPG was equilibrated for 16 h and then placed on top of PROTEAN IEF Cell (Bio-Rad, USA) and then IPG was focused to a total of 52,500 Volt hours (Vh) at 50 mA constant using a three-step focusing program. The focusing program included a rapid ramp to 500 V for 1 h, a linear ramp to 1,000 V over 1 h, and a 8,000 V step until 52,500 Vh were reached. Second dimension separation of proteins was carried out on 1.5-mm thick continuous 12.5% (w/v) acrylamide gel using the same gel buffer as in one-dimensional separation. The second dimensional electrophoresis, SDS-PAGE, was performed with PROTEAN II xi 2-D Electrophoresis Cell (Bio-Rad) under the conditions of constant current. For the first 30 min, the current was set at 20 mA per gel. The current was 40 mA per gel for the rest of electrophoresis time. The SDS-PAGE was developed until the bromophenol blue dye marker had reached at 0.5 cm from the bottom of the gel. After electrophoresis, the gel was 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).

Results and Discussion

Estimation of extracellular protein production

The extracellular protein concentration of *T. harzianum* was determined by the dye binding method of Bradford as function of incubation time (48-72 h), incubation temperature (25, 28 and 34 ˚C) and pH (4, 4.8, 5.5 and 6.5). The amount of protein was calculated using bovine serum albumin (BSA) as a standard and results are shown in Fig. 1. Protein concentration ranged from 5.87 to 234.13 µg/ml in supernatant of TFM at different conditions of fermentation. The maximum amount of extracellular protein content was obtained at pH > 5.5, within incubation time of 72-100 h at incubation temperature of 25 °C. Increasing fermentation incubation temperature resulted in decreased protein production. The lowest amount of protein production was observed at incubation temperature of 34 °C.

Estimation of enzymes activity

The results of cellulase activity of *T. harzianum* in TFM supernatant as function of incubation time (48-72 h), incubation temperature (25, 28 and 34 ˚C) and pH (4, 4.8, 5.5 and 6.5) with different substrates (Avicel, Carboxy methyl cellulose (CMC), Cellubiose and filter paper) are shown in Figs. 2, 3 and 4. These results indicate variations in the enzyme activity values of the *Trichoderma harzianum*. The activities of the enzymes are shown as international units (IU or U), in which, one unit of activity is defined as the amount of enzyme required to liberate 1 µmole of product per hour. The amount of reducing sugar released was estimated by the dinitrosalicylic acid method using glucose as the standard. CBH (exoglucanases) are classifed 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 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. Fig. 2a illustrates the Avicelase enzyme activity as function of incubation time and pH in constant incubation temperature of 25 °C. The lowest Avicelase activity in different incubation times of fermentation was observed in pH 5.5. Increasing the pH above 5.5 increased the activity of this enzyme in constant incubation temperature of 25 °C. The highest Avicelase

[DOR: 20.1001.1.22519041.2016.5.3.2.5]

activity was observed in pH 6.5, incubation time of 96 h and constant incubation temperature of 25 °C. CMCase activities have been analyzed using CMC as a substrate, and results are shown in Fig. 2b. The highest CMCase activity at incubation temperature of 25 °C was 7 U/ml in incubation time of 96 h and the pH above 5.5. EGs (CMCase) can randomly hydrolyze internal glycosidic bonds in cellulose chains. Fig. 2c illustrates the β-(1, 4) glucosidase enzyme activity as function of incubation time and pH at incubation temperature of 25 °C. Increasing incubation time of fermentation increased the activity of this enzyme at incubation temperature of 25 °C. The highest enzyme activity for all incubation times was observed at pH less than 4.8. The results for total cellulase (FPase) assay of *T. harzianum* at incubation temperature of 25 °C are shown in Fig. 2d. The results showed that the highest FPase was between 10-14 U/ml in different incubation times of fermentation and the pH above 5.5 at 25 °C. Fig. 3a illustrates the Avicelase enzyme activity as function of incubation time and pH in constant incubation temperature of 28 °C. Increasing the pH increased the activity of this enzyme at incubation temperature of 28 °C and the highest Avicelase activity was observed in pH above 5.5 and incubation time of 72 h. Fig. 3b illustrates the CMCase enzyme activity as function of incubation time and pH in constant incubation temperature of 28 °C. Increasing the incubation time of fermentation increased the activity of this enzyme and the highest CMCase activity was observed at the pH above 5.5 and incubation time of fermentation above 96 h. Fig. 3c illustrates the β- (1, 4) glucosidase enzyme activity as function of incubation time and pH at constant incubation temperature of 28 °C. Increasing incubation time of fermentation decreased the activity of this enzyme in different pH of fermentation media. At different pH of fermentation, the highest β-(1, 4) glucosidase activity was observed in incubation time between 48-72 h. The results for FPase assay of *T. harzianum* at constant incubation temperature of 28 °C are shown in Fig. 3d as function of incubation time and pH. The results showed that the lowest FPase was observed in pH 5.5 and the highest enzyme activity in different incubation times of fermentation was observed at the pH above 6. Fig. 4a illustrates the Avicelase enzyme activity as function of incubation time and pH in constant incubation temperature of 34 °C. Increasing the pH increased the activity of this enzyme at incubation temperature of 28 °C and the highest Avicelase activity was observed in pH above 5.5 and incubation time of 96 h. Fig. 4b illustrates the CMCase enzyme activity as function of incubation time and pH at constant incubation temperature of 34 °C. Increasing the pH of fermentation media decreased the activity of this enzyme and the highest CMCase activity was observed at the pH 4.8 and incubation time of fermentation between 72-96 h. Fig. 4c illustrates the β- (1, 4) glucosidaseenzyme activity as function of incubation time and pH in constant incubation temperature of 34 °C. Increasing incubation time of fermentation increased the activity of this enzyme in different pH of fermentation media. At different pH of fermentation, the highest β-(1, 4) glucosidase activity was observed in incubation time above 96 h. The results for filter paper assay of *T. harzianum* at constant incubation temperature of 34 °C are shown in Fig. 4d as function of of incubation time and pH. The results showed that the highest FPase was observed at the pH above 5.5 and incubation time of 96 h. The optimum incubation temperature range for cellulase production by *Trichoderma* sp. under SSF has been reported to be 25–28 °C (Chandra *et al*., 2010). However, Hanif *et al*. (2004) reported maximum cellulase production from *Aspergillus niger* at 30°C. The pH of the medium is one of the most critical factors affecting fungal growth, enzyme production and transport of various components across the cell membrane (Juhasz *et al*., 2004).

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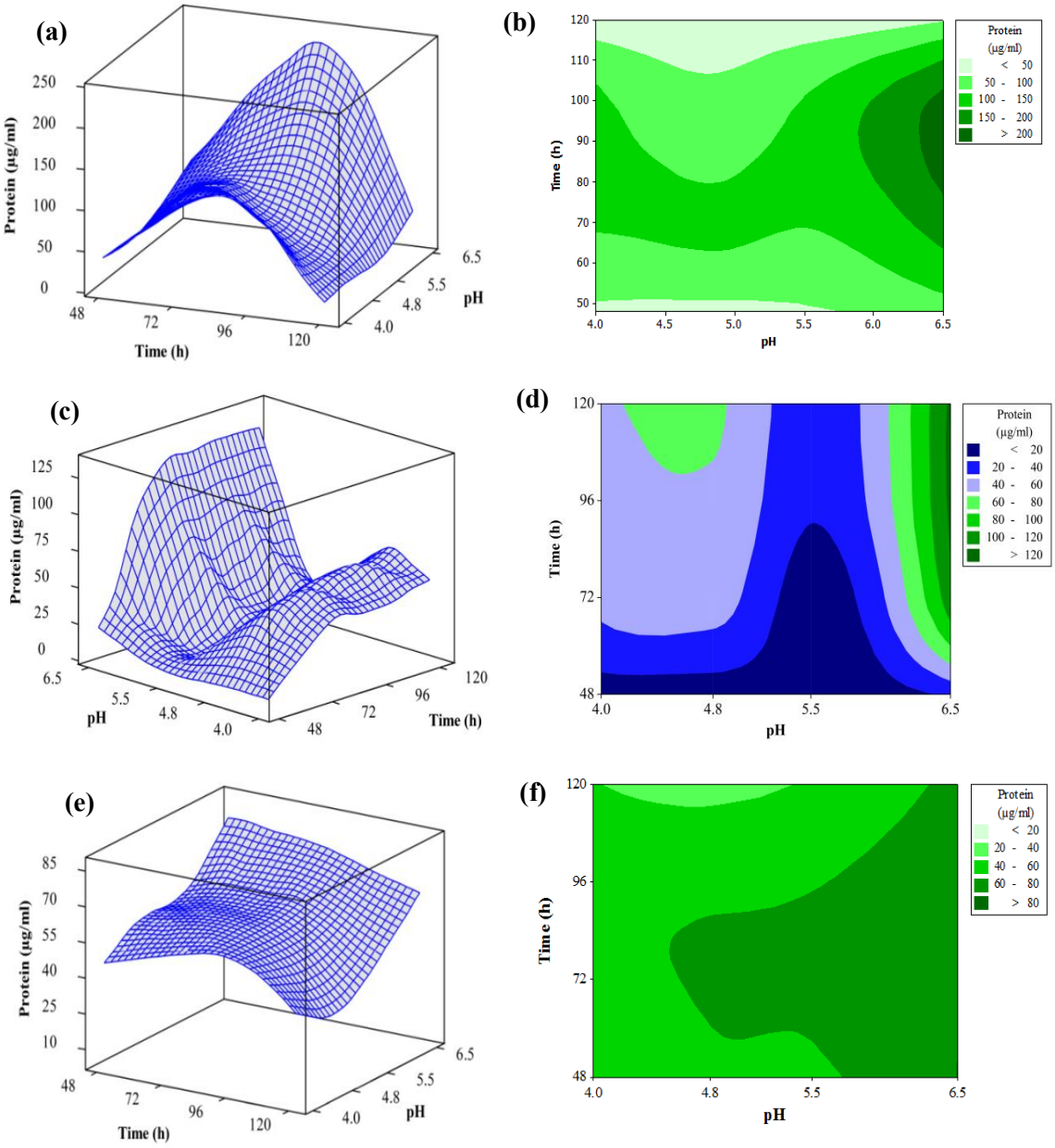

Figure 1 Surface plots and contour plots of extracellular protein (µg/ml) production by *Trichoderma harzianum* as functions of Incubation time (h) and pH at constant Incubation temperature of (a, b) 25 °C, (c, d) 28 °C and (e, f) 34 °C and 180 rpm.

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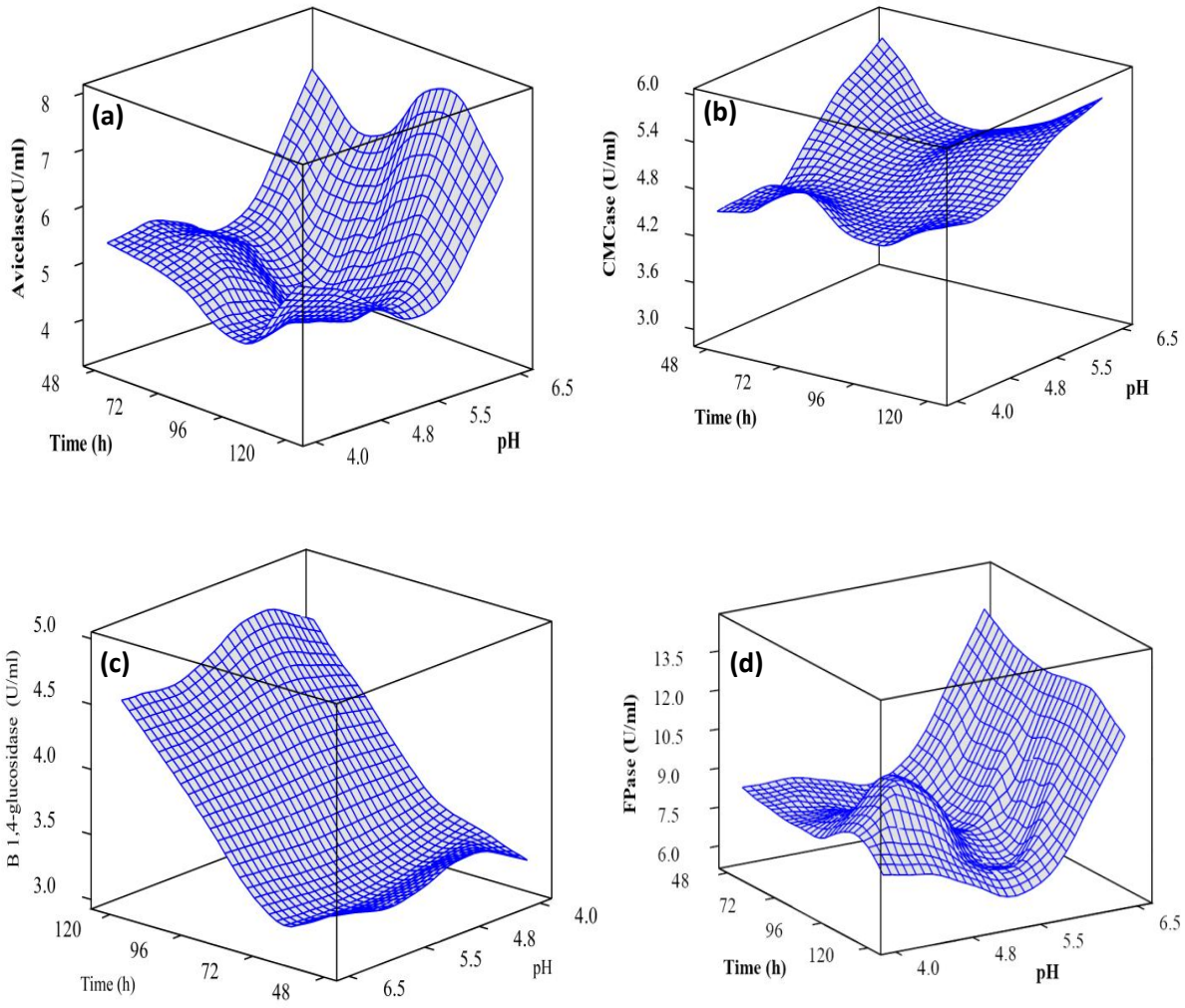

Figure 2 The optimization of cellulase enzyme production by *Trichoderma harzianum* as functions of Incubation time (h) and pH at constant Incubation temperature of 25 °C: (a) Avicelase (U/ml) or Cellobiohydrolase activity (CBH), (b) Endoglucanase (EG) or CMCase activity (U/ml) and (c) Cellubiase or β-1, 4- glucosidase activity (U/ml) and (d) Total cellulase or FPase activity.

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 *T. harzianum* as functions of Incubation time (h), pH and incubation temperature (˚C) are shown in Fig. 5. There are observable differences in the protein banding pattern ranging from 10 to 180 KDa. Several molecular bands were observed in the protein profiles, while for the TFM supernatant (not inoculated) there was no specific protein binding. Cel5A is an EG that belongs to GH family 5. The enzyme has an estimated molecular weight of 42 kilo Daltons (kDa), but has an apparent molecular weight of 48 kDa on a SDS-PAGE gel due to glycosylation. It has a pI of 5.5-5.6 (Shoemaker *et al*., 1983). This enzyme was observed in *T. harzianum* SDS-PAGE profiles in molecular

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weight of 42 KDa (Fig. 5 b, c and d). The highest Cel 5A production was observed only at the pH 6.5 and all incubation times of fermentation for incubation temperature of 28°C. This enzyme was Also, produced at the pH 6.5 and incubation time of fermentation 120 h for incubation temperature of 25 °C. 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* (Ilmen *et al*., 1997). Cel6A is a GH family 6 CBH. This enzyme has an estimated molecular weight of 47 kDa, 53 kDa on a SDS-PAGE, and it has a pI of 5.9 (Bhikhabhai *et al*., 1984; Fägerstam *et al*., 1977).

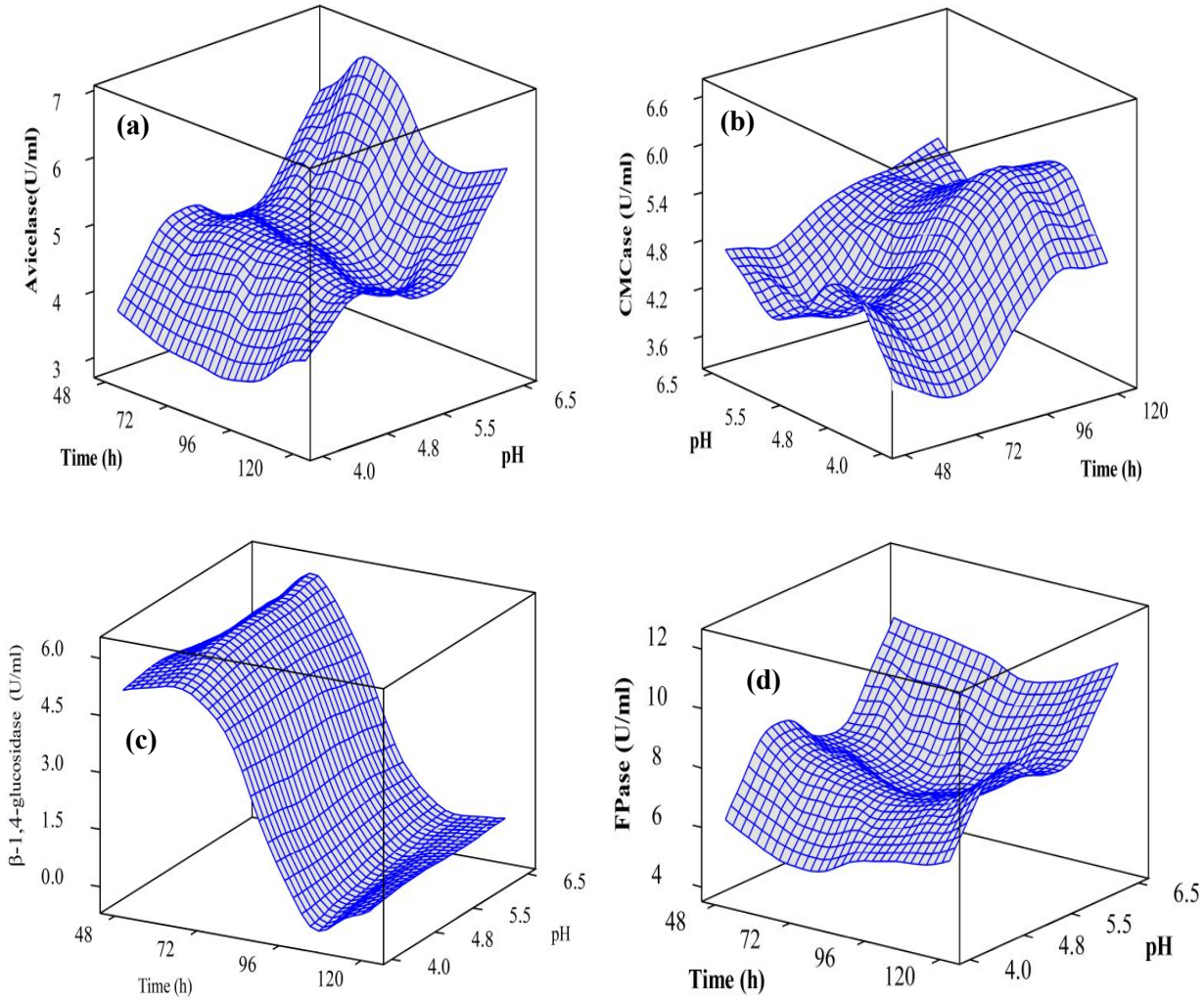

Figure 3 The optimization of cellulase enzyme production by *Trichoderma harzianum* as functions of Incubation time (h) and pH at constant Incubation temperature of 28 °C: (a) Avicelase (U/ml) or Cellobiohydrolase activity (CBH), (b) Endoglucanase (EG) or CMCase activity(U/ml) and (c) Cellubiase or β-1,4-glucosidase activity (U/ml) and (d) Total cellulase or FPase activity.

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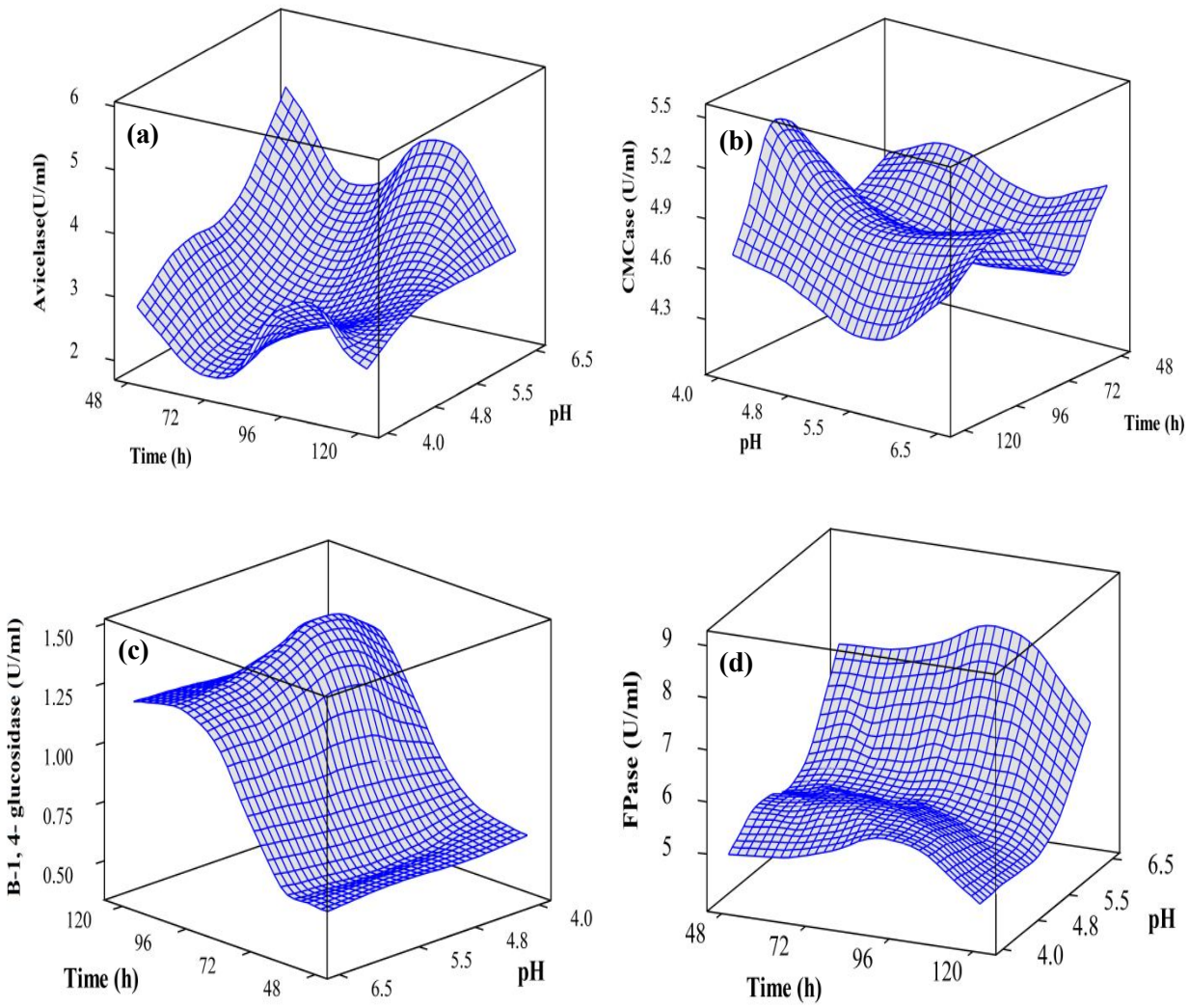

Figure 4 The optimization of cellulase enzyme production by *Trichoderma harzianum* as functions of Incubation time (h) and pH at constant Incubation temperature of 34 °C: (a) Avicelase (U/ml) or Cellobiohydrolase activity (CBH), (b) Endoglucanase (EG) or CMCase activity(U/ml) and (c) Cellubiase or β-1,4-glucosidase activity (U/ml) and (d)Total cellulase or FPase activity.

This enzyme was observed at incubation temperature of 25 °C, for incubation time of 48 and 72 h (Fig. 5 a) in molecular weight of 47 KDa and for pH 4 and 5.5 and in molecular weight of 51 KDa for pH 4.8 and 6.5. At this incubation temperature, for 72 hours, the expression of this enzyme significantly increased at pH 5.5. This enzyme was expressed in 96 h in pH 4.8 and 6.5 and was observed in much lower amount at pH 5.5. Also expression of this enzyme at the incubation time of 120 h was observed only at pH 6.5 (Fig. 5b). The expression of this enzyme at incubation temperature of 28 °C and pH 6.5 at all incubation times of fermentation was increased significantly (Fig. 5 c, d). The production of this enzyme at pH 4 and 4.8 and incubation temperature 28 °C was much less. The highest expression of this enzyme was observed at incubation temperature of 34 °C (Fig. 5 e, f).

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Fermentation at different incubation times, with an increase in the pH of the fermentation medium increased the expression of this enzyme. Optimum expression of this enzyme was observed at incubation temperature of 34 °C, pH 6.5 and incubation time of fermentation 120 h. 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 (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* (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). This enzyme was observed in molecular weight of 60 KDa for *T. harzianum* SDS-PAGE profiles that was considered as a Cel7A (CBH I) (Fig. 5). The expression of this enzyme at pH 4 and 5.5 significantly increased with increasing incubation time of fermentation. Moreover, the expression of this enzyme with fermentation at pH 4.8 and 6.5 was considerably decreased during incubation time of fermentation. Optimum expression of this enzyme was observed at incubation temperature of 28 °C, pH 6.5 and incubation time of fermentation 120 h (Fig. 5d). An increase in incubation temperature of fermentation from 28 °C to 34 °C leads to a significant reduction in the expression of this enzyme. Cel7A is the major cellulase produced by *T. harzianum*, and it has been estimated that 50-60% of total expressed cellulase in the fungus is Cel7A (Ilmen *et al*., 1997). It is probably the key enzyme used by the fungus for hydrolysis of crystalline cellulose. 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 (Divne *et al*., 1998; Barr *et al*., 1996). Cel12A is a GH family 12 EG. The enzyme has a molecular weight of 25 kDa, has a neutral pI of 7.5 (Ülker and Sprey, 1990; Hayn *et al*., 1993; Sprey and Bochem ., 1992). 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 having cellulose activity, has 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 in SDS-PAGE profile protein of *T. harzianum* with molecular weight of 25 KDa (Fig. 5 c, d).The highest Cel12A production was observed only at the pH 6.5 and all incubation times of fermentation for incubation temperature of 28°C. Also, this enzyme was produced very poorly at the pH 6.5 and incubation time of fermentation 120 h for incubation temperature of 25 °C. The weak bands were observed in molecular weight of 18 KDa that probably were related to xylanase II enzyme. 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 activity against β-glucan and xylan (Hayn *et al*., 1993; Karlsson *et al*., 2002). CEL 1A (BGL II) was observed in molecular weight of 100 KDa for SDS-PAGE profiles. This enzyme was expressed in all incubation times of fermentation in pH 4 and 5.5 at incubation temperature of fermentation 25 °C. The expression of this enzyme at pH 4 and 5.5 significantly increased with increasing incubation temperature of fermentation to 28 °C.

[DOR: 20.1001.1.22519041.2016.5.3.2.5]

Moreover, the expression of this enzyme with fermentation at pH 5.5 and incubation time of fermentation 96 hours was considerably decreased. Also, an increase in incubation temperature of fermentation from 28 °C to 34 °C leads to a significant reduction in the expression of this enzyme. β- Glucosidase hydrolyzes the soluble oligosaccharides, produced by cellulases, to glucose. The addition of β-glucosidases into the *T. reesei* cellulases 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.

2D SDS–PAGE and protein identification

Two-dimensional gel electrophoresis (2-DE) is considered as a powerful tool to separate and visualize hundreds of proteins at a time, which in combination with mass spectrometry (MS) offers a way to identify them. In this investigation, the total extracellular protein from the optimum culture supernatant (pH 6.5, incubation time of 72 h and incubation temperature of 28 °C) was separated by 2-DE. The protein map is shown in Fig. 6. The distribution of the protein spots indicates that most of the secreted proteins have an isoelectric point below 6 and a molecular mass above 20 kDa. We also quantified the number of protein spots using the Melanie 7 software, and more than 70 protein spots were detected on the 2-D gels after staining using Coomassie Brilliant Blue R-250. The majority of the accumulation of protein spots in the molecular weight range 20 to 75 KDa was observed. Spots with numbers 2 to 4, 7 to 12, 14 to 19, 21, 22, 25 to 28, 32, 33, 35 to 37 that had a spot intensity compared to other spots were marked. The highest concentration of protein was observed in spots of 11 and 12 with molecular weights of 63 and 60 KDa and Ip 4 and 4.8, respectively. Cel 7A (CBH I) and Cel 7B (EG) are enzymes that are placed in this area, rspectively. Cel7B is a GH family 7 EG. Cel7B 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). The cellobiohydrolase Cel7A and endoglucanase Cel7B were the most abundant of the cellulolytic enzymes secreted by *T. harzianum* (Fig. 6). However, as compared to the cellobiohydrolases, the endoglucanases were produced in low amounts. Also another sharp spot (No. 2) was located in molecular weight 60 KDa with Ip 5.2. Cel 6A is an enzyme that is consistent with this spot. In addition, some other components of the cellulolytic system of *T. harzianum*, such as endoglucanase III (Cel 12A), was identified on the 2-D gels. Spot number 36 having a molecular weight of 25 KDa and Ip 7.5 is consistent with the enzyme Cel 12A. Also, in this molecular weight range, xylanase enzymes (XYNI) with an isoelectric point 4.4 are visible. Among components of cellulases, β-1,4-cellobiohydrolases (CBH I and CBH II) dominated over secreted proteins which could be seen in 2-DE gel. Results from the 2-DE protocol were reproducible and the expression levels of proteins shown in the gel correlated well with specific activities of enzymes. The proteomic tool was proved to be useful for studying the composition of cellulases as a complicated enzyme system.

Conclusions

The results of present study showed that *T. harzianum* could secret a significant amount of cellulases to the medium under different conditions of fermentation on colloidal cellulose. The enzymatic degradation of waste cellulose by fungal enzymes has been suggested as a feasible alternative for the conversion of lignocellulosic materials into fermentable sugars and fuel ethanol. However, the most widely studied enzymatic system, namely *T. cellulases*, has shown several disadvantages. Attempts to use these enzymes in the degradation of cellulosic wastes have not been successful for several reasons such as: low enzymatic yields, low specific activities, end product inhibition of the enzymes. In conclusion, since any biotechnological process is likely to be based on crude extracts, it is important to increase any particular enzymes activity in culture supernatants through changes in the different physicochemical parameters of fermentation. Major parameters affecting the fermentation process for enzyme production were studied and

[DOR: 20.1001.1.22519041.2016.5.3.2.5]

optimal levels were identified. Through this optimization study, we have significantly increased the production of cellulose- degrading enzymes. This enhances the potential biotechnological application of the nonconcentrated enzyme system from *T. harzianum*. Based on this work, the optimum conditions for production of extracellular cellulolytic enzymes from *T. harzianum* can be summarized as:

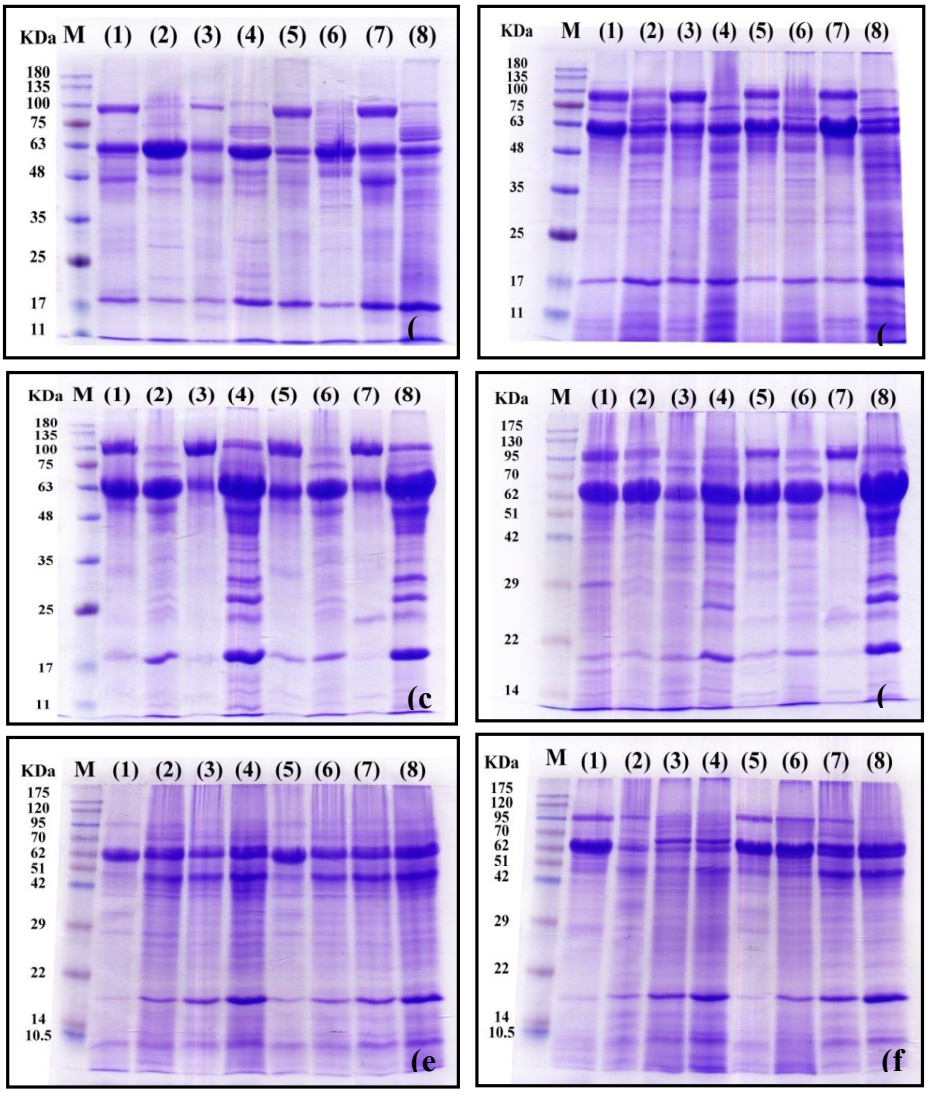

Figure 5 Profiles of proteins secreted into the culture supernatants of *Trichoderma harzianum* as functions of Incubation time (h), pH and Incubation temperature (°C): (a): Number of 1, 2, 3 and 4 for incubation time of 48 h and pH 4, 4.8, 5.5 and 6.5, respectively and number of 5, 6, 7 and 8 for incubation time of 72 h and pH 4, 4.8, 5.5 and 6.5, respectively at 25 °C; (b) incubation time of 96 h and 120 h at 25 °C, respectively; (c) incubation time of 48 h and 72 h at 28 °C, respectively; (d) incubation time of 96 h and 120 h at 28 °C, respectively; (e) incubation time of 48 h and 72 h at 34 °C, respectively; (f) incubation time of 96 h and 120 h at 34 °C, respectively; as a function of . "M" indicates a molecular weight marker(Vivantis^{TM}, prestained protein ladder, PR0602).

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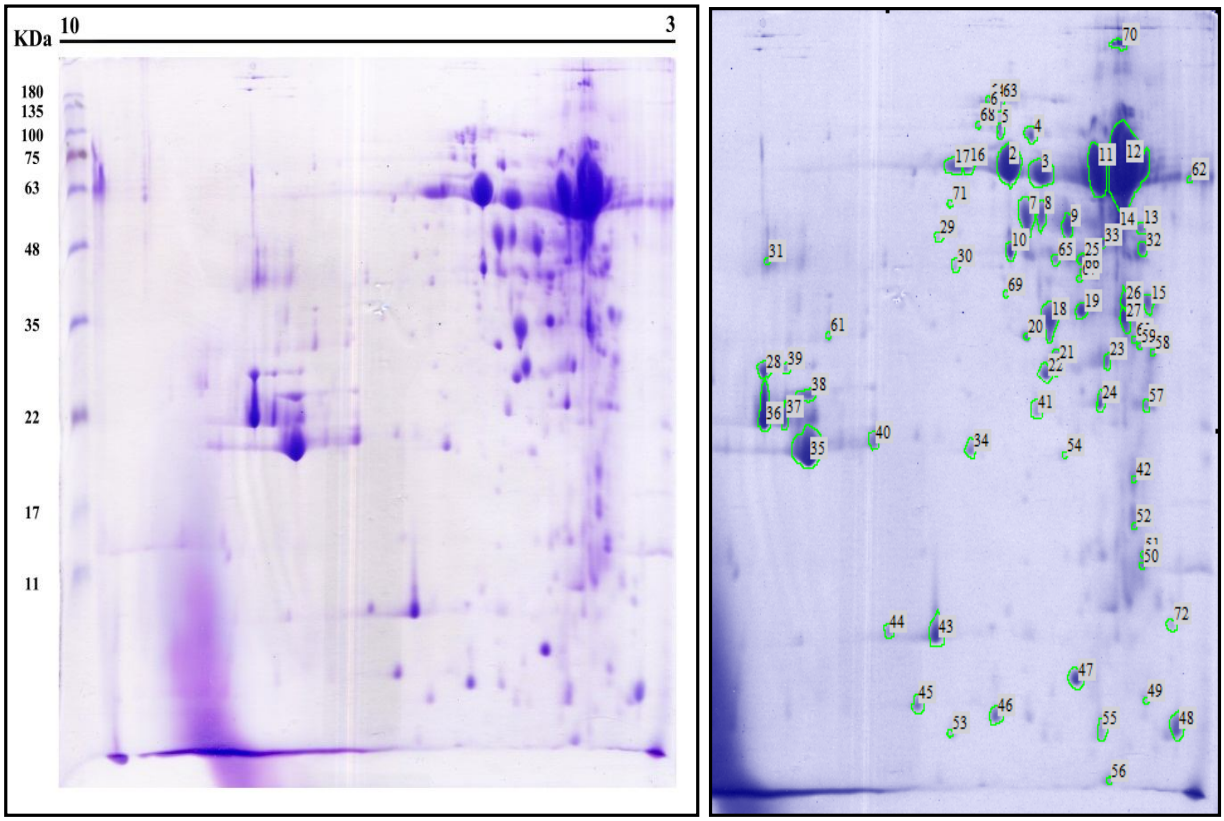

Figure 6 2D gel produced from the electrophoresis of protein (approximately 1200 µg) from the TFM supernatant of *Trichoderma harzianum* following growth in a cellulase-inducing liquid medium in optimum condition of cellulase production. Proteins were visualized on a 12.5% SDS-PAGE gel stained with Coomassie Brilliant blue R250.

1. The production of extracellular cellulolytic enzymes can bemaximized by increasing the incubation time of fermentation upto 72 h.
2. The optimum pH and incubation temperature for the production of extracellular cellulolytic enzymes by *T. harzianum* are at pH 6.5 and constant incubation temperature of 28 °C.
3. Finally, the results obtained provide new information on the effects of different complex cellulases on cellulose in the different physicochemical parameters of fermentation.

Acknowledgments

This study was supported by grants from the Radiation Application Research School (Project No. A88A099), Nuclear Science and Technology Research Institute (NSTRI), Atomic Energy organization of IRAN (AEOI).

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[DOR: 20.1001.1.22519041.2016.5.3.2.5]

تأثیر پارامترهای مختلف فیزیکوشیمیایی تخمیر در تولید آنزیم‌های سلولیتیک خارج‌سلولی تولید شده توسط قارچ *Trichoderma harzianum*

سمیرا شهبازی[1*]، حامد عسکری[1] و شیده موجرلو[2]

۱- پژوهشکده کشاورزی هسته‌ای، پژوهشگاه علوم و فنون هسته‌ای (NSTRI)، سازمان انرژی اتمی (AEOI)، کرج، ایران.
۲- گروه باغبانی وگیاه‌پزشکی، دانشکده کشاورزی، دانشگاه صنعتی شاهرود، شاهرود، ایران.
* پست الکترونیکی نویسنده مسئول مکاتبه: sshahbazi@nrcam.org
دریافت: ۲۴ فروردین ۱۳۹۵؛ پذیرش: ۸ خرداد ۱۳۹۵

چکیده: این مطالعه برای یافتن شرایط بهینه پارامترهای فیزیکوشیمیایی تخمیر(به‌عنوان مثال pH، دما و مدت زمان انکوباسیون) برای تولید آنزیم سلولاز در قارچ *Trichoderma harzianum* انجام شده است. غلظت پروتئین‌های خارج‌سلولی با استفاده از روش اتصال رنگ بردفورد اندازه‌گیری شد. میزان فعالیت آنزیم‌های اندوگلوکاناز (EG)، اگزوگلوکاناز یا سلوبیوهیدرولاز (CBH)، بتا گلوکوزیداز و سلولاز کل اندازه‌گیری گردید. وزن مولکولی آنزیم‌های سلولاز تولیدی با استفاده از آزمون SDS-PAGE مورد مطالعه قرار گرفت. برای شناسایی اجزای کاتالیزوری غالب در شرایط بهینه تولید آنزیم، سلولازها توسط تکنیک الکتروفورز دوبُعدی تفکیک شدند. شرایط بهینه تولید آنزیم سلولاز به شرح زیر مشخص شد: pH محیط برابر با ۶/۵، دمای انکوباسیون ۲۸ °C و مدت زمان تخمیر ۷۲ ساعت. پروفایل پروتئینی حاصل از آزمون SDS-PAGE چندین باند مربوط به آنزیم‌هایی مانند CBHs، EGs و BGLs را نشان داد. براساس این نتایج، قارچ *T. harzianum* دارای هر دو باند مربوط به آنزیم‌های (سلوبیوهیدرولاز) Cel7A و (اندوگلوکاناز) Cel7 می‌باشد. در نهایت، نتایج حاصل از تجزیه و تحلیل الکتروفورز دوبُعدی پروتئین‌ها نشان داد که پروفایل پروتئینی در شرایط بهینه تولید آنزیم دارای باندهای پروتئینی متعددی متعلق به آنزیم‌هایی مانند سلوبیوهیدرولاز و اندوگلوکاناز است و در اثر هم‌افزایی سلوبیوهیدرولاز و اندوگلوکاناز میزان بالایی از فعالیت سلولازی در این قارچ قابل مشاهده است.

واژگان کلیدی: *Trichoderma harzianum*، آنزیم سلولاز، SDS-PAGE و 2D-PAGE

[DOR: 20.1001.1.22519041.2016.5.3.2.5]