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Investigating Cellulase Producing Potential of Two Iranian *Thermoascus aurantiacus* Isolates in Submerged Fermentation

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Abstract

Cellulose is the most plentiful renewable biopolymer in nature which could be utilized by cellulolytic enzymes. Cellulases are among the most important groups of industrial enzymes which are widely consumed in biofuel production, pulp and paper, textile, and detergent industries. These enzymes can support a cleaner environment through reducing chemical processes in mentioned industries and agro-industrial waste management. Thermophilic filamentous fungi produce thermostable types of the enzymes with the property of hydrolysis the cellulose in higher temperatures with higher rates of reaction, decreased amounts of enzyme quantities and reduced risk of contamination by the mesophilic microorganisms. The cellulolytic capacity of two *Thermoascus aurantiacus* isolates (from Mashhad, Iran) was examined in a simple liquid state fermentation in different carbon and nitrogen sources, in comparison to the *Thermoascus aurantiacus* DSM 1831 as a reference fungus. Among different the tested sources, wheat bran and peptone led to the highest level of endoglucanase production by the isolated thermophilic fungi. The isolates showed higher cellulase activities, including endoglucanase, avicelase, and FPase, of the crude enzymes from the isolates in comparison to the reference fungus. Gene expression profiling revealed that changes in the cellulase mRNA levels are not correlated with the changes in protein activities during a 12-day period. This observation might be due to a complex process of enzymatic regulation of cellulases in response to the environmental signals.

Key words: Cellulolytic potential; Glucanase; β-glucosidase; *Thermoascus aurantiacus*; Thermophilic fungus

Introduction

Lignocellulosic biomass is the most plentiful renewable complex on earth that is made of cellulose (~50%), hemicellulose (~ 30%) and lignin (~ 20%) (Coral *et al.*, 2002). Cellulases, among the most important industrial enzymes, include three main groups of enzymes: endo-β-1,4- D glucanase (CMCase, EC 3.2.1.4), exo-β-1,4- D-glucanase (avicelase, EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). Synergistic activity of these groups of enzymes is required for complete biodegradation of cellulose to simple sugar glucose. Examples of industrial applications of cellulases are biofuel production, pulp and paper, textile and detergent industries (Sukumaran *et al.*, 2005; Singh *et al.*, 2007; Kuhad *et al.*, 2011). These enzymes can help to maintain cleaner environment. They have been replaced several chemical processes in mentioned industries and they could directly utilized in agro-industrial waste management (Wiseman, 1993; Uhlig, 1998; Colagar *et al*., 2004; Kuhad *et al.*, 2010). While burning these wastes leads to increasing air pollution, free carbon dioxide and global warming, biodegradation and bioconversion of these wastes suggest a novel market for bioenergy and enzyme production (Sánchez, 2009; Irshad *et al.*, 2012; Soliman *et al.*, 2013; Anwar *et al.*, 2014).

Fungi are famous organisms capable of degrading organic substances and particularly cellulosic materials (Lynd *et al.*, 2002). The interesting and promising purpose of low-cost production of thermophilic cellulases has been created due to efficient growth of some thermophilic fungi such as *Thermoascus aurantiacus* on lignocellulosic materials (Kalogeris *et al.*, 1998; Moretti *et al.*, 2012). Several studies showed that thermophilic fungus *T. aurantiacus* from ascomycetous class, is capable of producing different types of cellulolytic enzymes with high thermostability properties (Brienzo *et al.*, 2008). Comparison of cellulose degrading activity of a commercial mixture of cellulases Cellic CTec2 (Novozymes) and cellulases of *T.* a*urantiacus* ATCC 26904 supported the high potential of this fungus in efficient cellulose decomposition (McClendon *et al.*, 2012).

Thermophilic cellulases have attracted more attention for efficient degradation of lignocellulosic biomass. Using these thermostable enzymes has several advantages *i.e* occurring the hydrolysis process in higher temperatures would raise the rate of reaction, decrease the required enzyme quantities and reduce the risk of contamination by mesophilic microorganisms. Furthermore, this is more compatible with the high temperature of pretreatment processes and facilitates maintaining and transportation of cellulolytic enzymes (Sonnleitner and Fiechter, 1983; Viikari *et al.*, 2007).

Considering the importance of thermophilic cellulases, we isolated two strains of *T. aurantiacus* fungi from manure samples, found suitable conditions for their submerged cultures and studied their cellulase enzymatic activities and biotechnological potentials in this study.

Materials and Methods

Isolation of fungal strains

The manure samples were collected from Suran region (22 km west of Mashhad, 36º 26´38.44″, 59º 22´38.76″). The samples were taken from depth with about 50 ºC temperature, placed in polyethylene pockets and transferred to the laboratory quickly. One gram of manure was homogenized in 9.0 ml sterile dH_2O by shaking for 30 min at 100 rpm and 50 ºC. Then, different dilutions were prepared from the stock and transferred onto the selective medium plates containing (g/L): cellulose (or Carboxymethyl Cellulose, CMC), 1.0; KH_2PO_4, 1; $(NH_4)_2SO_4$, 0.5; $MgSO_4.7H_2O$, 0.5; urea, 0.5; $CaCl_2$, 0.5 and mineral solution (mg/L): $FeSO_4.7H_2O$, 7.5; $MnSO_4.H_2O$, 2.5; $ZnSO_4.7H_2O$, 3.6; $CoCl_2$, 3.7 and ampicillin, 100 (Hart *et al.*, 2002). Plates were incubated at 45 ºC for 24 h. All colonies were isolated by repeated sub-culturing on Potato Dextrose Agar (PDA) medium (QueLab). A circular shaped sample (1 mm in diameter) from each pure culture was transferred onto a new CMC plate for congo red staining (Sazci *et al.*, 1986). After 48 h, incubated CMC plates at 45 ºC were covered by 0.1% congo red solution (Merck) for 10 min and washed with NaCl solution (1 M) (Merck), so clear cellulolytic zones could be appeared. The best CMCase-producing fungal strains (7 cases) were selected for purification by single-spore processing, long-term maintaining and further studies, including species identification, quantitative cellulase studying at mRNA and protein levels.

Molecular species characterization

Fungal isolates were cultured in liquid Potato Dextrose Broth (PDB) (QueLab) at 45 ºC and 150 rpm for 48 h. Harvested mycelia were rinsed twice with phosphate buffered saline at 1000 rpm and then processed for genomic DNA extraction (Volossiouk *et al.*, 1995). Polymerase chain reactions (PCRs) were performed by using of ITS5 and ITS4 primers to amplify internal transcribed spacer regions of each isolate (Schoch *et al.*, 2012). PCR products were sequenced (Macrogen Company, South Korea), and the data were analyzed by BOLD Systems version 3 program. The MEGA-7 software was applied for dendrogram construction.

Quantitative enzyme activities

Microorganisms

Cellulase activities of the isolated *Thermoascus aurantiacous* strains (2 isolates) from Suran region were compared with *T. aurantiacus* Miehe (DSM 1831) as a reference fungus.

Inoculum preparation

Fungal strains were cultured on fungal complete medium (Michielse *et al.*, 2008), incubated at 45 ºC for 4-5 days, and after sporulation, the spore suspensions were made with concentration of 10^8 spore/ml. Then, inocula were prepared by adding 200 µl (2×10^7 spores) of a spore suspension to 10 ml PDB liquid medium in a 50 ml flask and incubating at 45 ºC and 150 rpm. After 48 h, these cultures were added to 100 ml of selective liquid media in 250 ml flasks.

Enzyme solutions

Fermented cultures (2-3 ml) were harvested from flasks at 2-day intervals and passed through filter papers. The media containing secreted enzymes were utilized for cellulase assays.

Influence of different nutrients on the CMCase activity

Effect of different carbon and nitrogen sources on CMCase production was tested using cellulose (Merck) and wheat bran as carbon sources and urea (QueLab), pepton water (Scharlau) and yeast extract (Scharlau) as nitrogen sources. The selective medium was utilized as main medium.

Optimum pH and temperature

The effects of pH and temperature on cellulase activities were examined by measuring the enzyme activities in the range of pHs 3-9 and temperatures between 55 ºC and 80 ºC.

Enzyme activity assays

Endoglucanase and exoglucanase assays

Endoglucanase (CMCase) and exoglucanase (avicelase) activities were measured using 2% (w/v) CMC (Sigma Aldrich) and avicel PH-101 (Merck) as substrates, respectively. Each reaction was mixture of 20 µl substrate suspension (in 50 mM sodium acetate buffer, pH 4.0 and 100 mM sodium acetate buffer, pH 6.0, respectively) and 20 µl crude enzyme medium. The reactions were incubated at 70 ºC for 30 min. 3,5-Dinitrosalicylic acid (DNS) solution, made according to Miller description (Miller, 1959), was used for measuring the produced glucose product. Adding 120 µl DNS reagent to each reaction and 5 min heating at 95 ºC was followed by preparing suitable dilutions (mixture of 30 µl of CMCase reaction and 150 µl dH_2O., 100 µl of avicelase reaction and 100 µl dH_2O) of reactions and reading optical density absorbencies at 540 nm (Miller, 1959; Zhang *et al.*, 2009; Kim *et al.*, 2012).

β-glucosidase assay

β-glucosidase activity was determined using p-Nitrophenyl β-D-glucopyranoside (pNPG) (Sigma Aldrich). For preparing each reaction, 10 µl of crude enzyme was mixed with 90 µl of pNPG solution (1 mg/ml) in sodium acetate buffer (100 mM) and incubated at 65 ºC for 30 min. Then, 100 µl reaction volume was added to 100 µl Na_2CO_3 solution (Merck) (2% w/v) in a 96 well microplate. The produced p-nitrophenol during reaction time was measured by spectrophotometer at 410 nm (Deshpande *et al.*, 1984; Zhang *et al.*, 2009).

Filter paper assay

Filter paper (FPase) assay was performed in microliter volumes. Each reaction consisting of 40 µl of 50 mM sodium acetate buffer (pH 5), a disk of filter paper Whatman no.1 (7 mm in diameter) and 20 µl of crude enzyme solution was incubated at 65 ºC for 60 min. Then, 120 µl DNS reagent was added to each reaction and heated at 95 ºC for 5 min. After cooling the reactions in ice-water bath, 36 µl of each reaction was mixed with 160 µl dH2O in a 96 well microplate and optical density absorbencies were determined at 540 nm (Xiao *et al*., 2004a). Enzyme activities were assayed (as U/ml culture medium) according to the relevant standard curves, one unit is represented amount of enzyme that releases one µmol of product per min.

Quantitative RT- PCR assay

The new isolates and the reference strain were cultured as described for enzyme activity assays. Mycelia were harvested on 2^{nd}, 4^{th}, 8^{th} and 12^{th} days, rinsed with PBS (Phosphate Buffered Saline) and grinded in liquid nitrogen for 5 min. Total RNA extractions were carried out using Riz Molecule Dana kit. Thermo Scientific kits were applied for DNase

treatment and cDNA synthesis. Relative expression levels of *eg*, *cbh* , *bgl1* and *bgl2* (GenBank accession nos. JX856135.1, AF421954.1, DQ114396.1 and EU263992.1, respectively) of the fungal strains were examined by quantitative real-time PCR technique. *β-tubulin* gene was considered as reference gene for data normalization of the experiments. Primers were designed using Allele ID softwere version 6 (Table 1), whereas described primers for *Talaromyces versatilis β-tubulin* gene were used for amplification of *β-tubulin* gene of *T. aurantiacus* strains (Llanos *et al.*, 2015).

Statistical analysis

Statistical program Prism version 6 was utilized for data analysis. Data were presented as mean±SEM (Standard Error of Mean). Statistically significant differences between control and other groups were considered as $p<0.05$, using two-way ANOVA and Dunnet test.

Table 1. List of primers utilized in polymerase chain reactions.

Primer name	Forward and Reverse Primers	Length of Amplicon	PCR condition	Reference
ITS5 ITS4	5′-TCCTCCGCTTATTGATATGC-3′ 5′-GGAAGTAAAAGTCGTAACAAGG-3′	600-700 bp	94ºC (5 min); 94ºC (45 s), 56ºC (30 s), 72 ºC (90 s) (40 cycles); 72ºC (10 min)	Schoch *et al*., 2012
eg	5′-AGCCTGACCGACCCATCTG-3′ 5′-CCTGAGCCACTGCGTTGC-3′	138 bp	94ºC (5 min); 94ºC (15 s), 65ºC (15 s), 72 ºC (15 s) (50 cycles)	This work
cbh	5′-GGGAAGCCAACAGCATCTCTAC-3′ 5′-TAAGGATTGAAGTCGCAGCCATC-3′	151 bp	94ºC (5 min); 94ºC (15 s), 65ºC (15 s), 72ºC (15 s) (50 cycles)	This work
bgl-1	5′-ACCTGACCATCGCCGTTCTC-3′ 5′-CGTGCTCGTAACCGTATTCATCC-3′	159 bp	94ºC (5 min); 94ºC (15 s), 65ºC (15 s), 72 ºC (15 s) (50 cycles)	This work
bgl-2	5′-ATCCGCAATACCGACCATAATACG-3′ 5′-CCCGAAGCCCTCCCAGTTG-3′	198 bp	94ºC (5 min); 94ºC (15 s), 65ºC (15 s), 72 ºC (15 s) (50 cycles)	This work
β-tub	5′-GTTCTGGACGTTGCGCATCTG-3′ 5′-TGATGGCCGCTTCTGACTTCC-3′	110 bp	94ºC (5 min); 94ºC (15 s), 65ºC (15 s), 72 ºC (15 s) (50 cycles)	Llanos *et al*., 2015

Results and Discussion

Isolation and identification of fungal strains

Thirty filamentous fungi were isolated from manure samples, followed by qualitative CMCase test on their pure cultures, which led to selection of 10 samples with clear zones between 7-8 cm on CMC plates after incubation at 45 ºC for 48 h. Identification of isolates was performed based on ITS sequences, from which two sequences were >99% identical to the *T. aurantiacus* fungus, whereas other sequences were identified as *Rhizomucor miehei* and *Rhizomucor pusillus* fungi. *T. aurantiacus* isolates were selected for more studies. Phylogeny tree was constructed using MEGA software version 7 (Fig. 1).

Effects of different nutrient substrates on endoglucanase activity

Endoglucanase (CMCase) is the main enzyme in complete cellulase mixture (Himmel *et al.*, 1999), therefor the isolates were screened based on CMCase activity and primary experiments were designed to improve the CMCase production by isolates. Isolate 2 was transferred to the liquid fermentations including wheat bran or cellulose as carbon sources; and urea, pepton or yeast extract as nitrogen sources, supplemented with $(NH_4)_2SO_4$, $MgSO_4$ and KH_2PO_4 in all cases.

Several studies suggested pH 3.8-5 as the optimum initial pH of culture medium for filamentous fungi (Prior *et al.*, 1992; Jain *et al.*, 2015), we also regulated pH of culture media at pH 5, although it seemed that pH could be varied significantly during the growth period (Mitchell and Lonsane, 1992).

Measuring the CMCase activity of the harvested crude enzymes prepared from 10-day cultures indicated that significant higher endoglucanase activity was observed in media containing wheat bran in comparison to cellulose containing media. The enzyme activity in media containing wheat bran supplemented with different nitrogen sources indicated

significant higher endoglucanase activity in media prepared with pepton in comparison to urea and yeast extract (Fig. 2). So, wheat bran and pepton were used as main constituents of the media for measuring cellulase activities in further experiments.

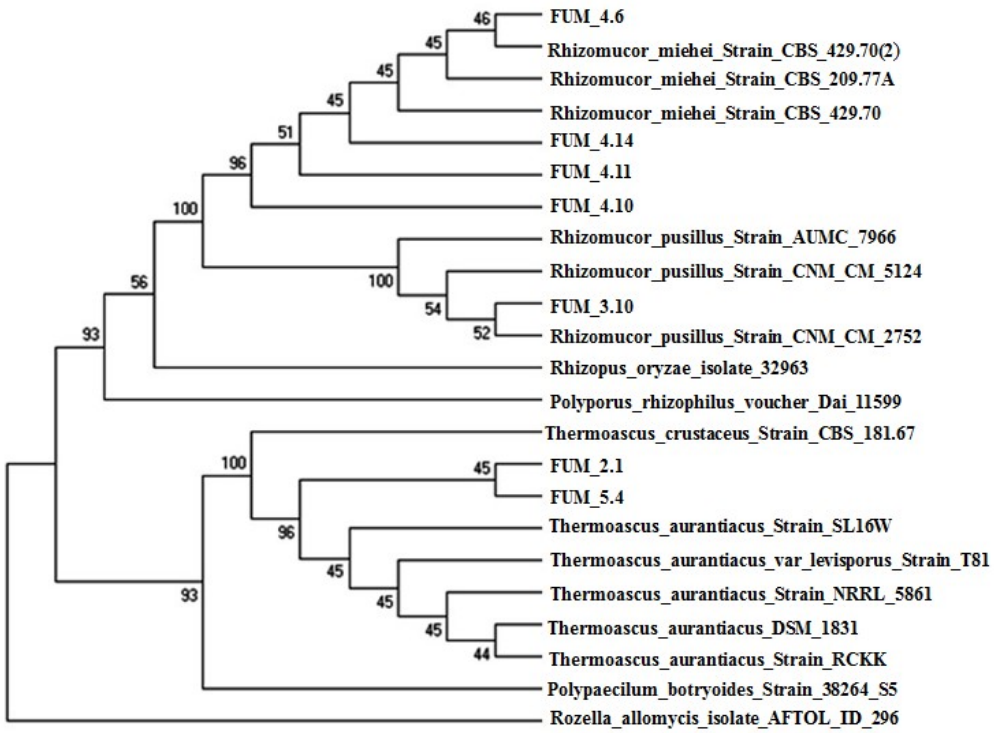

Fig. 1. Phylogeny of thermophilic fungal isolates: Phylogenetic tree of thermophilic cellulase-producing fungal isolates from suran region of Mashhad, Iran was constructed based on neighbor-joining method using of ITS sequences. Seven fungal isolates were characterized in this study. These isolates indicate as FUM_number. FUM_2.1 and FUM_5.4 are *Thermoascus aurantiacus* isolate 1 and 2, respectively. FUM_3.10 is *Rhizomucor pusillus* and FUM_4.6, FUM_4.10, FUM_4.11 and FUM_4.14 are *Rhizomucor miehei* strains. *Rozella allomycis* was utilized as the out group.

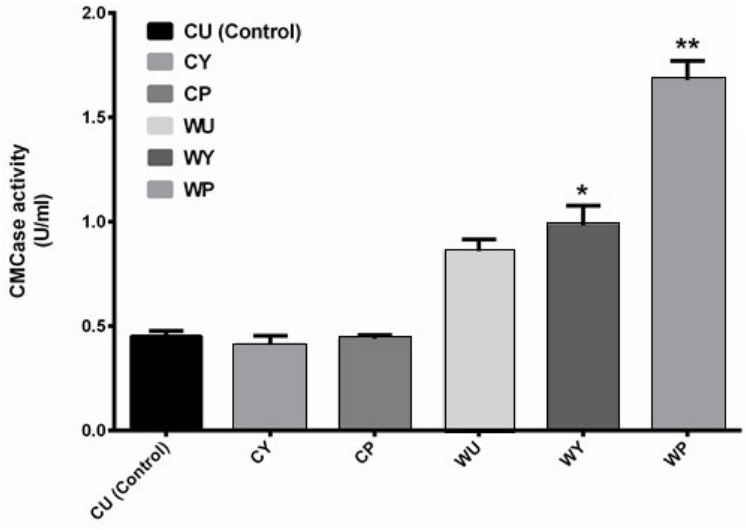

Fig. 2. Influences of nutrients on production of enzymes with CMCase activity. Effects of carbon and nitrogen sources on CMC hydrolysis by *Thermoascus aurantiacus* isolate 2 growing in submerged media at 45ºC and 150 rpm for 10 days. * and ** represent statistically significant differences between CU medium and other media at $p<0.05$ and $p<0.01$, respectively. C, cellulose., W, wheat bran., U, urea., Y, yeat extract., P, Pepton.

Kalogeris *et al*. studied the effects of various carbon and nitrogen sources on cellulase production by *T. aurantiacus* Miehei IMI 216529 and showed that culture in pepton led to better production of the cellulolytic enzymes as compared to the yeast extract and urea which is consistent with our results (Kalogeris *et al.*, 2003). Additionally, some reports revealed that organic nitrogen sources were suitable for cellulase production by *T. aurantiacus* (Grajek, 1986; Gomes *et al.*, 1994; Roche *et al.*, 1994).

Endoglucanase (CMCase) and exoglucanase (avicelase) activity test on *T. aurantiacus* showed that the best function happened at pHs 4 and 6 and at 70 ºC temperature, respectively (Figs. 3a and 3b).

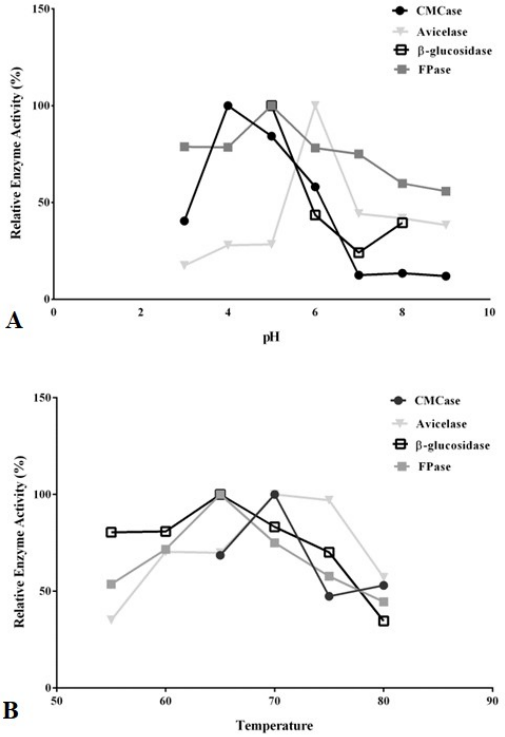

Fig. 3. Effects of different pHs and temperatures on cellulase activities. Effects of different pH 3 to 9 (a) and temperatures (55-80ºC) (b) on CMCase, Avicelase, β-glucosidase and FPase activities of *Thermoascus aurantiacus* isolate 2 growing in submerged media at 45ºC and 150 rpm for 7 days.

Previous studies on *T. aurantiacus* documented optimum pH 4.0-5.5 and optimum temperature 65-80 ºC for endoglucanase, while optimum pH in the range of 4.1-5 and temperature about 65 ºC were reported for exoglucanase (Brienzo *et al.*, 2008).

The maximum CMCase (7.15 U/ml, 715 U/g carbon source) and avicelase (0.22 U/ml) activities were obtained from isolate 2 on days 10 and 8, respectively. This was significantly better activity in comparison to the observed activities from the reference fungus strain

Enzyme activities

Endoglucanase and exoglucanase activities

(6.44 and 0.107 U/ml). Higher level of activities, for both enzymes, were observed in isolate 2 at all the time points examined as compared to the control one (Figs. 4a and 4b).

CMCaes activitiy of isolate 2 was higher than some other reported cases such as *T. aurantiacus* A-131 (5.8 U/ml), *T. aurantiacus* RCCK (88U/g) (Kawamori *et al.*, 1987; Jain *et al.*, 2015).

It seems that lower activity of avicelase in contrast to CMCase, as observed in our isolates, is a general trend among cellulase-producing fungi (Khandke *et al.*, 1989; Hong *et al.*, 2003).

β-glucosidase activity

The best β-glucosidase activity was observed at pH 5 and temperature 65 ºC for our isolate (Figs. 3a and 3b). Optimum pH and temperature in the range of 4.5-6 and 65-80 ºC have been reported for *T. aurantiacus* (Parry *et al.*, 2001; Hong *et al.*, 2006).

The reference fungus, *T. aurantiacus* (DSM 1831), produced the highest β-glucosidase enzyme activity (39.66 mU/ml on day 12). Significant differences between this strain and two isolates were observed throughout the fermentation period (Fig. 4c).

FPase activity

Optimum pH and temperature for FPase activity were observed at pH 5 and temperature 65ºC (Figs. 3a and 3b).

The highest FPase activity level was measured for isolate 1 on day 8 (0.375 U/ml) in contrast to the reference strain (0.139 U/ml). Comparing the FPase activity of the current isolates relative to the reference fungus revealed that these isolates presented better cellulase function during the period of fermentation (Fig. 4d).

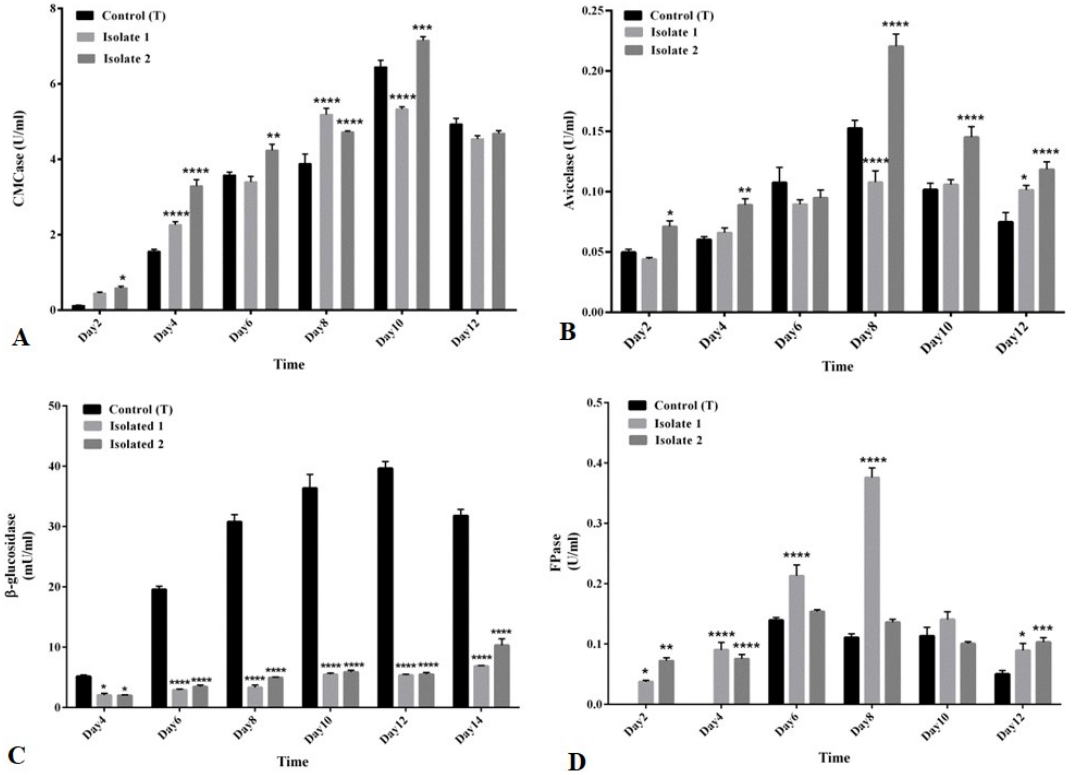

Fig. 4. Cellulase activities of *Thermoascus aurantiacus* strains. CMCase (A), Avicalase (B), β-glucosidase (C) and FPase (D) activities of *T. aurantiacus* isolates compared to control strain (*T. aurantiacus* DSM 1831) during a 12-day period. Fungi were growing in submerged media at 45ºC and 150 rpm. Enzyme activities were measured at optimum pHs and temperatures. Data were presented as mean±SEM. *, **, *** and **** represent statistically significant differences between control strain and two isolates at $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$, respectively.

β-glucosidase enzyme is required for converting the glucanase product, i.e. cellobiose, to glucose which itself induces other cellulase activities in turn, because cellobiose is an inhibitor for endo-and exoglucanase. On the other hand, glucose, can reduce cellulase production via feedback inhibition (Krogh *et al.*, 2010). Glucose also acts as an inhibitor of β -glucosidase and cellulases (Rao *et al.*, 1989; Xiao *et al.*, 2004b). Inhibitory effects of glucose on cellulase is stronger than its effects on β-glucosidase (Xiao *et al.*, 2004b). This reciprocal role of β-glucosidase on cellulase production and activity might be the reason for lower FPase (total cellulase) activity of reference *T. aurantiacus* in spite of much more production of β-glucosidase, in contrast to the isolate 2 in our study. However, there are some other compounds that could affect cellulases and β-glucosidase activities. Mannose, xylose and galactose, products of hemicellulase activities, significantly inhibit cellulase activities during cellulose degradation but have no effect on β-glucosidase activity (Xiao *et al.*, 2004b). It seems that complex collections of cellulase and hemicellulase products determine cellulase activities during the lignocellulosic material degradation.

Cellulase gene expression at mRNA level

Changing expression of four cellulase genes of the two isolates were examined at mRNA level using qRT-PCR on 2nd, 4th, 8th and 12th days (beginning, middle and final phases of the cellulase period study) while reference fungal strain was considered as control (Figs. 5a-d). During the period of study, enzyme activities of every fungal isolate increased gradually until reaching a top spot and then reduction of enzyme activities were observed. This pattern was not obvious at mRNA levels. Additionally, comparison of the cellulase mRNA levels between the isolates and the reference fungus were not consistent with the observed differences of corresponding cellulase enzyme activities.

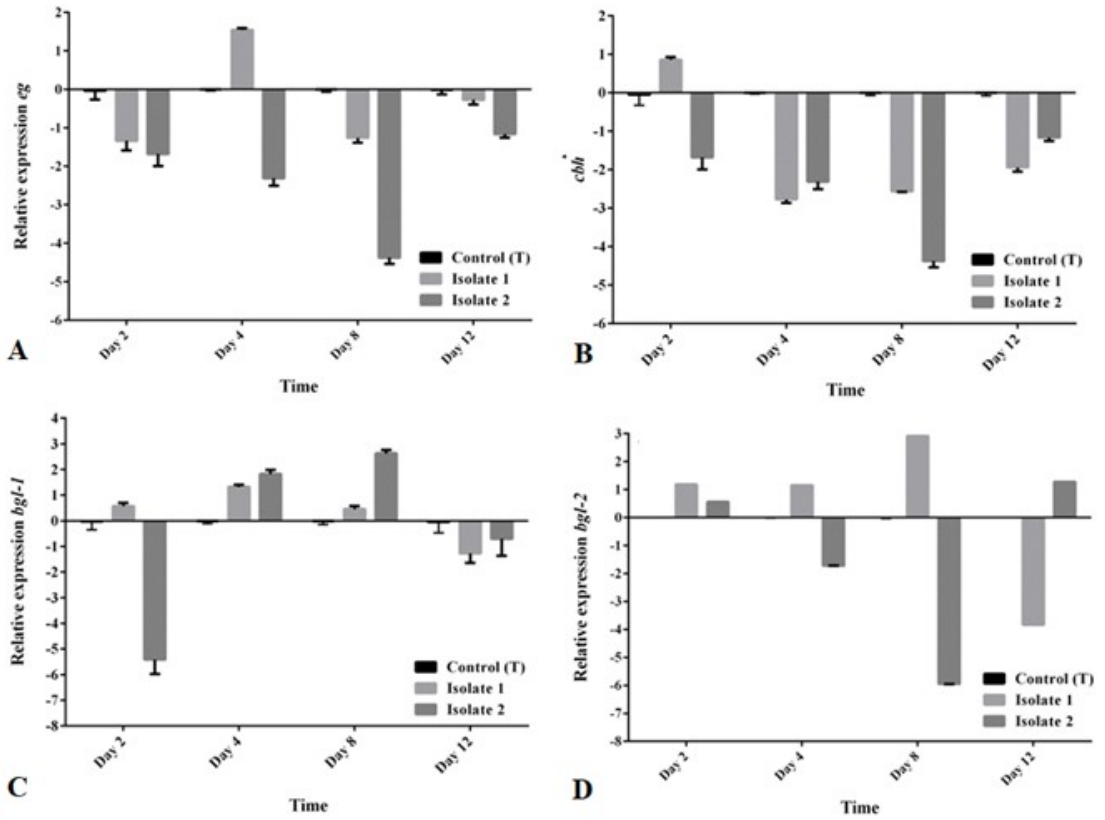

Fig. 5. Cellulase genes expression of *Thermoascus aurantiacus* strains at mRNA level: Relative expression levels of *eg* (A), *cbh* (B), *bgl-1* (C) and *bgl-2* (D) genes of *T. aurantiacus* isolates in comparison with control strain (*T. aurantiacus* DSM 1831) during a 12-day period. Fungi were growing in submerged media at 45ºC and 150 rpm. *β-tubulin* gene was used as reference gene for data normalization in qRT-PCR technique. Data were presented as mean±SEM.

Contrary to the cellulolytic activities, the mRNA levels for the corresponding genes did not show significant differences in the examined isolates. This while gene expression and protein secretion of cellulases are tightly regulated at the transcriptional level in filamentous fungi (Ilmen *et al.*, 1997; Gielkens *et al.*, 1999; Tian *et al.*, 2009). Some metabolites such as cellobiose (Mandels and Reese, 1960) and sophorose (Mandels *et al.*, 1962) acted as expression inducers of cellulase genes in *Trichoderma reesei*. In other words, mRNA abundances are not necessarily representatives of corresponding protein abundances and protein abundances are not always proxies for enzymatic activities of their products (Payne, 2015). For instance, Yoneda *et al*. found two glycovariants of β-glucosidase in fungus *Chaetomella raphigera* that showed different activities to cellobiose, stability to high temperature and inhibition by glucose. They proposed that O-glycosylation is an important factor in defining biochemical properties of these glycovariants (Yoneda *et al.*, 2014).

We intended to investigate the potential of our isolated fungi in cellulase genes processing to cellulase activities, so we designed qRT-PCR examinations in addition to enzyme activities of three fungal strains. It seems that our isolates are less successful in processing of β-glucosidase genes to enzyme activities. In the case of our isolates, lower levels of β-glucosidase activities were observed in spite of approximately equal levels of mRNAs. However, it is possible that other unidentified cellulase genes are present in *T. aurantiacus* genome which were not included in our qRT-PCR and have been functioning in enzymatic assays.

Conclusion

Iranian isolates of thermophilic fungus *T. aurantiacus* displayed maximum endoglucanase activities in a simple media

containing wheat bran and pepton as carbon and nitrogen sources, respectively. Our isolates showed higher levels of endoglucanase, exoglucanase and FPase activities compared to *T. aurantiacus* DSM 1831; whereas, their β-glucosidase activities were weaker than this reference fungus in spite of higher mRNA levels productions. It seems that our isolates are not capable of appropriate processing of β-glucosidase mRNAs to corresponding enzyme activities. However, this deficiency could be improved by co-culturing with other fungi or genetic manipulations.

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