Mycologia Iranica 11 (1): 77−92, 2024 **Original Article** DOI: 10.22092/mi.2024.365994.1283

Strain improvement of *Trichoderma afroharzianum* through induced gamma radiation mutation for cellulase and xylanase production enhancement

H. Askari
S. Shahbazi✉
Nuclear Agriculture School, Nuclear Science and Technology Research Institute (NSTRI), Atomic Energy Organization of Iran (AEOI), Karaj, Iran
S. Soleimanian-Zad
Department of Food Science and Technology, College of Agriculture, Isfahan University of Technology, Isfahan, 84156-83111, Iran Research Institute for Biotechnology and Bioengineering, Isfahan University of Technology, Isfahan, 84156-83111, Iran
M. Kadivar
Department of Food Science and Technology, College of Agriculture, Isfahan University of Technology, Isfahan, 84156-83111, Iran

Abstract: Cellulases and xylanases are hydrolytic enzymes that randomly cleave the β-1, 4 backbones of the cellulose and arabinoxylans of wheat flour and are widely used in the bakery industry as a dough texture improver in the formulations of flour products. Creating novel sources of a microbial strain using induced gamma irradiation can increase enzyme production for bakery industrial usage. According to this, Co^{60} gamma irradiation has been used to develop a mutant strain of *Trichoderma afroharzianum*. *Trichoderma* mutants were isolated, and the qualitative and quantitative screening were used to evaluate the extracellular enzyme production with the wheat bran waste as a substrate. The best *Trichoderma* mutant isolate was identified using the DNA barcoding method. The highest xylanase activities were observed in the superior mutant isolate of *Trichoderma afroharzianum* NAS107-M82, which is approximately 3.3 times higher than its parent strain. The electrophoretic pattern of proteins showed that the exo-glucanase I, endo-glucanase III, and the xylanase I enzymes hydrolyzed the wheat bran, synergistically. Overall, gamma irradiation-induced mutation could be an expedient technique to access such superior mutants for the bioconversion of wheat bran wastes to xylanase enzyme.

Submitted 15 May 2024, accepted for publication 19 June 2024
✉ Corresponding Author: E-mail: sshahbazi.aeoi@gmail.com

https://mij.areeo.ac.ir

Keywords: *Trichoderma*, Wheat bran, Cellulase-Xylanase, Gamma radiation, Mutation.

INTRODUCTION

Filamentous fungi such as *Trichoderma* spp. produce extracellular hydrolytic enzymes, such as endoglucanases (EGI/Cel7B, EGII/Cel5A, EGIII/Cel12A, EGIV/Cel61A, and EGV/Cel45A) and exoglucanases (the cellobiohydrolases, CBHI/Cel7A, and CBHII/Cel6A) that act synergitically to break down cellulose into cellobiose (glycosyl β-1,4-glucose) and subsequent hydrolysis to glucose by β-glucosidase) (Kunamneni et al., 2014), which cleave the β-1, 4-glycosidic bonds present in cellulose and its derivatives (Mach and Zeilinger, 2003). Several other enzymes by *Trichoderma* include β-mannase (EC 3.2.1.78), β-mannosidase (EC 3.2.1.25), α-L-arabinofuranosidase (EC 3.2.1.55), α-galactosidase (EC 3.2.1.22), acetylene xylan esterases (EC 3.1.1.72), laccases (benzendiol: oxygen oxidoreductases, EC 1.10.3.2), polygalacturonase (EC 3.2.1.15), pectin lyase (EC 4.2.2.10), and pectin esterase (EC 3.1.1.11) (Kunamneni et al., 2014). The cost of enzyme production can be significantly decreased by improving enzyme production by utilizing effective microorganisms, their mutation, and using agricultural waste as a substrate for fermentation. Effective microorganisms can improve production performance because the culture medium typically accounts for 25-50% of the total production costs (Shafei et al., 2011). Also, microorganisms that produce both cellulases and xylanases under the same culture conditions hold great significance due to their synergistic applications. Cost-effective substrates such as wheat bran can be used by a single microbial isolate to process multiple enzymes upstream in the same fermentation medium, which has great benefits for the industry.

Xylanase and cellulase production in filamentous fungi is much higher than that of actinomycetes, bacteria, and yeasts. These microorganisms release the enzyme directly without any negative side effects and without requiring the degradation of cell walls

hemicellulosic material in the environment. Induction of random mutations by physical mutagens such as UV, X-, and gamma radiation and chemical mutagens such as ethyl methane sulfonate have been used as useful tools to manipulate *Trichoderma* genetically (Catlett et al., 2003; Gohel et al., 2004).

Several studies have shown that gamma radiation can cause genetic diversity of filamentous fungi and induce positive (Abbasi et al., 2016; Lee et al., 2000; Shahbazi et al., 2014) or negative mutants (Zolan et al., 1988) of specific genes. For instance, a large number of the high-secreting mutants offer suitable strains for specific industrial objectives (Steensels et al., 2014), such as the overproduction of antibiotics (Barreiro et al., 2012; Kardos and Demain, 2011), and enhanced production of cellulase enzymes (Bischof et al., 2016; Dillon et al., 2006; Ribeiro et al., 2013), lipases (Karanam and Medicherla, 2008), citric acid (Javed et al., 2010) and bioethanol (Mobini-Dehkordi et al., 2008; Zhao et al., 2022).

According to the Food and Drug Administration (FAO), wheat was cultivated worldwide in 2023, producing approximately 785.1 million tons. (FAOSTAT, 2023). Wheat bran is primarily made up of cellulose and xylan, which is the abundant organic carbon source in the wheat flour industry. Bran's contribution to total wheat grain weight is between 13 and 19%, depending on the milling process (wet or dry) utilized for its extraction (Onipe et al., 2015), which currently has low commercial value, but can be used as the best carbon source for xylanase and cellulase production. Hemicellulose, which is typically present in higher concentrations on the outer surface of cellulose fibers but is also diffused into the interfibrillar space through fiber pores (Köhnke et al., 2010), has been suggested to act as a physical barrier that limits the accessibility of the cellulase enzymes to the cellulose (Pauly et al., 1999). The purified wheat bran contains high amounts of hemicellulose (Doner and Hicks, 1997), which can be used as the finest substrate for induction of xylanase-cellulase enzyme production by *Trichoderma* fungi.

Due to this enzymatic diversity of *Trichoderma* filamentous fungi and its ability to biodegradation of hemicellulosic waste and production of extracellular enzymes, in this study, we will approach to simultaneously produce xylanase and cellulase enzymes from wheat bran using superior gamma radiated mutants of *Trichoderma.* Therefore*,* the best *Trichoderma* isolate was screened, identified and treated with different doses of gamma irradiation to investigate the possible enhancement of xylanase and cellulase production by gamma radiation-induced mutation.

MATERIALS AND METHODS

Trichoderma isolates

In this study, twenty-four (T1 to T24) *Trichoderma* isolates (with NCBI accession numbers: MW718882, OM083873, MZ681938, MZ682034, OM083970, MW719569, MW714049, MW719590, MW719097, OM078503, MW719255, MW719475, MW719563, MW719876, MZ681867, MZ681937, OM084953, MW719878, MZ682163, MZ682208, OM074016, MZ682231, OM074000, MZ682234, respectively) were obtained from the fungal collection of the Plant Protection Department of the Nuclear Agriculture Research School, Alborz, Iran. The cultures were grown on potato dextrose agar (PDA, Merck, Germany) medium containing 100 mg. L^{-1} of chloramphenicol and stored at 4 °C for further studies.

Qualitative screening of best xylanase-producing isolates

All wild-type *Trichoderma* isolates were screened for their xylanase activity using the plate screening methods on *Trichoderma* complete medium (TCM or Mendel's mineral salts solution medium) 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; $CaCl_2.6H_2O$, 0.4; $FeSO_4.7H_2O$, 0.005; $MnSO_4$, 0.0016; $ZnSO_4$, 0.0014; $CoCl_2.6H_2O$, 0.002 and 0.2 ml. L^{-1}, Tween 80, supplemented with the addition of 17.5 g. L^{-1} of agar, 0.1% (v/v) Triton X-100, 4 gL^{-1} of sorbose and 0.5% w/v of arabinoxylan as a substrate. The medium was adjusted to pH 5.3 with pH meter. The plates were inoculated with mycelial plug (0.8 cm diameter), and incubated at 28 °C for 3 days followed by 18 h at 45 °C. The Petri dishes were flooded with 0.4% Congo red dye and after 10 minutes washed with 1M NaCl. For better visualization of the clear areas, acetic acid (5%) was flooded to the surface of the Petri dishes (Yopi et al., 2017). The best xylanase-producing isolate was determined by calculating its maximum hydrolysis capacity (HC). According to the definition, HC refers to the relationship between the diameter of the hydrolysis clearing zone surrounding the colony and the colony itself.

Gamma radiation-induced mutation of *Trichoderma*

After the determination of the best enzyme-producing isolate of *Trichoderma*, the spore suspensions were prepared from a seven-day-old PDA culture in sterile saline solution and irradiated using a gamma cobalt 60 source in Gammacell 220 irradiator

(MDS Nordion, Ottawa, Canada) at a dose rate of 4.5 kGy per hour, located at the radiation application research school, nuclear science and technology research institute (NSTRI) of the atomic energy organization, Tehran, Iran (Fig. 1). In triplicate, dose levels of 0, 100, 250, 500, 750, and 1000 Gy were used, and after the irradiation process, the spore suspensions were cultured on PDA media.

Fungal viability was used to estimate the gamma radiation D_{10} value. D_{10} value of gamma radiation represented the dose of gamma radiation that reduced the fungal viability of one log cycle or 90%. According to the previous study, gamma rays are inclined to create multiple mutations in one gene at a dose higher than the D_{10} value (Hidayati et al., 2021; Ottenheim et al., 2015). Determination of optimum dose of gamma irradiation for on-induced mutation was selected based on approximately 90-99 % (or 1-2 log cycle) reduction of spore germination on PDA medium. Based on the spore germination rate, the first single colonies of the fungus were isolated from the surface of the culture medium using a binocular microscope. The selected colonies from an optimum dose of gamma irradiation were subcultured on malt yeast glucose agar (MYGA) medium containing (gL^{-1}): malt extract, 5; yeast extract, 2.5; glucose, 10; agar, 17.5; and examined after 7 d of incubation at 28˚C for qualitative screening of xylanase producer mutant isolates similar to the method described above. Best xylanase-producing mutant isolates were determined based on the ratio between the hydrolysis capacity of mutant isolate (HCM), and the hydrolysis capacity of wild-type strain (HCW). The best mutant isolates of *Trichoderma* were maintained on MYGA medium.

Fig 1. Gammacell 220 irradiator used to induce mutation in *Trichoderma afroharzianum* NAS107

Quantitative screening of *Trichoderma* mutant isolates

One milliliter of spore suspension (1×10^7 spores/mL) was used as inoculum for the inoculation of 50 mL of TCM medium (pH 4.8) supplemented with 0.3% w/v glucose in 250 ml Erlenmeyer flasks, and placed in an orbital shaker (150 rpm) at 28 ˚C for 24 h. The fungal mycelia were collected and transferred to 50 ml of *Trichoderma* fermentation medium (TFM) containing TCM supplemented with 0.5% w/v wheat bran powder as a substrate at pH 5.3 to induce the production of xylanase enzymes. Growth conditions were as described previously, and triplicate flasks were harvested after an incubation time of 72 h at 28 °C. Estimation of extracellular protein and enzyme activity was assayed in TFM after centrifugation at 4500 ×g for seven min at 4 °C (Shahbazi et al., 2016).

Assay of extracellular protein production and enzyme activity

The extracellular protein content in the TFM supernatants was estimated in different mutant isolates of *Trichoderma* by the Bradford dye-binding method (Bradford, 1976). The amount of protein was calculated using bovine serum albumin (BSA) as a standard. The absorbance was read at 595 nm on a spectrophotometer (Jenway, USA). Exo-glucanase, endo-glucanase and β-glucosidase, total cellulase (Filter paperase or FPase), and xylanase activity were determined by measuring the amount of glucose or xylose released from substrates by the dinitrosalicylic acid (DNS) method with glucose or xylose as the standard (Ghasemi et al., 2019; Shahbazi et al., 2016).

Electrophoresis and molecular size determination of proteins

Protein samples (~ 200 µg) were precipitated from TFM supernatants with two volumes of cold acetone and kept at–20°C overnight. The molecular weight (kDa) of the proteins was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 5% (stacking) and 12.5% (separating) polyacrylamide gel based on Laemmli method (Laemmli, 1970). The proteins were separated at a constant amperage of 25 mA. 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). Estimation of the molecular weight of enzymes was performed using Gel-Pro Analyzer 6.0 densitometry software (Media Cybernetics, Inc.) using a protein marker belonging to Sinaclon Company (Gooruee et al., 2024). The molecular weight range was 11-245 kDa (Laemmli, 1970; Shahbazi et al., 2016).

Genotypic identification of best *Trichoderma* isolates and their mutant

Production of fungal biomass

Trichoderma cultures (wild-type strain and its best mutant isolate) were grown in potato dextrose broth at 28 ºC for 5 to 7 days at an agitation rate of 150 rpm. Mycelium was collected and frozen in liquid nitrogen and lyophilized.

DNA extraction, sequence alignment, and molecular phylogeny

The total genomic DNA extraction was done according to the protocol previously described by Abbasi et al., (2016). The ITS-rDNA and parts of the *TEF1*-α gene were amplified using the primer pairs ITS-1 (5'-CGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') and EF1-728F (5'-CATCGAGAAGTTCGAGAAGG-3'), and EF1-986R (5'-TACTTGAAGGAACC CTTACC-3'), respectively. The polymerase chain reaction (PCR) amplification was performed in 20 µl reaction volume containing 0.2 unit/µl Taq DNA polymerase (Sinaclon, Iran); 1.5 mM, $MgCl_2$; 0.2 µM of the primers, and 10 ng genomic DNA of each isolate. PCR amplification was carried out in a Bio-Rad thermocycler (USA), and PCR conditions for ITS-rDNA region were as: an initial denaturation step for 5 min at 93°C, followed by 35 cycles of denaturation at 93°C for 45 s, annealing at 57 °C for 30 s, extension at 72°C for 90 s, and a final extension step at 72°C for 5 min. TEF1-α were as: an initial denaturation step for 5 min at 94 °C, followed by 35 cycles of denaturation at 94°C for 60 s, annealing at 58°C for 60 s, and extension at 74 °C for 50 s, and a final extension step at 74 °C for 7 min. The PCR products were visualized using 1% (W/V) agarose gel electrophoresis stained with ethydium bromide in a TBE (Tris/Borate/EDTA, pH 8.3) buffer and were observed under UV using the gel-documentation system (BioRad, Gel Doc XR system). A 0.1-10-kb DNA ladder (Thermo Scientific™ O'RangeRuler DNA Ladder, Thermo Fisher Scientific, USA) was used as a molecular size marker for comparison. The amplified products were cleaned and sequenced using the same primers used for PCR amplification by Bio Magic Gen Co. ITS-rDNA and Tef1-α sequences were subjected to NCBI nucleotide BLAST (http://blast.ncbi.nlm.gov/Blast.cgi) to identify similar sequences of related species. The data sets used for phylogenetic analyses included 2 isolates of *T. afroharzianum* (wild type and its mutant isolate) and 26 reference sequences were assembled from GenBank (NCBI), namely: 2 *T. afroharzianum*, 4 *T. guizhouense,* 2 *T. lentiforme,* 3 *T. harzianum*, 2 *T. pleoroticola*, 2 *T. virense*, 2 *T. citrinoviride*, 2 *T. longibrachiatum*, 2 *T. hamatum*, 2 *T. atroviride*, 2 *T. caerulescens* and *Porotocrea farinosa* which was selected as the out-group taxon.

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.65694272 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004), and are in the units of the number of base substitutions per site. This analysis involved 28 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 2559 positions in the final dataset. The Phylogenetic tree was constructed using the MEGA version 10.0.5 (Kumar et al., 2018).

Statistical analysis

All experiments had three replications for 24 isolates. Differences in the extracellular protein production and qualitative, and quantitative xylanase activities were analyzed using a completely randomized design. The results were subjected to analysis of variance (ANOVA) with means compared by the Duncan test and HSD Tuky test ($p<0.05$), using the SPSS (ver. 16) statistical software.

RESULTS AND DISCUSSION

Pure cultures (24 *Trichoderma* isolates) were prepared by the single spore isolation method. All isolates presented white mycelium of spongy consistency in the early days of cultivation on the PDA culture medium that spread throughout the plate and consequently had a yellowish-green to dark green color after 3-5 days of incubation at 25 ºC.

The initial screening of the *Trichoderma* wild-type strains was done first based on the high xylanase enzyme activity because the xylan forms a sheath on each cellulose microfibril and it might be affected by the depolymerization of cellulose by cellulases within the fiber.

Qualitative screening of the best xylanase-producer strain

Figure 2 shows the qualitative evaluation of xylanase enzyme production in the studied 24 different isolates of *Trichoderma* by the plate screening method. The results of hydrolysis capacity in different isolates

showed a statistically significant difference ($p<0.05$). The highest hydrolysis capacity was observed in the T7 isolate, which was selected for gamma radiation-induced mutation. Hydrolysis plays a crucial role in enzyme plate screening by enabling the identification and characterization of enzymes with hydrolytic activities on various substrates, including xylan. Hydrolysis can indeed enhance the efficiency of enzyme plate screening by providing a means to identify enzymes with specific substrate specificities, thus improving biocatalyst performance.

Gamma radiation-induced mutation

The results of gamma radiation showed that the spore viability of T7 strain was significantly decreased with increasing in radiation dose (Figure 3). High doses of gamma radiation cause more DNA damage and lead to fungal death. According to the previous study, gamma rays are inclined to create multiple mutations in one gene at a dose higher than the D_{10} value (Hidayati et al., 2021; Ottenheim et al., 2015). The D_{10} value represents the radiation dose required to reduce the viability of fungi by 10%. The logarithmic reduction of viable spore population as a function of radiation dose can be described by the present equation in Figure 3. According to the results shown in Figure 3, the irradiation in a dose of 250 Gy resulted in an approximately 90-99 % (or 1.825 log cycle) reduction of spore germination on the PDA medium. Therefore, the dose of 250 Gy was chosen as the optimal dose of gamma radiation-induced mutation.

Screening of the best xylanase-cellulase-producing mutant isolates

Qualitative screening of mutant isolates

118 pure cultures were obtained from an optimum dose of gamma radiation (250 Gy), for further investigations regarding the activity of xylanase by using single spore isolation method. To identify the best mutant isolates, the xylanase production on qualitative plate screening containing xylan (psyllium hydrocolloid) as a carbon source was studied. Of the 118 mutant isolates, 17 isolates were selected based on the ratio of HCM/HCW more than 1.25 (Table 1). Also, M17 and M79 isolates were selected as mutants with hydrolysis capacity similar to and weaker than the wild-type strain (control), respectively.

Table 1 presents the results of comparing the mean hydrolysis capacity of mutant isolates (19 mutants) with the wild-type strain. Results showed statistically significant differences at $p<0.05$. The highest hydrolysis capacity in qualitative screening tests was observed in mutant isolates of M56, M114, M89, M17, M62, and M44, respectively.

These results suggest that xylanase or cellulase enzymes, which affect the structure of arabinoxylan, may be secreted into the culture medium by the mutant isolates. Therefore, xylanase and cellulase enzyme activity measured in the supernatant of TFM could be a better indicator (criterion) of the ability of mutant isolates to hydrolyze wheat bran.

Quantitative screening of mutant isolates

The results of extracellular protein concentration are shown in Table 2. Mutant isolates showed statistically significant differences at $p<0.05$ in extracellular protein production. The extracellular protein concentration varied from 56.97 to 185 µg/mL. The highest extracellular protein concentration was obtained in the TFM supernatant of the M82 isolate and the other mutant isolates had lower concentrations than this isolate.

Extracellular proteins play a crucial role in fermentation processes by contributing to various aspects of microbial metabolism and product formation. Extracellular proteins in *Trichoderma* play a crucial role in enzyme production and interactions with other organisms. These proteins include hydrolytic enzymes such as cellulases and xylanase, which are essential for degrading complex polysaccharides and organic compounds. Overall, the extracellular proteins of *Trichoderma* exhibit enzymatic activities that are vital for their biological functions and industrial applications.

The results of enzyme activity are presented in Table 2. The isolates showed statistically significant differences at $p<0.05$. The highest xylanase activity was obtained in the M82 mutant isolate (5.61 ± 0.48 U/mL), which was approximately 3.3 times higher than the wild-type, and other mutant isolates. The results showed that the induced mutation in M82 mutant isolate has led to the development of a xylanase hyper-producing system, further increasing xylanase yields for efficient biomass degradation.The results of cellulase activity are shown as international units (U). One unit of cellulase activity (Exo-, endo-glucanase, β-glucosidase, and total cellulase) is definite as the amount of enzyme that liberates reducing sugars equivalent to 1.0 µmol of glucose for a 1-hour reaction.

The enzyme system for conversion of cellulose to glucose involves at least three types of cellulases including exo-glucanase, endo-glucanase, and β-glucosidase. Cellobiohydrolases (CBH) act as exo-glucanases to release the disaccharide cellobiose. The highest exo-glucanase activity (U/mL) was obtained in

mutant isolates of M82, and M44, respectively. These results showed that these mutant isolates, probably have a high ability to decompose crystalline regions of lignocellulosic materials. Exo-glucanase activity in the mutant isolate of M82 was approximately 1.78 times higher than the wild-type strain. Exo-glucanases or cellobiohydrolases (CBH I and II) cleave the accessible reducing and non-reducing wheat bran cellulose chain ends to liberate cellobiose and glucose molecules (Zhang and Lynd, 2004).Also, the highest endo-glucanase (EG) activity was observed in mutant isolates of M82, and M69, respectively. EGs can randomly hydrolyze internal glycosidic bonds in cellulosic cell walls of wheat bran, especially in the amorphous regions of the cellulose chain. EG activity in the mutant isolate of M82 was approximately 1.48 times higher than the wild-type strain. Also, the mutant isolates of M62, M73, M72, M86, and M82 showed high β-glucosidase activity compared to other isolates, respectively.

Overall, the total cellulase system contains exo-glucanases, endo-glucanases, and β-D-glucosidases, all of which hydrolyze the crystalline cellulosic cell wall of wheat bran synergistically. The most commonly studied type of synergy is between EGs and exo-glucanases, and it is a quantitative necessity for the hydrolysis of crystalline cellulose. The highest FPase or total cellulase activity (U/mL) was found in mutant isolates of M82. However, the mutant isolate of M82 could be the best superior mutant of cellulase producer, because of its higher cellulase activity. A higher level of total cellulase activity in M82 isolate could be attributed to its higher exo-glucanase activity and EG activity compared to other mutant isolates and the synergistic action of these enzymes on the cellulose chain (Table 2). Studies have shown that the synergistic interaction between endo- and exocellulases enhances the de-polymerization of cellulose chains, leading to improved bioconversion efficiency (Zajki-Zechmeister et al., 2022). Additionally, the supplementation of β-glucosidases (BG) in cellulase cocktails has been found to significantly enhance synergism, resulting in higher hydrolysis yields and improved recyclability of enzymes during the saccharification process (Fernandes et al., 2023, 2022). Understanding the degree of synergism thresholds between different cellulolytic enzymes is essential for formulating effective enzyme cocktails for biomass degradation, as not all interactions between endo- and exo-cellulases are synergistic, highlighting the importance of selecting compatible enzyme combinations for optimal cellulose hydrolysis (Mafa et al., 2021; Zajki-Zechmeister et al., 2022).

The electrophoretic pattern of proteins

The variation and molecular weight distribution of enzyme-rich proteins in different superior mutants are shown in Figure 4 (a, b). The presence of diverse protein groups within the range of 11 to 135 kilodaltons (kDa), indicates the diversity of enzymes with various molecular weights (Mw), which can contribute to the hydrolyzation of wheat bran cell wall compounds. The proteins with the molecular weight of 17, 21, 32, and 43 kDa belong to a group of enzymes called xylanase (Xyl I, II, III, and IV, respectively) (Lappalainen et al., 2000; Parkkinen et al., 2004; Tenkanen et al., 1992; Torronen, 1997; Xu et al., 1998). The most Xyl I (17 kDa) was observed in the TFM supernatant of M76, and M11 mutant isolates, respectively. However, the higher xylanase activity in M82 (Table 2) may be due to the presence of the Cel12A enzyme, which, besides acting in the cellulose chain as a endoglucanase, has acted against beta-1,3-glucan and beta-1,4-xylan hydrocolloids (Hayn et al., 1993; Karlsson et al., 2002). The results showed that the Cel12A protein band with a weight of 26 kDa was highest in the M82 isolate, and this was confirmed by testing the xylanase enzyme activity. Also, the highest concentration of xylanase IV (43 kDa) was observed in M17, M73, M44, M11, M49, M51, M89, M51, M82, M69, and M86 mutant isolates, respectively.

Previous studies have shown that the *Trichoderma* species can be produced at least two cellobiohydrolases, Cel6A (CBH II, Mw 56-62 kDa), and Cel7A (CBH I, Mw 66 kDa; EC 3.2.1.91) (Teeri et al., 1983, 1987), and five endo-glucanases, Cel5A (EG II, Mw 48 kDa) (Saloheimo et al., 1988), Cel7B (EG I, Mw 50-55 kDa) (Penttilä et al., 1986), Cel12A (EG III, Mw 25 kDa) (Okada et al., 1998; Ward et al., 1993), Cel45A (EG V, Mw 23 kDa)(Saloheimo et al., 1994), and Cel61A (EG IV, Mw 34 kDa; EC 3.2.1.4)(Saloheimo et al., 1997).

Trichoderma mutant isolates of M82 (lane 16), M76 (lane 13), M49 (lane 6), M44 (lane 5), and M17 (lane 3) had two exo-glucanase enzyme bands called cellubiohydrolase I and II (CBH I and CBH II). The highest optical density of CBH protein bands (CBH I and II) was observed in mutant isolates M82. Also, the highest secreted proteins of CBH II (62 kDa) and Cel5A (EG II, 48 kDa) were observed in the mutant isolate M82 (Figure 4b), as confirmed by the results obtained from exo-glucanase and endo-glucanase enzyme activity, respectively (Table 2).

The Cel3A protein band, also known as BGLI, with a molecular weight of 73 kilodaltons, was present in the profiles of M82 mutant isolate of *Trichoderma*. Also, Cel1A, also known as BGLII,

has a molecular weight ranging from about 95-105 kDa. This enzyme was observed only in the protein profile of M11, M49, M51, M14, M79, M80, M82, M86, M87 mutant isolates.

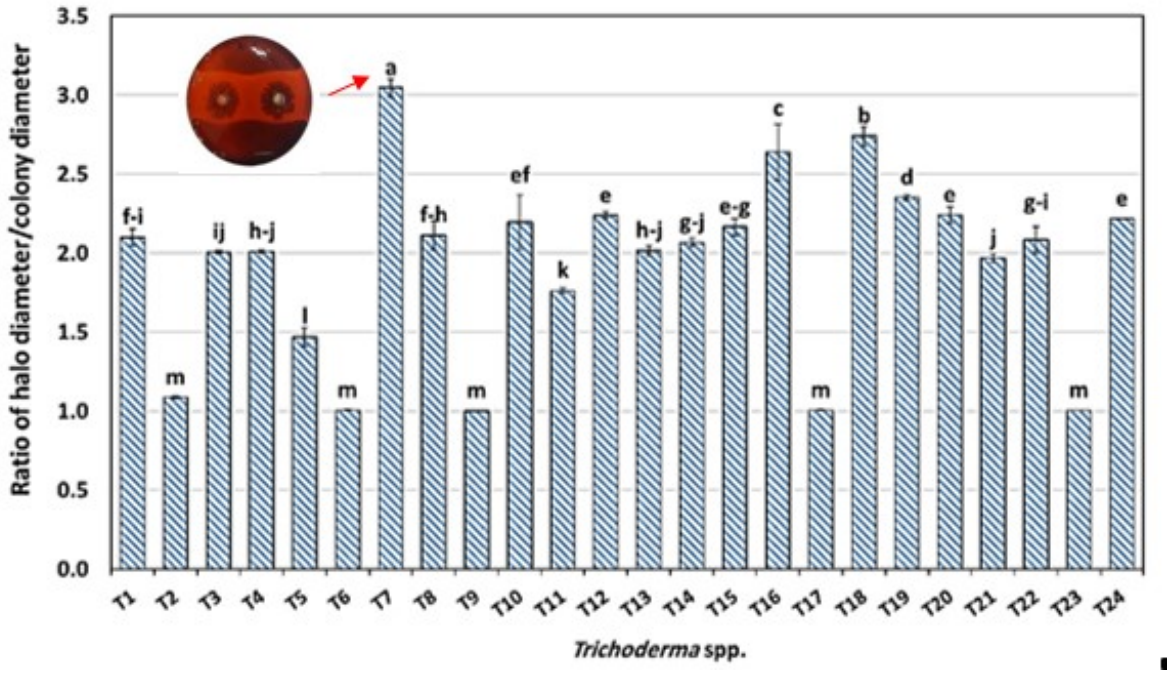

Fig 2. Qualitative evaluation of different *Trichoderma* isolates for their xylanase activity using the plate screening methods. T1-T24: different wild type *Trichoderma* isolates. Different letters in each column indicate a statistically significant difference between different *Trichoderma* isolates.

Table 1. Maximum hydrolytic capacity (HC) value of *Trichoderma* mutant isolates on plate screening media.

Strains	Hydrolysis capacity (HC)	Ratio $HC_{Mutant}/HC_{Wild\ type}$
Wild type	$3.34^{h} \pm 0.02$	$1.00^{l} \pm 0.00$
M 10	$4.37^{e} \pm 0.00$	$1.31^{gh} \pm 0.01$
M 11	$4.24^{f} \pm 0.00$	$1.27^{i} \pm 0.01$
M 17	$4.88^{bc} \pm 0.05$	$1.46^{c} \pm 0.02$
M 44	$4.79^{cd} \pm 0.03$	$1.44^{d} \pm 0.02$
M49	$4.46^{e} \pm 0.01$	$1.34^{f} \pm 0.01$
M 51	$4.45^{e} \pm 0.00$	$1.33^{fg} \pm 0.01$
M 56	$5.25^{a} \pm 0.04$	$1.57^{a} \pm 0.02$
M 62	$4.85^{c} \pm 0.00$	$1.45^{cd} \pm 0.01$
M 69	$3.60^{g} \pm 0.02$	$1.08^{k} \pm 0.00$
M 72	$4.37^{e} \pm 0.05$	$1.31^{gh} \pm 0.01$
M 73	$4.42^{e} \pm 0.02$	$1.32^{f-h} \pm 0.00$
M 76	$4.12^{f} \pm 0.01$	$1.23^{j} \pm 0.01$
M 79	$4.70^{d} \pm 0.01$	$1.41^{e} \pm 0.01$
M 80	$4.35^{e} \pm 0.01$	$1.30^{h} \pm 0.01$
M 82	$4.45^{e} \pm 0.02$	$1.33^{fg} \pm 0.01$
M 86	$4.16^{f} \pm 0.03$	$1.25^{j} \pm 0.02$
M 87	$2.86^{i} \pm 0.01$	$0.86^{m} \pm 0.01$
M 89	$4.91^{bc} \pm 0.04$	$1.47^{c} \pm 0.02$
M 114	$5.00^{b} \pm 0.05$	$1.50^{b} \pm 0.02$

* Different letters in each column indicate a statistically significant difference ($p<0.05$).

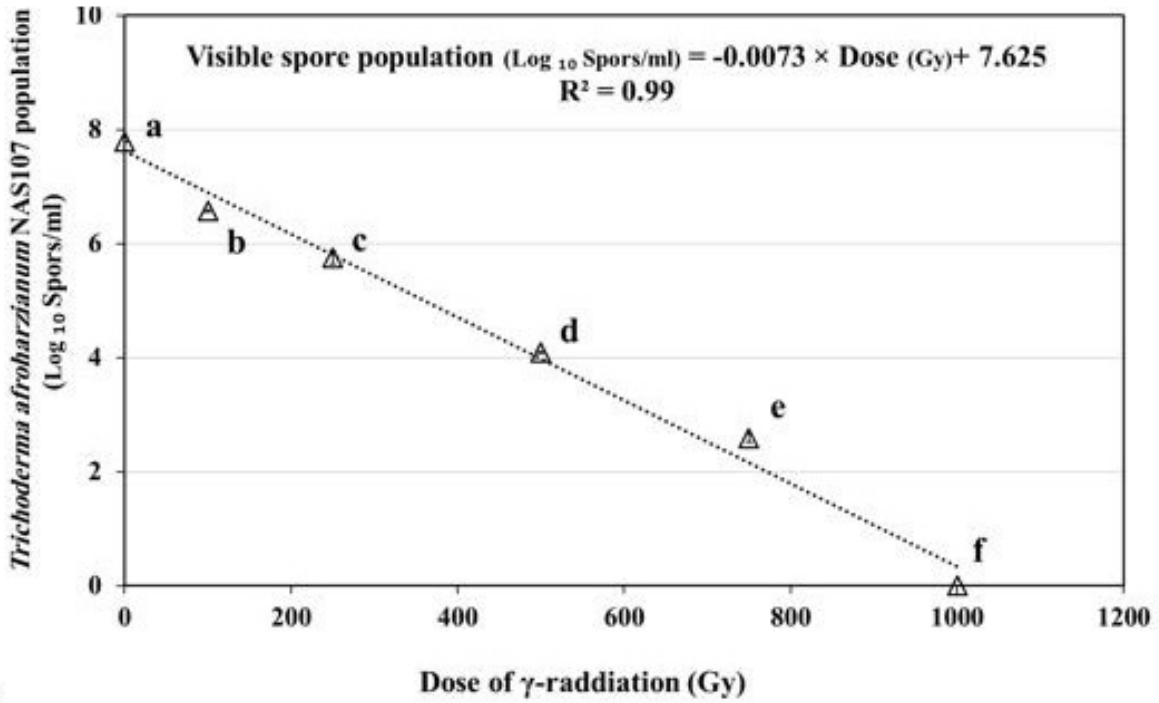

Fig 3. Effect of gamma radiation doses on the spore viable population of *Trichoderma afroharzianum* NAS107

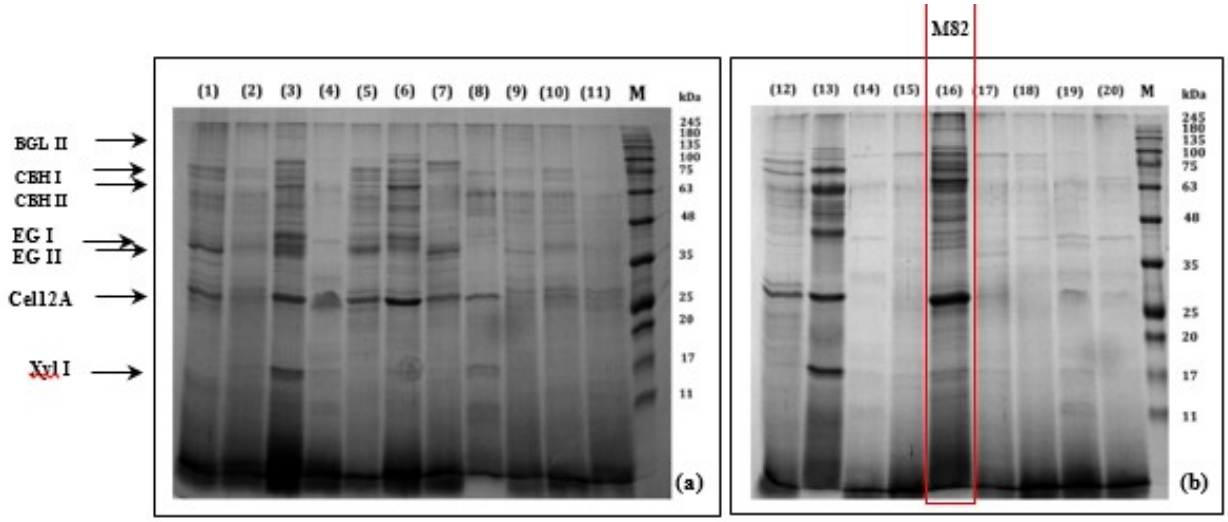

Fig 4. Electrophoretic pattern (SDS-PAGE) of extracellular proteins of the wild-type and different superior mutant isolates: a:1-11 indicates wild-type strain T. afroharzianum NAS107 and M10, M11, M17, M44, M49, M51, M56, M62, M72, and M73 isolates respectively. b: 12-14 indicates the wild-type strain T. afroharzianum NAS107 and M76, M79, M80, M82, M86, M87, M89, and M114, respectively. (M) Protein molecular weight marker in the range of 11-245 Kda.

Molecular identification of the best *Trichoderma* strain and its mutant isolates

Molecular identification of *Trichoderma afroharzianum* involves utilizing genetic markers like actin, calmodulin, rDNA (ITS), and translation elongation factor 1-α (tef1-α) for accurate species differentiation (Hussein and Saadullah, 2023; Marques et al., 2022). DNA extraction was performed from wild-type strain T7 and its superior mutants of M82 and

M44. The quantity and quality of extracted DNA were examined by spectrophotometry and agarose gel electrophoresis (Figure 5-a). PCR amplification of the the genomic regions, (ITS-rDNA and TEF1-α) produced amplicon sizes of 650 and 350 bp, respectively (Figure 5-b, c). The forward sequence of PCR products (ITS-rDNA and TEF-1α) were registered in the GenBank database at the National Center for Biotechnology Information (NCBI) and the accession numbers are presented in their phylogenetic tree.

Using bioinformatics tools, *T. afroharzianum* NAS107 was identified for *Trichoderma* sp. T7 and its mutant isolate and the phylogenetic relationship of our *Trichoderma* and other isolates are shown in Figure 6. We studied the sequence of ITS-rDNA/TEF1-α regions in our *Trichoderma* strains and compared them to six other groups of *Trichoderma* using the MEGA 10.0 software and maximum likelihood method at 1000 replications for each bootstrap. While this number characterizes only a few percent of the *Trichoderma* species designated today, the isolates selected are included in the phylogenetic clade Harzianum.

The superior isolate of *Trichoderma* T7 isolate and its mutant isolate of M82 were deposited in the microbial collection of Iran Biological Resources Center (IBRC) with the accession numbers IBRC-M 30594 (*T. afroharzianum* NAS107), and IBRC-M 30594 (*T. afroharzianum* NAS107-M82). The molecular characterization of *Trichoderma* species, like *T. afroharzianum* NAS107, through ITS-rDNA and TEF1-α sequencing, provides valuable insights into their diversity, behavior, and potential applications in biological control and enzyme production.

Trichoderma afroharzianum, known for its mycoparasitic and biocontrol abilities, has been studied in various regions, including Iran. Research in Iran has revealed the presence of *T. afroharzianum* alongside other *Trichoderma* species like *T. harzianum*, *T. virens*, *T. brevicompactum*, and *T. longibrachiatum* in different habitats such as pistachio orchards (Mirkhani and Alaei, 2015; Naeimi et al., 2011). Phylogenetic analyses have positioned *T. afroharzianum* within the genus *Trichoderma*, showing a close relationship with *T. harzianum*, *T. simmonsii*, and *T. lixii* (Özkale et al., 2024; Özkale kaya et al., 2022). Furthermore, the characterization of the mitochondrial genome of *T. afroharzianum* has contributed to understanding the phylogeny and evolution of the order *Hypocreales*, confirming its place within this fungal order (Özkale et al., 2024). These findings highlight the diversity and evolutionary relationships of *Trichoderma* species, including *T. afroharzianum*, in different geographic regions like Iran.

CONCLUSIONS

A complex of extracellular enzymes is secreted by *Trichoderma*, which is one of the most well-known filamentous fungi for the degradation of wheat bran waste and other hemicellulose materials. Various organisms with different characteristics produce xylanase-cellulase complexes that are utilized in industrial applications, such as in the food and baking industry. We evaluated different isolates of *Trichoderma* for their hydrolysis potential of arabinoxylan compounds and wheat bran through quantitative and qualitative screening. Among them, the Trichoderma isolate T7 was selected as the best wild-type strain.

Traditionally, morphological identification was used for *Trichoderma* phenotypic and taxonomic studies. New types of *Trichoderma* have been discovered, but they look very similar and it is hard to tell them apart just by their morphology. For instance, *Trichoderma* types usually grow quickly and make many branchy conidiophores. They also produce cylindrical to nearly subglobose structures called phialides and ellipsoidal, and globose spores called conidia. Furthermore, its appearance properties may be altered under various environmental conditions (Samuels et al., 2002). Therefore, studying DNA sequences has helped us to classify and understand a new way of how fungi evolve and are related to one another. Thus, DNA extraction was performed from wild-type strain T7 and its superior mutants of M82 and M44, and was identified using ITS-rDNA and TEF1-α sequences as *T. afroharzianum*. Sequence analysis of the ITS-rDNA region and TEF-1α is widely used for the precise reorganization of *Trichoderma* species (Druzhinina et al., 2005).

Efficient mutation or improvement of microbial strains is a fundamental requirement for any commercial fermentation process of enzymes. In most cases, mutation influences the overall economics of enzyme production because it allows an organism to carry out a biochemical process more efficiently.

Gamma radiation was used to induce mutations in the best wild-type isolate (*T. afroharzianum* NAS107) to generate mutants that produce more xylanase-cellulase. Although there are numerous studies on the application of gamma radiation to an induced mutation in other microorganisms, this is the first time that *T. afroharzianum* mutants were produced using gamma radiation mutagenesis. Gamma radiation can lead to deactivation by destroying the DNA structure of microorganisms (Smith and Pillai, 2004), while sometimes incomplete inhibition by less cellular alteration leads to the induced mutation of cells (Ross et al., 2003). *Trichoderma's* DNA can be directly affected

a high dose of gamma irradiation, leading to growth or reproduction inhibition (Tauxe, 2001). The DNA can be damaged and the functioning of the living organism can be affected by free radicals when ionizing beams interact with water particles in *Trichoderma* cells. Moreover, other factors such as the number or density of spores, can also affect the sensitivity or tolerance of *Trichoderma* to gamma radiation (Mobini-Dehkordi et al., 2008; Zhao et al., 2022).

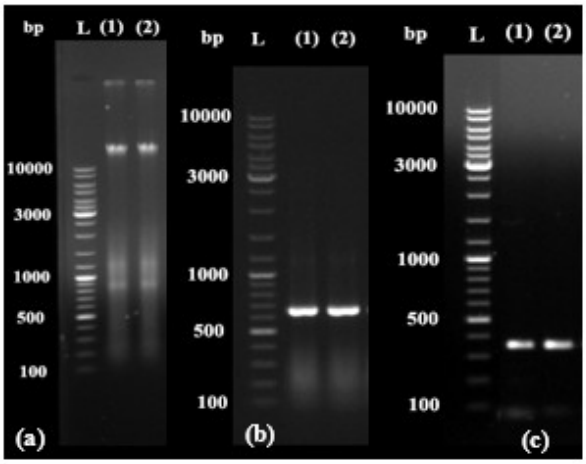

Fig 5. Garose gel electrophoresis of DNA extraction (a), PCR products amplification of the fungal isolates: The internal transcribed spacer (ITS) regions of the ribosomal DNA (b), and the translation elongation factor 1-α encoding gene (TEF1-α) (c); L: Molecular weight ladder (100 bp-10 kb, Thermo Scientific™ O'RangeRuler DNA Ladder); (1, and 2): DNA or PCR product of the *Trichoderma* sp. T7 and its mutant isolate NAS107-M82, respectively.

Our results showed that, statistically significant changes in xylanase-cellulase production of *T. afroharzianum* NAS107 after irradiated treatment were influenced by doses of the ray. Similarly, the study of Gherbawy exhibited that the low dose of gamma irradiation (1 MCi for 10 min), improved the virulence of *Aspergillus niger* by more polygalacturonase, cellulase, and protease production, while the high dose of radiation (1 MCi for 20 min and 30 min) was inhibited fungi growth (Gherbawy, 1998). Our investigation revealed that the mutants exposed to gamma radiation had increased levels of xylanase and cellulase activity compared to the wild-type strain.

The research conducted on various fungi and bacteria exposed to gamma radiation consistently demonstrated an increase in enzyme activity, particularly xylanase and cellulase, in mutant strains compared to the wild types. Studies on *Trichoderma* mutants showed enhanced production of chitinase and cellulase enzymes, crucial for biological control of plant diseases (Ghasemi et al., 2019; Sahu et al., 2023). Similarly, investigations on filamentous fungi exposed to gamma irradiation revealed mutants with significantly higher cellulase secretion, leading to improved cellulose hydrolysis capacity (Diep et al., 2020). These findings collectively support the notion that gamma radiation-induced mutagenesis can effectively enhance xylanase and cellulase activity in various fungal and bacterial strains, offering potential applications in bioprocessing and biocontrol strategies.

The highest extracellular enzyme activity was observed in mutant isolates of *T. afroharzianum* NAS107-M82, in which its xylanase and cellulase activities were approximately 3.3 times higher than wild-type strain. In this mutant isolate, exo-glucanase (CBH I, cell 7A), endo-glucanase (EG III, Cel 12A), and xylanase (Xyl I) hydrolyzed the wheat bran, synergistically.

Different strains of *Trichoderma* have xylanase activity, which is influenced by xylanases I, II, III, and IV, as well as cellulases that digest xylan. Xylanases I and II are small proteins that belong to a group of enzymes called Family 11 of glycosyl hydrolases. They weigh around 17 and 20 kDa and have pI 5.5 and 9, respectively (Tenkanen et al., 1992; Torronen, 1997). Xylanase III (Mw of 32 kDa and pI 9.1) belongs to the Family 10 glycosyl hydrolase and was first identified in *T. reesei* PC-3-7. Xylanase I works best at a pH of 4.0-4.5, while xylanase II works best at a pH between 4.0 and 6.0. The optimum pH for xylanases III and IV (pI 7.0, 43 kDa) activity was observed at pH 6.0–6.5 and 3.5–4.0, respectively (Zhi et al., 1999). According to the results of Table 2, the mutant isolate of NAS107-M82 in this study could be the superior mutant that can produce xylanase, with high specific activity, and optimal temperature and pH of 45ºC and 5, respectively.

The entire group of enzymes that break down the cellulose structure of wheat bran is called the cellulase system. This system includes CBHes, EGes, and cellobiases, which work synergically to break down the wheat bran. Synergism between endo-glucanases and exo-glucanases is the foremost type of synergy, which was observed in *T. afroharzianum* NAS107 (or T7 isolate) and its mutant isolates; and it is among the foremost quantitatively imperative for hydrolysis cellulose. Also, wheat bran cellulose crystallinity plays a critical role in its enzymatic hydrolysis. The presence of amorphous and crystalline regions in wheat bran cellulose structure induces the *T. afroharzianum* NAS107 to produce endo-glucanases and exo-glucanases, to act synergistically with each other. In addition to substrate properties, fermentation conditions

and potential mutant isolates for enzyme production also affected the extent of synergy observed. In *T. afroharzianum* NAS107-M82, we observed that the gamma radiation-induced mutation led to the creation of a mutant isolate, could produce a high amount of exo-glucanase and endo-glucanase enzymes, and led to synergy in total cellulase activity. Based on the SDS-PAGE profile of proteins (Fig. 4), it was determined that *T. afroharzianum* NAS107-M82 has both enzyme bonds of CBH I and CBH II. *Trichoderma afroharzianum* NAS107-M82 had a high concentration of exo-glucanase activity because of the high level of cellobiohydrolases being secreted.

The activity of CBH I and CBH II results in a gradual decrease in the degree of polymerization (DP) of wheat bran cellulose and produces cellobiose. This mutant isolate had the highest production of CBH II and Cel5A (EG II) proteins, leading to maximum synergy of total cellulase. Comparing cellulase activity in different mutant isolates, especially *T. afroharzianum* NAS 107-M82, showed that optimal wheat bran degradation is usually obtained with a large amount of exo-enzyme and a minor amount of endo-enzyme (Zhang and Lynd, 2006). The synergistic action between CBH and or 'endo-exo' synergistic model causes to high production of small oligosaccharides and mainly cellobiose. Initially, internal β-1, 4-glycosidic bonds in the chains at the surface of the wheat bran cellulosic fibers randomly hydrolyzed and thereby free reduced and non-reduced chain ends for hydrolysis by CBH I and II produced. The superiority of cellulase activity in *T. afroharzianum* NAS107-M82 is due to the presence of CBH and EG enzymes and their cooperative behavior or synergism of them. Wheat bran is a suitable substrate for *T. afroharzianum* NAS107-M82 to produce xylanase-cellulase, as indicated by the enzyme activity results.

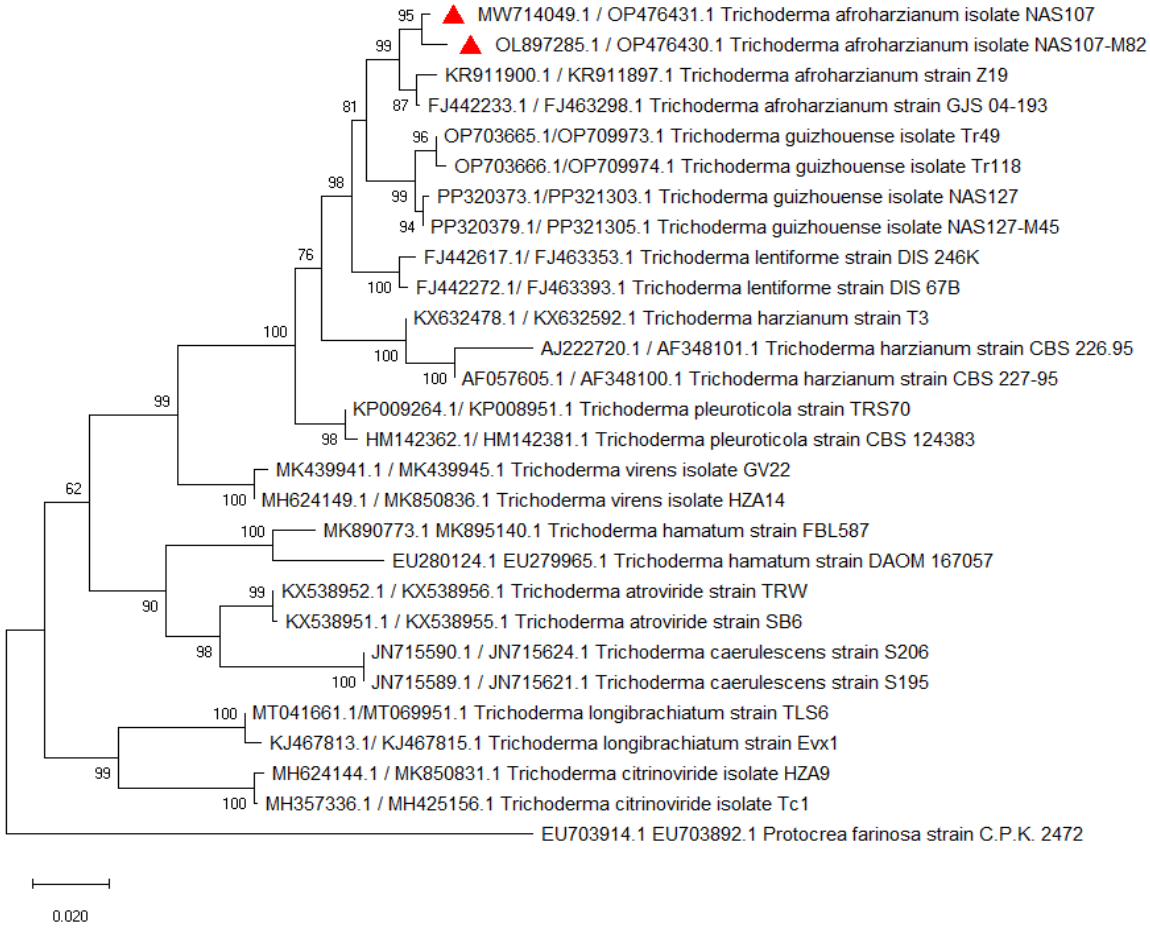

Fig 6. Phylogenetic relationship of *Trichoderma afroharzianum* NAS107 and its mutant of *T. afroharzianum* NAS107-M82 using the internal transcribed spacer (ITS) and the translation elongation factor 1-α encoding gene (TEF1-α) regions gene nucleotide sequence alignment using maximum likelihood method at 1000 replications for each bootstrap value using the MEGA 10.0, symbols in red triangle.

Table 2. Extracellular protein concentration (µg/ml) and enzyme activity (U/ml) of different mutant isolates of *Trichoderma* in the supernatant of the TFM containing wheat bran as a substrate after 72 h fermentation at 28ºC and 150 rpm.

Strains	Soluble Protein (µg/ml)	Enzyme activity (U/ml)				
		Xylanase	Exo-glucanase	Endo-glucanase	β-glucosidase	Total cellulase
W. T.*	87.89 ± 3.77 d-f	1.70 ± 0.06 ij	1.04 ± 0.01 b-d	0.79 ± 0.04 b-d	0.45 ± 0.00 l	2.24 ± 0.40 b-d
M 10	78.95 ± 6.65 gh	2.00 ± 0.04 hi	0.82 ± 0.02 de	0.60 ± 0.08 gh	3.00 ± 0.60 e	1.98 ± 0.36 c-e
M 11	116.84 ± 6.64 b	5.18 ± 0.14 b	0.82 ± 0.05 de	0.79 ± 0.11 b-d	1.89 ± 0.22 gh	1.97 ± 0.05 c-e
M 17	56.97 ± 1.94 j	1.63 ± 0.16 j	0.73 ± 0.07 de	0.68 ± 0.00 d-g	1.59 ± 0.15 hi	1.60 ± 0.05 d-g
M 44	81.58 ± 0.00 f-h	3.04 ± 0.06 ef	1.27 ± 0.06 b	0.66 ± 0.02 e-h	2.25 ± 0.18 fg	2.69 ± 0.05 b
M49	115.00 ± 0.71 b	2.60 ± 0.17 g	1.17 ± 0.70 bc	0.83 ± 0.01 bc	0.78 ± 0.03 kl	2.70 ± 0.18 b
M 51	93.42 ± 4.77 c-e	2.06 ± 0.25 h	0.89 ± 0.05 c-e	0.72 ± 0.08 c-f	2.07 ± 0.15 fg	1.84 ± 0.03 c-f
M 56	93.16 ± 4.71 c-e	3.87 ± 0.43 d	0.75 ± 0.05 de	0.73 ± 0.05 c-e	2.44 ± 0.01 f	1.92 ± 0.00 c-f
M 62	84.21 ± 0.53 fg	4.63 ± 0.24 c	0.69 ± 0.09 c-e	0.49 ± 0.04 i	6.04 ± 0.59 a	1.29 ± 0.17 fg
M 69	88.16 ± 1.32 d-f	5.21 ± 0.51 b	0.80 ± 0.02 de	0.87 ± 0.07 b	3.21 ± 0.05 e	2.13 ± 0.75 b-e
M 72	93.29 ± 7.49 c-e	2.94 ± 0.23 e-g	0.90 ± 0.08 de	0.56 ± 0.01 hi	4.88 ± 0.32 c	1.53 ± 0.04 e-g
M 73	94.47 ± 9.38 cd	1.72 ± 0.05 ij	0.74 ± 0.01 de	0.61 ± 0.00 f-h	5.42 ± 0.13 b	1.96 ± 0.04 c-e
M 76	75.13 ± 7.05 h	2.70 ± 0.18 fg	0.81 ± 0.01 de	0.66 ± 0.05 e-h	1.37 ± 0.05 ij	1.65 ± 0.19 d-g
M 79	86.45 ± 9.70 ef	2.87 ± 0.31 fg	0.75 ± 0.07 de	0.73 ± 0.12 c-e	4.89 ± 0.31 c	1.52 ± 0.19 e-g
M 80	78.95 ± 3.33 gh	2.23 ± 0.07 h	0.75 ± 0.03 de	0.60 ± 0.04 gh	1.85 ± 0.07 gh	1.92 ± 0.06 c-f
M 82	185.00 ± 0.26 a	5.61 ± 0.48 a	1.81 ± 0.12 a	1.17 ± 0.10 a	3.28 ± 0.19 e	4.68 ± 1.12 a
M 86	87.89 ± 9.20 d-f	2.68 ± 0.14 g	0.63 ± 0.02 e	0.57 ± 0.02 g-i	4.28 ± 0.44 d	1.63 ± 0.08 d-g
M 87	97.89 ± 1.41 c	3.24 ± 0.41 e	1.22 ± 0.11 b	0.80 ± 0.05 bc	0.95 ± 0.06 jk	2.33 ± 0.12 bc
M 89	67.11 ± 6.13 i	3.70 ± 0.05 d	0.74 ± 0.01 de	0.60 ± 0.06 gh	1.06 ± 0.08 jk	1.11 ± 0.07 g
M 114	81.58 ± 1.41 f-h	4.78 ± 0.49 c	0.81 ± 0.10 de	0.73 ± 0.09 c-e	0.81 ± 0.08 kl	1.59 ± 0.26 d-g

* Wild type

** Different letters in each column indicate a statistically significant difference ($p<0.05$).

Finally, the results display the possibility of improving the *T. afroharzianum* for xylanase-cellulase production through mutation with gamma radiation. In simple words: Gamma irradiation has the potential to generate beneficial mutations in fungi, which could result in improved industrial varieties. According to our study, a mutant isolate of *T. afroharzianum* NAS 107-M82 can be a successful extracellular enzyme producer candidate for the bioconversion of wheat bran wastes into xylanase-cellulase enzymes. Also, this study highlighted the significance of wheat bran as a substrate for *Trichoderma* fermentation in producing valuable extracellular proteins like cellulases and xylanase. Overall, gamma radiation emerges as a promising tool for strain improvement across various organisms, offering enhanced traits and productivity. In other fungi, this technique can be utilized to create new mutants with various uses.

ACKNOWLEDGMENTS

This study was supported by Project No. TGP-A-00-001 from the Nuclear Science and Technology Research Institute (NSTRI), Atomic Energy Organization of IRAN (AEOI).

REFERENCES

Abbasi, S., Safaie, N., Shams-Bakhsh, M., Shahbazi, S., 2016. Biocontrol activities of gamma induced mutants of *Trichoderma harzianum* against some soilborne fungal pathogens and their DNA fingerprinting. Iran. J. Biotechnol. 14, 260–269. https://doi.org/10.15171/ijb.1224

Barreiro, C., Martín, J.F., García-Estrada, C., 2012. Proteomics shows new faces for the old penicillin producer *Penicillium chrysogenum*. J. Biomed. Biotechnol. 2012, 105–109. https://doi.org/10.1155/2012/105109

Bischof, R.H., Ramoni, J., Seiboth, B., 2016. Cellulases and beyond: The first 70 years of the enzyme producer *Trichoderma reesei*. Microb. Cell Fact. 15, 1–13.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Catlett, N.L., Lee, B.-N., Yoder, O.C., Turgeon, B.G., 2003. Split-marker recombination for efficient targeted deletion of fungal genes. Fungal Genet. Rep. 50, 9–11.

Diep, T.B., Thi Thom, N., Dang Sang, H., Xuan An, T., Van Binh, N., Minh Quynh, T., 2020. Effect of gamma irradiation on the viability and cellulase production of some filamentous fungi. Vietnam J. Biotechnol. 18, 341–348.

Dillon, A.J.P., Zorgi, C., Camassola, M., Henriques, J.A.P., 2006. Use of 2-deoxyglucose in liquid media for the selection of mutant strains of *Penicillium echinulatum* producing increased cellulase and β-glucosidase activities. Appl. Microbiol. Biotechnol. 70, 740–746.

Doner, L.W., Hicks, K.B., 1997. Isolation of hemicellulose from corn fiber by alkaline hydrogen peroxide extraction. Cereal Chem. 74, 176–181.

Druzhinina, I.S., Kopchinskiy, A.G., Komoń, M., Bissett, J., Szakacs, G., Kubicek, C.P., 2005. An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. Fungal Genet. Biol. 42, 813–828.

FAOSTAT, F.A.O., 2023. Food and Agriculture Organization of the United Nations-Statistic Division https://www. fao. org/faost at/en/# data.

Fernandes, C.G., Sawant, S.C., Mule, T.A., Khadye, V.S., Lali, A.M., Odaneth, A.A., 2022. Enhancing cellulases through synergistic β-glucosidases for intensifying cellulose hydrolysis. Process Biochem. 120, 202–212.

Fernandes, C.G., Sawant, S.C., Mule, T.A., Khadye, V.S., Odaneth, A.A., 2023. Synergistic β-glucosidases for improving cellulases recyclability and biomass enzymatic saccharification in wheat straw. Biomass and Bioenergy 175, 106881.

Ghasemi, S., Safaie, N., Shahbazi, S., 2019. Enhancement of lytic enzymes activity and antagonistic traits of *Trichoderma harzianum* using γ-radiation induced mutation edible and medicinal mushrooms: genetic diversity and molecular systematics View project Manipulating the plant microbiome to disease. J. Agric. Sci. Technol. 21, 1035–1048.

Gherbawy, Y.A.M.H., 1998. Effect of gamma irradiation on the production of cell wall degrading enzymes by *Aspergillus niger*. Int. J. Food Microbiol. 40, 127–131.

Gohel, V., Megha, C., Vyas, P., Chhatpar, H.S., 2004. Strain improvement of chitinolytic enzyme producing isolate *Pantoea dispersa* for enhancing its biocontrol potential against fungal plant pathogens. Ann. Microbiol. 54, 503–515.

Gooruee, R., Hojjati, M., Behbahani, B.A., Shahbazi, S., Askari, H., 2024. Extracellular enzyme production by different species of *Trichoderma* fungus for lemon peel waste bioconversion. Biomass Convers. Biorefinery 14, 2777–2786.

Hayn, M., Steiner, W., Klinger, R., Steinmüller, H., Simer, M., Esterbauer, H., 1993. Basic research and pilot studies on the enzymatic conversion of lignocellulosics. Bioconversion For. Agric. Plant Residues 33–72.

Hidayati, F.L.N., Sardjono, Giyatmi, Cahyanto, M.N., 2021. Enhancement of Indigenous Fungal Cellulase Production by Gamma Rays, in: IOP Conference Series: Materials Science and Engineering. IOP

Publishing, p. 012004. https://doi.org/10.1088/1757-899x/1192/1/012004

Hussein, L.F., Saadullah, A.A.M., 2023. DNA Marker Identification of *Trichoderma* and *Fusarium* Level Species, in: IOP Conference Series: Earth and Environmental Science. IOP Publishing, p. 12175.

Javed, S., Asgher, M., Sheikh, M.A., Nawaz, H., 2010. Strain improvement through UV and chemical mutagenesis for enhanced citric acid production in molasses-based solid state fermentation. Food Biotechnol. 24, 165–179.

Karanam, S.K., Medicherla, N.R., 2008. Enhanced lipase production by mutation induced *Aspergillus japonicus*. African J. Biotechnol. 7, 2064–2067.

Kardos, N., Demain, A.L., 2011. Penicillin: The medicine with the greatest impact on therapeutic outcomes. Appl. Microbiol. Biotechnol. 92, 677–687. https://doi.org/10.1007/s00253-011-3587-6

Karlsson, J., Siika-Aho, M., Tenkanen, M., Tjerneld, F., 2002. Enzymatic properties of the low molecular mass endoglucanases Cel12A (EG III) and Cel45A (EG V) of *Trichoderma reesei.* J. Biotechnol. 99, 63–78.

Köhnke, T., Lund, K., Brelid, H., Westman, G., 2010. Kraft pulp hornification: A closer look at the preventive effect gained by glucuronoxylan adsorption. Carbohydr. Polym. 81, 226–233.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35, 1547–1549.

Kunamneni, A., Plou, F.J., Alcalde, M., Ballesteros, A., 2014. *Trichoderma* Enzymes for Food Industries, in: Biotechnology and Biology of Trichoderma. Elsevier, pp. 339–344.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lappalainen, A., Siika□Aho, M., Kalkkinen, N., Fagerström, R., Tenkanen, M., 2000. Endoxylanase II from *Trichoderma reesei* has several isoforms with different isoelectric points . Biotechnol. Appl. Biochem. 31, 61–68.

Lee, Y.K., Chang, H.H., Kim, J.S., Kim, J.K., Lee, K.S., 2000. Lignocellulolytic mutants of *Pleurotus ostreatus* induced by gamma-ray radiation and their genetic similarities. Radiat. Phys. Chem. 57, 145–150.

Mach, R.L., Zeilinger, S., 2003. Regulation of gene expression in industrial fungi: *Trichoderma*. Appl. Microbiol. Biotechnol. 60, 515–522.

Mafa, M.S., Pletschke, B.I., Malgas, S., 2021. Defining the frontiers of synergism between cellulolytic enzymes for improved hydrolysis of lignocellulosic feedstocks. Catalysts 11, 1343.

Marques, E., DE OLIVEIRA, D.R., Santos, F.H.C., DE CASTRO, K.H.M., Silva, M.R., Abreu, V.P., DA CUNHA, M.G., 2022. Antagonism and molecular identification of *Trichoderma* isolated from rhizosphere of medicinal plants. J. Biol. Control 36, 7–16.

Mirkhani, F., Alaei, H., 2015. Species diversity of indigenous *Trichoderma* from alkaline pistachio soils in Iran. Mycol. Iran. 2, 22–37.

Mobini-Dehkordi, M., Nahvi, I., Zarkesh-Esfahani, H., Ghaedi, K., Tavassoli, M., Akada, R., 2008. Isolation of a novel mutant strain of *Saccharomyces cerevisiae* by an ethyl methane sulfonate-induced mutagenesis approach as a high producer of bioethanol. J. Biosci. Bioeng. 105, 403–408.

Naeimi, S., Khodaparast, S., Javan-Nikkhah, M., Vágvölgyi, C., Kredics, L., 2011. Species pattern and phylogenetic relationships of *Trichoderma* strains in rice fields of Southern Caspian Sea, Iran. Cereal Res. Commun. 39, 560–568. https://doi.org/10.1556/CRC.39.2011.4.11

Okada, H., Tada, K., Sekiya, T., Yokoyama, K., Takahashi, A., Tohda, H., Kumagai, H., Morikawa, Y., 1998. Molecular characterization and heterologous expression of the gene encoding a low-molecular-mass endoglucanase from *Trichoderma reesei* QM9414. Appl. Environ. Microbiol. 64, 555–563.

Onipe, O.O., Jideani, A.I.O., Beswa, D., 2015. Composition and functionality of wheat bran and its application in some cereal food products. Int. J. Food Sci. Technol. 50, 2509–2518.

Ottenheim, C., Werner, K.A., Zimmermann, W., Wu, J.C., 2015. Improved endoxylanase production and colony morphology of *Aspergillus niger* DSM 26641 by γ-ray induced mutagenesis. Biochem. Eng. J. 94, 9–14.

Özkale, E., Doğan, Ö., Budak, M., Korkmaz, E.M., 2024. Mitogenome evolution in *Trichoderma afroharzianum* strains: for a better understanding of distinguishing genus. Genome 67, 139–150.

Özkale kaya, E., Dogan, Ö., Budak, M., Korkmaz, E.M. 2022. An Insight into Mitochondrial Genomes of *Trichoderma afroharzianum* Strains: A Comparative and Evolutionary Analysis. SSRN Electron. J. https://doi.org/10.2139/ssrn.4139369

Parkkinen, T., Hakulinen, N., Tenkanen, M., Siika-aho, M., Rouvinen, J., 2004. Crystallization and preliminary X-ray analysis of a novel *Trichoderma reesei* xylanase IV belonging to glycoside hydrolase family 5. Acta Crystallogr. Sect. D Biol. Crystallogr. 60, 542–544.

Pauly, M., Albersheim, P., Darvill, A., York, W.S., 1999. Molecular domains of the cellulose/xyloglucan network in the cell walls of

higher plants. Plant J. 20, 629–639. https://doi.org/10.1046/j.1365-313X.1999.00630.x

Penttilä, M., Lehtovaara, P., Nevalainen, H., Bhikhabhai, R., Knowles, J., 1986. Homology between cellulase genes of *Trichoderma reesei*: complete nucleotide sequence of the endoglucanase I gene. Gene 45, 253–263.

Ribeiro, O., Magalhães, F., Aguiar, T.Q., Wiebe, M.G., Penttilä, M., Domingues, L., 2013. Random and direct mutagenesis to enhance protein secretion in *Ashbya gossypii*. Bioengineered 4, 322–331. https://doi.org/10.4161/bioe.24653

Ross, A.I.V., Griffiths, M.W., Mittal, G.S., Deeth, H.C., 2003. Combining nonthermal technologies to control foodborne microorganisms. Int. J. Food Microbiol. 89, 125–138.

Sahu, S., Mahilang, A., Agrawal, T., IRRI, U.S.S., Kotasthane, A.S., Zaidi, N.W., 2023. Gamma-ray Induced Putative Mutants derived from *Trichoderma atroviride* (T-14) Stimulating Plant Growth through Enhanced Siderophore production, Phosphate solubilisation, ACCd activity and IAA production.

Saloheimo, A., Henrissat, B., Hoffrén, A. □M, Teleman, O., Penttilä, M., 1994. A novel, small endoglucanase gene, egl5, from *Trichoderma reesei* isolated by expression in yeast. Mol. Microbiol. 13, 219–228.

Saloheimo, M., Lehtovaara, P., Penttilä, M., Teeri, T.T., Ståhlberg, J., Johansson, G., Pettersson, G., Claeyssens, M., Tomme, P., Knowles, J.K.C., 1988. EGIII, a new endoglucanase from *Trichoderma reesei*: the characterization of both gene and enzyme. Gene 63, 11–21.

Saloheimo, M., Nakari-SETÄLÄ, T., Tenkanen, M., Penttilä, M., 1997. cDNA cloning of a *Trichoderma reesei* cellulase and demonstration of endoglucanase activity by expression in yeast. Eur. J. Biochem. 249, 584–591.

Samuels, G.J., Dodd, S.L., Gams, W., Castlebury, L.A., Petrini, O., 2002. *Trichoderma* species associated with the green mold epidemic of commercially grown Agaricus bisporus. Mycologia 94, 146–170.

Shafei, M.S., Mohamed, T.A., Elsalam, I.S.A., 2011. Optimization of extracellular lipase production by *Penicillium chrysogenum* using factorial design. Malays. J. Microbiol. 7, 71–77.

Shahbazi, S., Askari, H., Mojerlou, S., 2016. The impact of different physicochemical parameters of fermentation on extracellular cellulolytic enzyme production by *Trichoderma harzianum*. J. Crop Prot. 5, 397–412.

Shahbazi, S., Ispareh, K., Karimi, M., Askari, H., 2014. Gamma and UV radiation induced mutagenesis in *Trichoderma reesei* to enhance cellulases enzyme activity. Int. J. Farming Allied Sci. 3, 543–554.

Smith, J.S., Pillai, S., 2004. Irradiation and food safety. Food Technol. 58, 48–55.

Steensels, J., Snoek, T., Meersman, E., Nicolino, M.P., Voordeckers, K., Verstrepen, K.J., 2014. Improving industrial yeast strains: Exploiting natural and artificial diversity. FEMS Microbiol. Rev. 38, 947–995.

Tamura, K., Nei, M., Kumar, S., 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc. Natl. Acad. Sci. U. S. A. 101, 11030–11035.

Tauxe, R. V., 2001. Food safety and irradiation: Protecting the public from foodborne infections. Emerg. Infect. Dis. 7, 516–521.

Teeri, T., Salovuori, I., Knowles, J., 1983. The molecular cloning of the major ceuulase gene from *Trichoderma reesei*. Bio/Technology 1, 696–699.

Teeri, T.T., Lehtovaara, P., Kauppinen, S., Salovuori, I., Knowles, J., 1987. Homologous domains in *Trichoderma reesei* cellulolytic enzymes: Gene sequence and expression of cellobiohydrolase II. Gene 51, 43–52.

Tenkanen, M., Puls, J., Poutanen, K., 1992. Two major xylanases of Trichoderma reesei. Enzyme Microb. Technol. 14, 566–574.

Torronen, A.K., 1997. The two major endo-1, 4-beta-xylanases from *Trichoderma reesei*: Characterization of both enzymes and genes.

Ward, M., Wu, S., Dauberman, J., Weiss, G., Larenas, E., 1993. Cloning, sequence and prelimary analysis of small, high pI endoglucanase (EGIII) from *Trichoderma reesei*, in: Trichoderma Reesei Cellulases and Other Hydrolases. Foundation of Biotechnical and Industrial Fermentation Research, pp. 153–158.

Xu, J., Takakuwa, N., Nogawa, M., Okada, H., Morikawa, Y., 1998. A third xylanase from *Trichoderma reesei* PC-3-7. Appl. Microbiol. Biotechnol. 49, 718–724.

Yopi, Tasia, W., Melliawati, R., 2017. Cellulase and xylanase production from three isolates of indigenous endophytic fungi, in: IOP Conference Series: Earth and Environmental Science. IOP Publishing, p. 12035.

Zajki-Zechmeister, K., Eibinger, M., Nidetzky, B., 2022. Enzyme synergy in transient clusters of endo- and exocellulase enables a multilayer mode of processive depolymerization of cellulose. ACS Catal. 12, 10984–10994.

Zhang, Y.H.P., Lynd, L.R., 2006. A functionally based model for hydrolysis of cellulose by fungal cellulase. Biotechnol. Bioeng. 94, 888–898.

Zhang, Y.H.P., Lynd, L.R., 2004. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: Noncomplexed cellulase systems. Biotechnol. Bioeng. 88, 797–824.

Zhao, S., Tan, M.Z., Wang, R.X., Ye, F.T., Chen, Y.P., Luo, X.M., Feng, J.X., 2022. Combination of genetic engineering and random mutagenesis for improving production of raw-starch-degrading enzymes in *Penicillium oxalicum*. Microb. Cell Fact. 21, 272.

Zhi, C.W., Clarkson, K.A., Morgan, A.J., 1999. Xylanase From *Acidothermus Cellulolyticus*. U.S. Patent 5,902,581.

Zolan, M.E., Tremel, C.J., Pukkila, P.J., 1988. Production and characterization of radiation-sensitive meiotic mutants of *Coprinus cinereus*. Genetics 120, 379–387.

بهبود سویه قارچ *Trichoderma afroharzianum* از طریق جهش ناشی از تشعشع گاما برای افزایش تولید سلولاز و زایلاناز

حامد عسکری[1]، سمیرا شهبازی[1]✉، صبیحه سلیمانیان زاد[2و3]، مهدی کدیور[2]

1-پژوهشکده کشاورزی هسته‌ای، پژوهشگاه علوم و فنون هسته‌ای، سازمان انرژی اتمی ایران، ایران

2-گروه علوم و صنایع غذایی، دانشکده کشاورزی، دانشگاه صنعتی اصفهان، اصفهان، ایران

3-پژوهشکده زیست فناوری و مهندسی زیستی، دانشگاه صنعتی اصفهان، اصفهان، ایران

چکیده: سلولازها و زایلانازها به عنوان آنزیم‌های هیدرولیتیک دسته بندی می‌شوند که بطور تصادفی پیوندهای بتا-1و4 را در سلولز و آرابینوکسیلان‌های آرد گندم می‌شکنند و به طور گسترده در صنایع نانوایی به عنوان بهبوددهنده بافت خمیر در فرمولاسیون محصولات مبتنی بر آرد استفاده می‌شود. از این‌رو، پرتو گاما کبالت 60 برای تولید سویه جهش‌یافته قارچ *Trichoderma afroharzianum* استفاده شد. موتانت‌های قارچ تریکودرما جدا شدند و هر دو روش غربالگری کمی و کیفی، برای ارزیابی تولید آنزیم‌های خارج سلولی با استفاده از سبوس گندم به عنوان سوبسترا استفاده شد. جدایه موتانت بهینه با استفاده از روش خط شناسه DNA شناسایی شدند. جدایه موتانت قارچ *Trichoderma afroharzianum*- NAS107-M82 بالاترین فعالیت آنزیم زایلاناز را نشان داد، که تقریباً 3/3 برابر بیشتر از سویه مولد آن بود. تجزیه و تحلیل الکتروفورتیک پروتئین‌ها نشان داد که آنزیم‌های اگزو گلوکاناز I، اندو گلوکاناز III و زایلاناز I به طور هم‌افزایی سبوس گندم را هیدرولیز می کنند. به طور خلاصه، جهش ناشی از تابش گاما می تواند یک استراتژی سودمند برای به دست آوردن چنین جهش یافته‌های برتر با هدف تبدیل زیستی ضایعات سبوس گندم به آنزیم های زیلاناز و سلولاز باشد.

کلمات کلیدی: قارچ *تریکودرما*، سبوس گندم، سلولاز، زایلاناز، پرتو گاما، موتاسیون، الکتروفورز.

مکاتبه کننده: سمیرا شهبازی Email: sshahbazi.aeoi@gmail.com

تاریخ دریافت: ۱۴۰۳/۰۲/۲۶ تاریخ پذیرش: ۱۴۰۳/۰۳/۳۰