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Increasing the efficiency of sugar beet pulp saccharification by *Trichoderma reesei* superior mutants for bioethanol production

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ABSTRACT

Sugar beet pulp is one of the by-products of sugar industries used to produce cellulase enzyme, *Enzymatic Saccharification*, and alcohol due to its high lignocellulosic content percentage. *Trichoderma spp*. is an important fungus which produces a wide range of cellulolytic enzymes. In this study, cellulase enzyme was produced by placing sugar beet pulp on *Trichoderma* fermentation media together with 21 gamma-irradiated *T. reesei* mutants and then shaking at 180 rpm for 72 h at 28°C. All isolates were screened for cellulolytic enzyme production. *Trichoderma r M5* mutant had the highest level of endo-glucanase, total cellulase, and exo-glucanase enzyme activity among all mutants and primary parental isolates. It also had optimum ß-glucosidase activity. The protein profile of *T. r M5* mutant was analyzed using SDS-PAGE test. *Trichoderma r M5* had different enzymatic bands with variable molecular weight related to EG IV, Cel 3C, Cel 3D, Cel 3A, Cel 7A, Cel 6A, Cel 5A, and Cel 61A enzymes. Results showed that *T. r M5* mutant had the highest efficiency for sugar beet pulp saccharification among all mutants. *S*ugar beet pulp saccharification was carried out within 1 h using enzymes produced by this mutant. The amount of alcohol production from sugar released by industrial yeast stains *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* was evaluated. Alcohol production in *T. r M 5* was 1.5-2 times as great as that of its parent, *T. reesei*.

Keywords: Bio-ethanol, cellulase, *Kluyveromyces marxianus*, mutation, saccharification, *Saccharomyces cerevisiae*, *Trichoderma reesei*

INTRODUCTION

Cellulose is the main structure in the cell wall of plants, algae and fungi (Cannon and Anderson 1991). Cellulose is the most abundant biopolymer on Earth so that 180 billion tons of this biopolymer is annually produced in nature (Zhao 2007). Cellulases (a mix of complex enzymatic systems) act cumulatively to hydrolyze celluloses of agricultural wastes into simple glucose units. Cellulases are produced by cellulolytic fungi such as *Chaetomium*, *Fusarium*, *Myrothecium* and *Trichoderma* species. Other species including *Penicillium* and *Aspergillus* can also produce cellulase (Jun *et al*., 2011). Trichoderma species produce at least two exoglucanases (cellobiohydrolases) including Cel 6A (CHB II) and Cel 7A (CBH I), five endoglucanases including Cel 5A (EG II), Cel 7B (EG I), Cel 12A (EGIII), Cel 45A. EG V, and Cel 61A (EG17), and two β-glucosidases including Cel 1A (BGL II), and Cel 3A (BGL I) (Grishutin 2004, Foreman *et al*. 2003). During an enzyme hydrolysis process, all three groups of enzymes (cellobiohydrolases, endoglucanases, and β-glucosidases) act to break cellulose (Lynd *et al*. 2002). If only a single group of enzymes is involved in the hydrolysis, the hydrolysis process may be disrupted. To have an effective enzymatic hydrolysis process, it is essential for all three groups of cellulolytic enzymes to act together

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(commonly known as synergy). The synergy between cellulolytic enzymes shows that the hydrolysis degree of a mixture of enzymatic compounds outweighs the hydrolysis degree obtained from single enzymes. Synergism between the various groups of cellulose-hydrolyzing enzymes has been extensively studied and documented (Zhang and Lynd 2004). In sugar factories, in addition to sugar, high quantities of molasses, filter cake, pulp (in sugar beet factories), and bagasse (in sugarcane factories) are also produced. Although these are factory waste, each one is an appropriate substrate for the production of enzymes, organic acids, solvents, vitamins, antibiotics, ethanol, paper, particle board, animal feed, etc. Sugar beet pulp contains large amount of raw fiber and is composed of pectins, celluloses, and hemicelluloses in almost equal amounts. Pulp has a very low amount of lignins and, therefore, it is highly degradable. Sugar beet pulp is a rich source of carbohydrate compounds, including pectin (19%), araban (21%), cellulose (23%), and other sugars (14%). Given the high percentage of carbohydrate constituents, sugar beet pulp enzymatic degradation provides a rich source of inexpensive fermentable sugar compounds for the industrial production of, inter alia, bioethanol. Presently, cellulose is gaining an increasing importance due to its applicability in the development of bioethanol production technology. Most studies on cellulase enzymes have focused on fungal cellulolytic systems, and fungi are mainly used for commercial production of cellulase enzymes. Among all fungi, especially those of Trichoderma genus, *Trichoderma* spp. is well-known for the production of cellulase enzymes with relatively high enzymatic activity. Cellulase derived from Trichoderma is resistant to chemical inhibitors and contains all compounds required for the hydrolysis of cellulose crystalline. Since the fungal cellulolytic activity was first reported, extensive efforts have been made on genetic improvement of Trichoderma isolates and optimization of their culture condition in attempts to enhance the efficiency of cellulase production and to attain new genotypes with higher potential to produce enzymatic complex. Cellulase production challenges include proper bio-processes, appropriate inexpensive substrate and environment condition, and fermentation inducer which are the main questions of this research aimed at shedding light on these questions. The present study was seeking to assess the production rate of cellulase enzyme complex by gamma-irradiated *T. ressei* mutants mediated with sugar beet pulp as fermentation substrate and to find out the best mutant isolate for beet pulp saccharification. Then, the superior isolate was used to enzymatically hydrolyze beet pulp, and the feasibility of alcohol production from this hydrolyzed substrate by two yeast species (*Saccharomyces cerevisia* and *Cluyveromyces marxianus*) was evaluated.

MATERIALS AND METHODS

Preparation of T. reesei wild fungus and mutant

Trichoderma reesei was provided in lyophilized form by Fungi and Bacteria Collection Center of the Iranian Research Organization for Science and Technology encoded as 5142. The fungi were transferred into potato dextrose broth liquid culture medium under aseptic condition and kept at 28°C for 72 h. Then, spores in a vegetative form were transferred to PDA medium and incubated under abovementioned condition. The culture plates were incubated for 7 days and the generated spores were collected with saline solution. Their population was set at 1×10^6 spores/mL, and were used for mutation induction. The criterion for appropriate uptake dose to induce non-lethal mutation in spores was about 40-50% germination of spores after irradiation. On the other hand, the dosage should not reduce fungal growth rate as compared with maternal type (Ahari 2009). It has been already shown that at 250 Gy dosage, 47.5% of spores germinate, so this has been selected as the appropriate irradiation dosage (Moradi *et al*. 2010). irradiation was carried out with a Gammacell Cobalt-60 irradiator – with a total activity of 2500 Ci and a dose rate of 0.23 Gy/s in Nuclear Agriculture Research Center of Karaj, Atomic Energy Organization of Iran. A serial dilution was prepared from the spore suspension and it was cultured in PDA medium. The germinated spores were transferred to a fresh culture medium and a number of 21 mutant isolates were selected with respect to their morphological differences and the Mendel test in order to be assessed for their sugar beet pulp saccharification potential.

Cellulase enzyme production

The isolates of mutant and wild *T. reesei* fungi were cultured on MYG agar culture medium containing 5 g L^{-1} of malt extract, 2.5 g L^{-1} of yeast extract, 10 g L^{-1} of glucose, and 20 g L^{-1} of agar and were incubated at 28°C. Using saline solution, a spore suspension was prepared with a population of 10^7-10^8 spore mL^{-1} from 7-day-old spore-

containing plates using a hemocytometer. The initial culture of spore suspension was performed in Trichoderma complete medium (TCM) containing 1 g L^{-1} Bacto Peptone, 0.3 g L^{-1} urea, 2 g L^{-1} KH_2PO_4, 1.4 g L^{-1} $(NH_4)_2SO_4$, 0.3 g L^{-1} $MgSO_4.7H_2O$, 0.3 g L^{-1} $CaCl_2.6H_2O$, 0.005 g L^{-1} $FeSO_4.7H_2O$, 0.002 g L^{-1} $MnSO_4$, 0.002 g L^{-1} $ZnSO_4$, 0.002 g L^{-1} $CoSO_4.7H_2O$, and 2 mL L^{-1} Tween® 80. The pH of TCM was set at 4.8 and it was mixed with 0.3% (w/v) glucose. The fermentation was carried out in TCM in 250-mL Erlenmeyer containing 50 mL of TCM shaken at 180 rpm at 28°C for 24 h after which the spores entered the mycelium vegetative phase. The mycelia were separated from TCM by centrifuging at 4500 rpm for 7 min. To induce the generation of cellulolytic enzymes, the mycelia rinsed with saline solution were poured into a 500-mL Erlenmeyer containing 50 mL Trichoderma fermentation medium (TFM), 0.3 g L^{-1} urea, 2 g L^{-1} KH_2PO_4, 1.4 g L^{-1} $(NH_4)_2SO_4$, 0.3 g L^{-1} $MgSO_4.7H_2O$, 0.3 g L^{-1} $CaCl_2.6H_2O$, 0.005 g L^{-1} $FeSO_4.7H_2O$, 0.002 g L^{-1} $MnSO_4$, 0.002 g L^{-1} $ZnSO_4$, 0.002 g L^{-1} $CoSO_4.7H_2O$, and 2 mL Tween® 80. The medium pH was set at 4.8 and it contained 5% (v/w) wet pulp of sugar beet. The growth condition was similar to the previous condition (shaken at 180 rpm and 28°C for 72 h). Then, the fungal mycelia were separated by centrifuging at 4500 rpm for 7 min, and the supernatant was used to measure extracellular protein and enzymatic activity (Wen *et al*., 2005).

Measurement of extracellularly produced protein concentration in TFM medium and determination of enzymatic activity

The protein of the supernatant of TFM was measured by the Bradford method. Three mL of the Bradford reagent was poured into a test tube and 150 µL of fermented TFM supernatant was added. The sterile supernatant of TFM was used as a control. The absorption of the samples was read at 595 nm with a spectrophotometer and it was compared with the standard diagram plotted for Bovine serum albumin (BSA) pure protein to calculate the protein content of the TFM supernatant (in mg mL^{-1}). The activities of the enzymes Avicelase, carboxymethyl cellulose, cellobiose, and total cellulose were measured by the amount of glucose released from the substrates Avicel, carboxymethyl cellulose, cellobiose, and Whatman filter paper Grade 1 using DNS method and glucose as the standard (Nidetzky and Steiner 1993). The reaction mix was composed of 0.5 mL of 0.5% (w/v) solution of each substrate in 0.05 M sodium citrate (ph 4.8) buffer and 0.5 mL of the supernatant of TFM. Samples were placed in Bain-marie at 50°C for 60 min and the enzymatic reaction was stopped by adding 3 mL of dinitrosalicylic acid solution. Samples were well mixed and then, they were placed in Bain-marie for 5 min, and immediately after that, they were cooled down. After the dilution, their absorbance was read at 540 nm with a spectrophotometer. Each unit of enzymatic activity was defined as the amount of enzyme that can release 1 µM of glucose per hour. Also, to determine total cellulase activity, 1×6 cm stripes of Whatman filter paper Grade 1 was applied as the substrate.

Electrophoresis and determination of molecular weight of enzymes

Electrophoresis test was performed by Laemmli (1970) using stacking gel 4% and resolving gel 12.5%. To prepare protein, first 5 mL of the supernatant of TFM was mixed with 5 mL of cold acetone (-20°C) and its protein precipitation after centrifuging at 4500 rpm for 7 minutes was collected. After the removal of acetone from the samples, it was added with 100 µL twice distilled water and was well mixed. Then, they were added with 100 µL of the sample buffer, were placed in Bain-marie for 5 min, and 20 µL of the samples were injected in each well. Electrophoresis test was performed at constant 20 mAh and the electrophoresis gel was stained with Coomassie Brilliant Green R-250 and was bleached with bleaching agent containing methanol:acetic acid:water at a ratio of 8:1:1.

Bioethanol production

Bioethanol was produced with two yeasts – *K. marxianus* and *S. cerevisiae*. Beet pulp moisture content was set at 65% and pH 4.8 using TFM and it was inoculated with mycelia of *T. reesei* and *T. r M5* under aseptic conditions with similar bulk cell volume at 28°C for 72 h. Then, 50 mL of this culture medium was added with 0.05 M sodium citrate buffer and was placed in Bain-marie at 50°C for 1 h. After sugar beet pulp was hydrolyzed with the enzyme produced by Trichoderma, it was inoculated with *K. marxianus* and *S. cerevisiae*, and its alcohol content was measured with an alcoholometer after 48 h in Bain-marie at 35°C and mixing rate of 180 rpm.

Statistical analysis

All results were subjected to analysis of variance (ANOVA) and mean comparison with

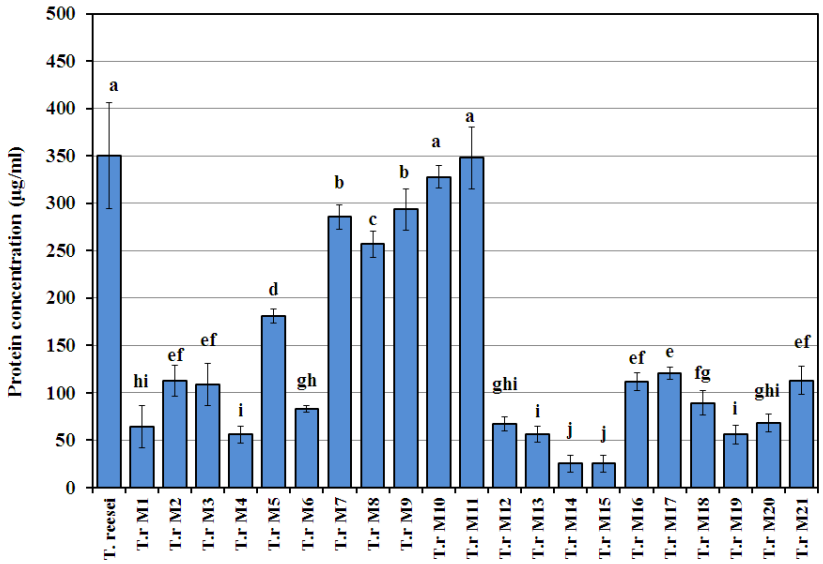

Figure 1. Extracellular protein content (µg mL^{-1}) of *T. reesei* and its mutant isolates in supernatant of the fermentation medium 72 h after incubation at 28°C and 180 rpm

Duncan's multiple range test at $p < 0.05$ level. The statistical analyses were performed using SPSS (Ver. 13) statistical software.

RESULTS

Extracellular protein content of enzymatic activity

Figure 1 displays the amount of extracellular protein in wild fungus and 21 mutant isolates of *T. reesei*. Protein content varied in the range of 5.57-4675 µg mL^{-1}. The highest protein content was related to the wild *T. reesei* and the lowest was observed in the supernatant of *T. r M15* fermentation medium.

In total, it was found that the concentration of extracellular protein produced in TFM differed significantly ($p < 0.05$) among studied fungi level. The mutant isolates *T. r M9*, *T. r M10*, and *T. r M11* did not show significant differences versus their wild parent in terms of extracellular protein production in TFM supernatant. Results of the cellulase enzymes activities in TFM supernatant using different substrates (carboxymethyl cellulose, Avicel, cellobiose and filter paper) are illustrated in Figure 2. Results imply the diversity in enzymatic activity among mutant isolates of Trichoderma. These values, also, exhibited significant ($p < 0.05$) differences. Enzymatic activity is expressed in international unit U in which each unit of enzymatic activity is defined as the amount of enzyme required for the release of 1 µM product per hour. The amount of reduced sugar (glucose) release is measured with dinitrosalicylic acid method. The present study used carboxymethyl cellulose which is an ionic derivative of cellulose (CMC as water-soluble substrate) to determine endo-glucanase activity called CMCase. It has an amorphous structure due to its ionic nature and endo-glucanase enzymes randomly hydrolyze β-glucosidase (1,4) bands inside the molecules of it. Also, cellobiohydrolase (exo-glucanases) were measured with commercial Avicel (also called cellulose microcrystal or hydrocellulose) because it has a low degree of cellulose polymerization and has several amorphous regions unavailable to the attack by endo-glucanases. Enzymes that show relatively higher activity on Avicel but lower activity on carboxymethyl cellulose are defined as exo-glucanase (Maki *et al*. 2009). The activity of β-glucosidase was measured with cellobiose substrate. The activity of the enzyme carboxymethyl cellulose was tested on three wild species of Trichoderma with generated carboxymethyl cellulose which results are shown in Figure 2. The highest activity of carboxymethyl cellulose was 7.12 U mL^{-1} observed in the supernatant of *T. r M5* fermentation medium and its lowest activity was as low as 0.62 U mL^{-1} observed in the supernatant of *T. r M20* fermentation medium. Avicelase activity was measured with pure Avicel and results are illustrated in Figure 2.

The highest activity of Avicelase was 5.39 U mL^{-1} observed in the supernatant of *T. r M5* fermentation medium. Its lowest activity was estimated to

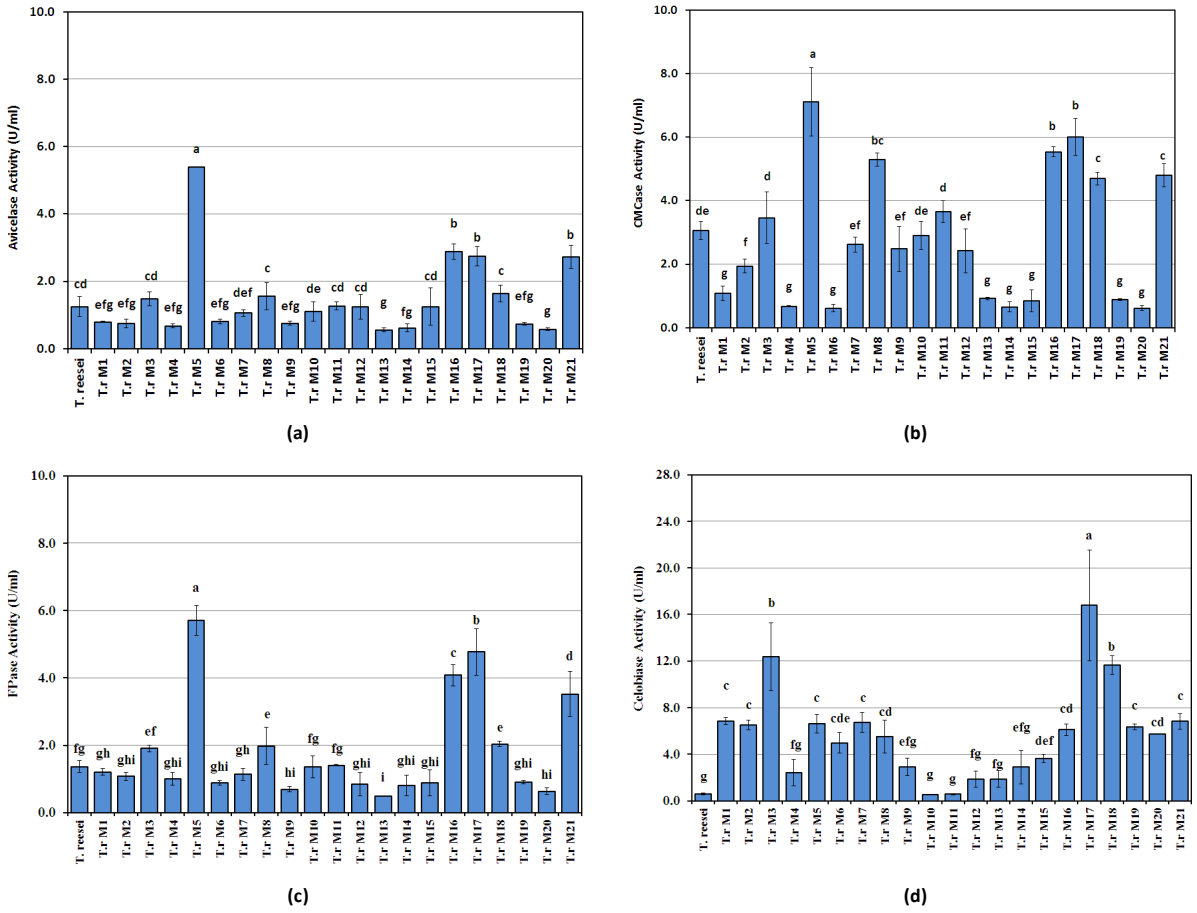

Figure 2. The activity of enzymes (a) Avicelase, (b) carboxymethyl cellulase, (c) total cellulase, and d) cellobiose (U mL^{-1}) of *T. reesei* and its mutant isolates

be 0.56 U mL^{-1} in the supernatant of *T. r M13* culture medium. Figure 2 represents the results of measuring total cellulase enzyme activity using Whatman filter paper in the supernatant of the fermentation environment of *T. reesei* and its mutant isolates. Results showed that the highest total cellulase activity was 5.71 U mL^{-1} related to the enzymes released by *T. r M5* mutant isolate. The activity of the enzyme β-glucosidase (or cellobiose) was also measured with cellobiose substrate which results are presented in Figure 2c. All results were statistically significant ($p < 0.05$). The highest activity of the enzyme β-glucosidase was observed in *T. r M17* mutant isolate (Figure 2d).

Electrophoresis and molecular weight of enzymes

T. r M5 mutant isolate was found to be the best isolate for the hydrolysis of sugar beet pulp. Its extracellular protein profile in TFM supernatant was examined with the SDS-PAGE test. Results are depicted in Figure 3. There are numerous molecular bands in the protein profile whilst non-inoculated TFM supernatant lacked any specific protein bands. The lowest molecular weight was related to the enzyme Cel61A (EG IV) in which a strong band emerged at 34.14 kDa. In the protein profile of the SDS-PAGE gel of *T. r M5* (Figure 3), a

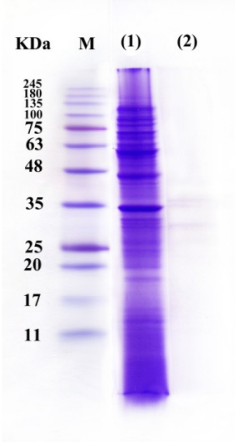

Figure 3. Protein profile of cellulase enzymes. (1) Supernatant of TFM inoculated with *T. r M5* mutant, (2) supernatant of non-inoculated TFM, and (M) protein marker.

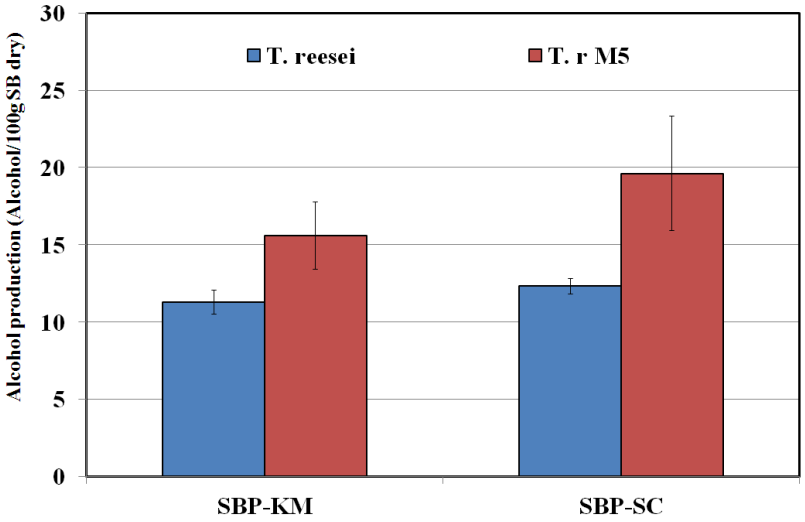

Figure 4. Comparison of alcohol production rate (alcohol percentage per 100 g dry matter of sugar beet pulp) using *Cluyveromyces marxianus* and *Saccharomyces cerevisiae* yeasts after sugar beet pulp saccharification using *T. reesei* and *T. r M5*

strong band of the enzyme Cel5A was observed at the molecular weight of 46.25 kDa. Cel6A was observed at the molecular weight of 59.19 kDa. Also, Cel7A was observed at the molecular weight of 63 kDa in protein profile of the SDS-PAGE gel. The enzymatic band Cel7B was only seen in the protein profile of *T. reesei* at the molecular weight of 54 kDa. Also, Cel3A (BGLI) with the molecular weight of 71.57 kDa was obtained from the protein profile of *T. r M5*. Cel 3D (BGL) with the molecular weight of 78.67 kDa was observed in the protein profile of *T. r M5*. Cel 3C (BGL) and EG VI were observed at the molecular weights of 91.91 and 119.44 kDa, respectively.

Bioethanol production

The sugar content was measured at 0.22 g mL^{-1} in sugar beet and at 0.007 g mL^{-1} in pulp. Results showed that samples treated with sugar beet pulp and *T. reesei* mutant (which were exposed to yeast after 48 h of hydrolysis) produced 19.61 and 15.59% alcohol in *S. cerevisiae* and *C. marxianus* fermentation treatment, respectively. In two other treatments in which fresh sugar beet was directly used without *T. reesei* treatment, 45.37 and 46.94% alcohol was obtained in culture medium treated with *S. cerevisiae* and *C. marxianus*, respectively. This means that the alcohol produced with *S. cerevisiae* yeast in the medium derived from fresh sugar beet was 2.31 times as great as the one produced with *S. cerevisiae* yeast in the medium derived from pulp. The alcohol production with *C. marxianus* yeast in the medium derived from fresh sugar beet was 3.01 times as great as that of *C. marxianus* yeast in the medium derived from pulp. Nonetheless, the sugar content of sugar beet was measured to be 31.4 times as great as that pulpimplying the high potential for enzymatic hydrolysis of the crystalline (amorphous) structure of sugar beet pulp in *T. rM5* mutant.

Figure 4 depicts the rate of alcohol production for pulp hydrolyzed with the enzymes of *T. reesei* and *T. r M5* using the yeasts *S. cerevisiae* and *C. marxianus*. Alcohol production rate was higher in saccharification with *T. r M5* than that of *T. reesei* wild fungus and fermentation with other yeasts (by 1.5-2 times), implying the higher efficiency of this mutant isolate in the enzymatic decomposition of sugar beet pulp and the release of more reduced sugars for fermentation.

Results showed that samples treated with pulp and *T. reesei* mutant, which were subjected to fermentation after 48-hour hydrolysis, produced 12.51 and 11.45% alcohol in the treatment with *S. cerevisiae* yeast and *C. marxianus* yeast, respectively. With respect to two other treatments in which fresh sugar beet was not directly used and were not treated with *T. reesei*, 45.37 and 46.94% alcohol was obtained in *S. cerevisiae*- and *C. marxianus*-treated culture medium, respectively. This means that the alcohol produced with *S. cerevisiae* in the medium derived from fresh sugar beet was 3.96 times as great as that produced with *S. cerevisiae* in the medium derived from the pulp. Also, alcohol produced with *C. marxianus* in the medium derived from fresh sugar beet was 4.09 times as great as that produced with *S. cere-*

visiae in the medium derived from the molasses. This was despite the fact that sugar content of sugar beet was measured to be 31.4 times as great as that of its pulp.

Since the culture medium had an equal amount of 20 g dry matter, the following results were obtained after fermentation. In *T. r M5* treatment of sugar beet pulp with *S. cerevisiae*, 62.09% of dry matter was converted into alcohol. However, this was 49.17% for the pulp treated with *C. marxianus*. In the treatment of the parental type of *T. reesei* pulp with *S. cerevisiae*, 59.96% of dry matter was converted into alcohol while this was 47.77% for fermentation with *C. marxianus*. In the two treatments in which fresh sugar beet was applied, *C. marxianus* fermented 69.38% of the dry matter into alcohol and *S. cerevisiae* converted 68.43% of dry matter into alcohol.

DISCUSSION

Sugar beet pulp is a byproduct of sugar industries with a significant role in sugar beet processing industry. This lignocellulosic material is of lower commercial value than the fermentation substrates commonly used in the industry and it is mainly used as animal feed. Furthermore, it proper for processing in industry due to its availability and high decomposable lignocellulose content. Given the increased costs of the fermentation process for bioethanol production, the production of cellulolytic enzymes forms a key step in the hydrolysis of lignocelluloses like sugar beet pulp and their usage as a fermentation substrate. Multiple species of Trichoderma produce cellulolytic enzymes. This fungus has been subjected to extensive studies to induce mutation for the increase of cellulase enzymes production. In addition to enzyme production, this fungus has other uses, e.g., in feed production, pharmaceutical and textile industries. The present study used sugar beet pulp in the fermentation medium and used 21 gamma-irradiated *T. reesei* mutants to produce cellulase enzyme. Also, the potential of all isolates to produce cellulolytic enzymes were assessed.

Results of the extracellular protein measurement showed that studied samples differed significantly ($p < 0.05$). Given the protein nature of the extracellular enzymes secreted by the tested fungi, useful information could be derived about the rate of the production of these enzymes by measuring the concentration of extracellular proteins. The measurement of the extracellular protein concentration is not always an easy task because multiple parameters may affect final results (Adney *et al*. 1995, Zaia *et al*. 1998). There are three major parameters which may influence the measurement of protein concentration in the samples: (i) all protein measurement methods are based on quantitative differences and principles; (ii) the presence of non-protein compound in enzyme solution or reaction medium may be a source of error if it is involved in the results of quantitative methods; and (iii) the presence of non-cellulase proteins in enzyme preparation process can complicate the interpretation of the data on specific enzymatic activity. These differences may also be given rise by the fact that different enzymatic isolates have different initial structures. Furthermore, they differ in the degree of glycosylation. Thus, these parameters are reflected in the response of proteins derived from different Trichoderma species. Among all mutant isolates and wild parental isolate, the activity of the enzymes endo-glucanase, exo-glucanase, and total cellulase were the highest in *T. r M5* mutant isolate. This isolate exhibited a good level of β-glucoside activity. Total cellulase includes the activity of the enzymes endo-glucanase, exo-glucanase, and β-glucosidase which hydrolyze crystalline cellulose in a synergy. Most studies have focused on the synergism between endo-glucanases and exo-glucanases. The synergy of endo-exo enzymes has been most frequently reported on substrates treated for reducing crystallization such as homogenized Avicel (Henrissat *et al*. 1985) and ground SolkFlok (Fan *et al*. 1981). Cellulose crystallization plays a key role in enzymatic hydrolysis. It is generally believed that cellulose structure is divided up into two regions: an amorphous region that is readily hydrolyzed by enzymes and crystalline region which hydrolysis is difficult by enzymes. This provides us a good understanding of the observed kinetics for cellulose hydrolysis in which enzymes hydrolyzing rapidly illustrate compound with more of the amorphous region, and in a slow hydrolysis process, more crystalline region are seen in the compound. Results lead us to the conclusion that fresh sugar beet pulp has more crystalline structure than amorphous region and is more prone to degradation by endo-glucanases. In addition to substrate characteristics, evaluation conditions influence the extent of synergy. It has been reported that endo-exo synergy increases with an increase in enzyme level below saturation point, but its further increase after saturation point resulting in the synergy loss (Watson *et al*. 2002). Total cellulase activity is always measured with

insoluble substrates including pure cellulose substrates, such as Whatman filter paper Grade 1, cotton lint, microcrystalline cellulose, bacterial cellulose, algae cellulose, and the cellulose-containing substrates such as stained celluloses, α-cellulose, and primed lignocelluloses. Avicel contains some amorphous region and soluble cellodextrins that can act as a substrate for both exo-glucanases and endo-glucanases. No absolutely excellent substrates exist to test exco-glucanases activity in cellulase mixtures (Wood and Bhat 1988). However, this substrate cannot be appropriate to determine CBH II activity in *T. reesei* since it does not have an effective exo-glucanase activity for the substrate (Van Tilbeurgh *et al*. 1982, 19850. Among cellulose substrates, Avicel possesses the highest rate of terminal to internal chains of available β-glucoside bands. The enzymes CBH I and CBH II can break numerous bands when absorbed even before both substrate complex and enzyme separation (Valjamae *et al*. 1998). Therefore, the performance of CBH I and CBH II results in a gradual reduction of cellulose polymerization degree (Srisodsuk *et al*. 1998). Previous studies reported the specific activity of the CBH II enzyme as almost twice as great as the activity of the CBH I enzyme (Nidetzky *et al*. 1994, Medve *et al*. 1994). Higher Avicelase activity in *T. r M5* mutant is likely to be related to the higher ability of this fungus in degrading crystalline areas. The activity of endo-glucanases is often measured against the hydrolysis of cellulose solution derivatives, such as carboxymethyl cellulose. There are no reports on the weak relationship between carboxymethyl cellulase activity and its ability to hydrolyze insoluble cellulose, even for isolated endo-glucanases (Klyosov 1988, 1990). Among three *T. viride* endo-glucanases isolated by Shoemaker and Brown (1978), the one that had the highest rate of Avicel hydrolysis had the lowest rate of carboxymethyl cellulase activity. Klyosov (1990) clearly pointed to the activity of endo-glucanase of many measured microorganisms on carboxymethyl cellulose and showed the lack of any relationship with the activities against insoluble cellulose. The rate of soluble reduced sugar production by EG I versus CBH I was ≥1 for amorphous cells, ≤1 for Avicel, and ≤1 for bacterial microcrystalline cellulose (BMCC) and cotton (Zhang and Lynd 2004). The relatively low rate of reduced sugars release by EG I on crystalline cellulose is compatible with most reduced ends produced by the activity of endo-glucanases in solid phase and does not necessarily imply the lower rate of β-glucoside bands breaking. The efficient hydrolysis of crystalline cellulose by cellulase requires the synergistic performance of endo-glucanases and cellobiohydrolases as reviewed by Teeri and Koivula (1995). This synergistic performance is well recognizable in *T. r M5* mutant. The maximum synergy is usually obtained from high quantities of exo-enzymes and low quantities of endo-enzymes (Reinikainen 1994). The protein profile of *T. r M5* mutant isolate was examined by SDS-PAGE test. This isolate had numerous enzymatic bands with different molecular weights which were related to the enzymes EG IV, Cel 3C, Cel 3D, Cel 3A, Cel 7A, Cel A, Cel 5A, and Cel 61A that hydrolyzed sugar beet pulp in a synergy. Cel5A is an endo-glucanase from the family of 5-glucohydrolases which molecular weight has been estimated at 42 kDa. However, it has an apparent molecular weight of 48 kDa on the SDS-PAGE gel. The isoelectric point of this enzyme is in the range of 5.5-5.6 (Shoemaker and Brown 1978). It has been estimated that among enzymes expressed in *T. reesei*, 5-10% of total cellulase expression is accounted for Cel5A enzyme (Ståhlberg 1991, Ilmen *et al*. 1997). Cel6A is a cellobiohydrolase from 6-glucohydrolases family. It has a molecular weight of 47 kDa, a weight of 52 kDa on the SDS-PAGE gel, and the isoelectric point of 5.9 (Fägerstam and Pettersson 1980, Bhikhabhai et al. 1984). However, its molecular weight has been also reported to be in the range of 50-58 kDa. The enzyme Cel6A (CBH II) is an enzyme which breaks glycoside bands from the non-reduced end of the chain (Barr *et al*. 1996, Boisset *et al*. 2000). There are also some reports about its endo-glucanase activities (Nutt *et al*. 1998). Among all enzymes secreted by *T. reesei*, 17-20% of total expressed cellulase enzymes are related to Cel6A (Ståhlberg 1991, Ilmen *et al.* 1997). Cel7A, or CBH I, is a cellobiohydrolase from 7-glucohydrolases family and is the first recognized cellulase enzyme of *T. reesei* (Wey *et al*. 1994). The apparent molecular weight of Cel7A is 52 kDa, its molecular weight is 66 kDA on the SDS-PAGE gel, and its isoelectric point is 4.3 (Fägerstam *et al.* 1977, Shoemaker *et al.* 1983). Cel7A is the most cellulase expressed by *T. reesei* so that it accounts for 50-60% of total expressed cellulase (Ståhlberg 1991; Ilmen *et al.* 1997). However, in the present study, it was expressed to a lower extent than Cel6A due to the characteristics of the fermentation substrate. This enzyme is likely to play a key role in the hydrolysis of the crystalline cellulose. Cel7A is an efficient enzyme in the hydrolysis of glycoside bands in cellulose and pre-

fers to start hydrolysis from the reduced end of the chain (Barr *et al*. 1996, Divne *et al*. 1998). Cel7B is an endo-glucanase from 7-glucohydrolases family with estimated molecular weight of 48 kDa that is 50-55 kDa on SDS-PAGE gel and an isoelectric point of 4.5 (Shoemaker *et al*. 1983, Bhikhabhai *et al*. 1984). Cel7B hydrolyzes glycoside chains in cellulose with a specific mechanism. In *T. reesei*, the expression of Cel7B accounts for 6-10% of the total expressed cellulase (Ståhlberg 1991, Ilmen *et al.* 1997). β-glucosidases produce glucose by hydrolyzing the dissolved oligosaccharides. It has been documented that the addition of β-glucoside from *T. reesei* to cellulose hydrolysis process can improve the performance of saccharification (Xin *et al*. 1993). β-glucosidase hydrolyzes the cellobiose that is an inhibitor of cellulase enzyme activity. Our results showed that *T. r M5* mutant isolate was the best isolate for saccharification of sugar beet pulp. The rate of alcohol production in saccharification treatment with *T. r M5* was about 1.5-3 times as great as that of *T. reesei* wild species and fermentation with the tested yeasts. All in all, it can be concluded that the mutation of *T. reesei* fungus resulted in the production of *T. r M5* mutant isolate which was highly capable of secreting cellulolytic enzymes to degrade sugar beet pulp. Assays indicated that the saccharification of sugar beet pulp with this isolate produced a higher percentage of alcohol than that produced through fermentation with *C. marxianus* and *S. cerevisiae*. So, this isolate can be applied in the industry for cellulose decomposition and alcohol production. This isolate is kept at Fungi and Bacteria Collection in Plant Protection and Food Maintenance Research Group, Nucleo-Agriculture Research Center of Atomic Energy Organization, Iran.

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