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Enhancement of xylanase enzyme production by induced gamma radiation mutation of Trichoderma

Samira Shahbazi[a,*], Hamed Askari[b], Sabihe Soleimanian-Zad[b,c], Mahdi Kadivar[b]

[a] *Nuclear Science and Technology Research Institute (NSTRI), Atomic Energy Organization of Iran (AEOI), Tehran, Iran*
[b] *Department of Food Science and Technology, College of Agriculture, Isfahan University of Technology, Isfahan, 84156-83111, Iran*
[c] *Research Institute for Biotechnology and Bioengineering, Isfahan University of Technology, Isfahan, 84156-83111, Iran*

HIGHLIGHTS

- Creating genetic diversity in Iranian sources of Trichoderma strains using gamma irradiation for induced mutation.
- Identification novel mutant with significant increased xylanase/cellulase enzyme production potential.
- Optimizing the method of genetic modification of beneficial microbes (*Trichoderma*) widely used in the industry.
- Production of new xylanase-cellulase complex enzyme product by NAS107M82 mutant.

ABSTRACT

Xylanase enzymes produced by *Trichoderma afroharzianum* have significant industrial applications, including animal feed, food, biofuel, and textile industries. Creating novel sources of *Trichoderma* strains using induced gamma irradiation mutation can increase enzyme production. In this research, Co-60 gamma irradiation has been used to develop a mutant strain of *T.afroharzianum*. *T.afroharzianum* mutants were isolated, and qualitative and quantitative screenings were used to evaluate the production of the extracellular enzymes with wheat bran waste as a substrate. The best *T.afroharzianum* mutant was identified using the DNA barcoding method. The highest xylanase activities were observed in the mutant, *T.afroharzianum* NAS107-M82, which is approximately 3.3 times higher than its parent strain. The electrophoretic analysis of the mutant secreted proteins indicated that the combined action of exo-glucanase I, endo-glucanase III, and xylanase I enzymes effectively hydrolyzed the wheat bran. This research collectively emphasizes the varied potential of xylanases derived from gamma-radiated mutants of *T. afroharzianum* for a range of industrial and agricultural applications, underscoring their significance in enzyme production.

KEYWORDS
Gamma radiation
Trichoderma afroharzianum
Mutation
Xylanase
Cellulase

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1 Introduction

Industrial enzymes play a crucial role in various industries globally, including Iran. The application of enzymes in industrial processes such as manufacturing, bioenergy, biomaterials, detergents, textiles, food, animal feed, leather, and pulp and paper industries has been well-documented (Cipolatti et al., 2021; Lobedanz et al., 2000; Uhlig, 1998).

The development of recombinant DNA technology and protein engineering has further enhanced the production and customization of enzymes (Lobedanz et al., 2000; Uhlig, 1998). Furthermore, random mutagenesis by physical (such as UV, X, and gamma radiation) and chemical (such as ethyl methane sulfonate) mutagens have been used as useful tools to manipulate industrial microorganisms genetically (Catlett et al., 2003; Gohel et al., 2004). Gamma radiation mutagenesis has been successfully employed to enhance enzyme production in fungi such as *Trichoderma* (Askari et al., 2024; Bagheri et al., 2018; Ghasemi et al., 2019).

Filamentous fungi such as *Trichoderma* afroharzianum, are known for their significant role in industrial enzyme production, specifically cellulases and xylanase (Mach and Zeilinger, 2003). These enzymes, including endoglucanases (EG I, EG II, EG III, EG IV, and EG V) and exoglucanases (CBHI and CBHII), work synergistically to break down cellulose into cellobiose and further hydrolyze it into glucose with the help of β-glucosidase (Dong et al., 2022; Kunamneni et al., 2014). *T. afroharzianum* is known to produce a

*Corresponding author: sshahbazi@aeoi.org.ir

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Figure 1: Gammacell 220 irradiator used to induce mutation in *Trichoderma* afroharzianum.

wide array of enzymes with diverse functions, including β-mannase, β-mannosidase, $\alpha - L$-arabinofuranosidase, α-galactosidase, acetylene xylan esterases, laccases, polygalacturonase, pectin lyase, and pectin esterase (Manzar et al., 2022; Nandini and Geetha, 2021). The ability of T. afroharzianum to produce these enzymes efficiently is attributed to genetic mutations that enhance enzyme secretion, as revealed by integrative transcriptome and proteome profiling analyses (Dong et al., 2022). The presence of these enzymes highlights the multifaceted capabilities of T. afroharzianum, making them valuable assets in sustainable agriculture and biotechnological advancements.

Studies have shown that gamma mutagenesis of *Trichoderma* reesei strains led to a significant increase in endoglucanase and xylanase activities, with up to an 8-fold rise in xylanase activity observed (Kostyleva et al., 2019, 2018). Several studies have shown that gamma radiation can cause genetic diversity of filamentous fungi and induce positive (Abbasi et al., 2016; Askari et al., 2024; Lee et al., 2000; Shahbazi et al., 2014) or negative mutants (Zolan et al., 1988) of specific genes.

Enhancing enzyme production through the utilization of effective microorganisms, their genetic modification, and the incorporation of agricultural waste as fermentation substrates can lead to a substantial decrease in the costs associated with enzyme production. Effective microorganisms can improve production performance because the culture medium typically accounts for 25-50% of the total production costs (Shafei et al., 2011).

Due to this enzymatic diversity of *Trichoderma* filamentous fungi and its ability to biodegradation of hemicellulosic waste and production of extracellular enzymes, this study investigated *Trichoderma* mutation as part of the technological plan for enzyme production (Project No. TGP-A-00-001) at the Atomic Energy Organization of Iran. Therefore, the best *T. afroharzianum* strain 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.

2 Materials and Methods

2.1 *Trichoderma strains preparation*

In this study, twenty four *Trichoderma* strains were obtained from the fungal collection of the Plant Protection Department of Nuclear Agriculture Research School, Alborz, Iran. Five-day cultures of purified fungi on Potato Dextrose Agar (PDA, Merck, Germany) containing 100 mg.L^{-1} chloramphenicol were stored at 4 °C for later use.

2.2 *Qualitative screening for the best xylanase-producing strain*

All wild-type *Trichoderma* strains were screened for xylanase activity using the plate screening method on *Trichoderma* Complete Medium (TCM or Mendels mineral salts solution medium) contained (g.L^{-1}): Bactopeptone, 1.0; urea, 0.3; KH_2PO4, 2.0; $(NH4)_2SO_4$, 1.4; MgSO4. $7H_2O$, 0.3; $CaCl_2.6H_2O$, 0.4; FeSO4.$7H_2O$, 0.005; $MnSO_4$, 0.0016; $ZnSO_4$, 0.0014; $CoCl_2.6H_2O$, 0.002, and 0.2 mL Tween 80, 17.5 g.L^{-1} agar, 0.1% (v/v) Triton X-100, 4 g.L^{-1} sorbose, and 0.5% w/v of arabinoxylan (psyllium hydrocolloid) as a substrate. pH was adjusted to 5.3. Inoculated plates were incubated at 28 °C for 3 days followed by 18 h at 45 °C. The petri dishes were flooded with 0.4% Congo red, and washed with 1M NaCl after 10 minutes. Acetic acid (5%) was then flooded to the surface of the Petri dishes for better visualization of clear zones (Tasia et al., 2017). The best xylanase-producing strain was screened according to hydrolysis capacity (the ratio of the clear zone to colony diameter) (Askari et al., 2024).

2.3 *Gamma radiation induced-mutation of Trichoderma*

After isolation of the best enzyme-producing strain of *Trichoderma*, washed spore suspensions were prepared from 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). The applied dose levels were 0, 100, 250, 500, 750, and 1000 Gy (in triplicate) and

after the irradiation process, the spore suspensions were cultured on PDA medium.

Determination of the optimum gamma dose for induced mutation was based on a 1-2 log cycle reduction in spore germination on PDA. The wild type and selected colonies from the optimum dose were subcultured on PDA and examined after 7 d of incubation at 28 °C for qualitative screening of xylanase producer mutants similar to the method described above. Best xylanase-producing mutants were screened based on the ratio of the hydrolysis capacity of the mutant (HCM) and the hydrolysis capacity of the wild-type strain (HCW) (Askari et al., 2024).

2.4 *Quantitative screening of Trichoderma mutants*

The mutants were maintained on MYG agar. One milliliter-washed spore suspension (1×10^7 spores $m.L^{-1}$) was used as inoculum of the 50 mL TCM medium. Seed culture was produced in *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; $(NH4)_2SO_4$, 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 with pH 4.8 and supplemented with 0.3% w/v glucose. Cultures were produced in 50 mL of TCM in 250 mL Erlenmeyer flasks shaken at 150 rpm at 28 ° for 24 h. To induce the production of xylanase enzymes, washed mycelia were transferred to 50 mL of *Trichoderma* fermentation medium (TFM) which contained TCM supplemented with 0.5% w/v wheat bran powder as a substrate at pH 5.3. Triplicate flasks were harvested after an incubation time of 72 h at 28 °. Estimating extracellular protein and enzyme activities were assayed in TFM after centrifugation at 4500 ×g for seven min at 4 ° (Shahbazi et al., 2016).

2.5 *Assay of extracellular protein production and enzyme activity*

The extracellular protein content in the TFM supernatants was estimated in different mutant strains according to the Bradford method (Bradford, 1976). The absorbance was read at 595 nm on a spectrophotometer (Jenway, USA). Exo-glucanase, endo-glucanase and β-glucosidase, total cellulase (Filter paper-ase 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).

2.6 *Electrophoresis and molecular size determination of proteins*

Aliquots of 2 mL of TFM supernatants with two volumes of cold acetone were 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) (Laemmli, 1970). The proteins were separated at a constant amperage of 25 mA. The gels were stained with Coomassie Brilliant Blue R-250 in methanolacetic acidwater (5:1:4, v/v), and decolorized in methanolacetic 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 an 11-245 kDa protein marker (Sinaclon) (Gooruee et al., 2024; Laemmli, 1970; Shahbazi et al., 2016).

2.7 *Genotypic identification of best Trichoderma strains and their mutant*

2.7.1 Production of fungal biomass

Trichoderma cultures (wild-type strain and its best mutant) were grown at 28 °C in potato dextrose broth for 5 to 7 days at 150 rpm. Mycelia were collected and frozen in liquid nitrogen and lyophilized.

2.7.2 DNA extraction, sequence alignment, and molecular phylogeny

The total genomic DNA extraction was done according to the protocol previously described by Abbasi and others (Abbasi et al., 2016). 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 ITS-1 primer (5'-CGTAGGTGAACCTGCGG-3') and ITS-4 primer (5'-TCCTCCGCTTATTGATATGC-3') for amplification of the sequences of internal transcribed spacers 1 and 4 (ITS-rDNA region) (Papzan et al., 2021) or EF1-728F primer (5'-CATCGAGAAGTTCGAGAAGG-3'), and EF1-986R primer (5'-TACTTGAAGGAACC CTTACC-3') for amplification of the translation elongation factor 1-α encoding gene (TEF1-α) (Mirkhani and Alaei, 2015), and 10 ng genomic DNA of each strain. PCR amplification was carried out in a Bio-Rad thermocycler (USA) programmed for ITS-rDNA region as follows: An initial denaturation 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, and extension at 72 °C for 1.5 min, and the amplification was completed with one cycle of final extension at 72 °C for 5 min. Also, PCR amplification of the TEF1-α region was programmed as follows: An initial denaturation for 5 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and extension at 74 °C for 50 s, and the amplification was completed with one cycle of final extension at 74 °C for 7 min. PCR amplicons were resolved by agarose gel electrophoresis and afterward, the obtained sequences were aligned using MEGA X. Most of the sequences analyzed here alongside our sequences were obtained by searching BLAST. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1992). 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

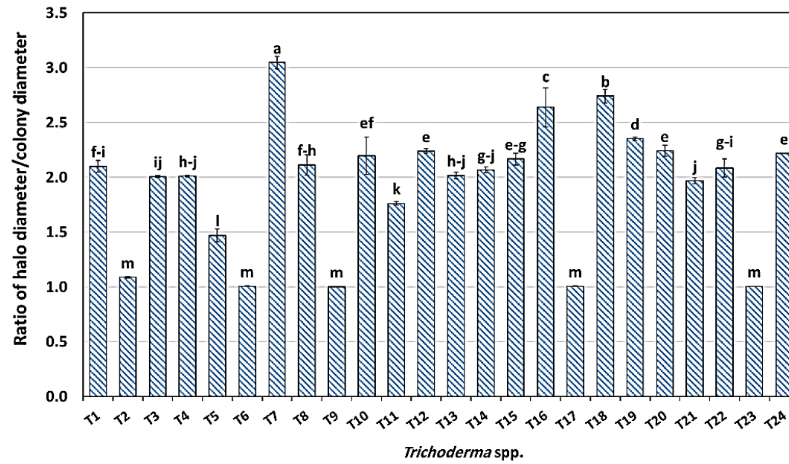

Figure 2: Qualitative evaluation of different *Trichoderma* strains for their xylanase activity using plate screening methods. Synthetic solid medium stained with the dye Congo red (0.4% w/v) after incubating at 28 °C for 3 days followed by 18 h at 50 °C.

Maximum Composite Likelihood method (Tamura et al., 2004), and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

2.8 Statistical analysis

All experiments had three independent replications for each species. Differences in the mycelial growth rate, size of conidia for each *Trichoderma* strain on PDA media, extracellular protein production, and qualitative, and quantitative xylanase activities were analyzed using a completely randomized design, and results were submitted to analysis of variance (ANOVA) with means compared by the Duncan test and HSD Tukey test ($p<0.05$), using the SPSS (ver. 16) statistical software.

3 Results

The relationship between *Trichoderma* morphology and the production of cellulase and xylanase enzymes is crucial for optimizing enzyme production. The pure cultures (24 *Trichoderma* strains) were prepared by the single spore isolation method. All strains presented white mycelium of spongy consistency in the early days of cultivation on the PDA that spread throughout the plate and consequently had a yellowish-green to dark green color after 3-5 days of incubation at 25 °C.

3.1 Qualitative screening of the best xylanase-producer strain

Qualitative screening of the best xylanase-producer *Trichoderma* involves evaluating strains for their xylanase-production capabilities. The results of quantitative screening highlighted the diverse enzymatic capabilities of *Trichoderma* strains. Figure 2 shows the qualitative evaluation of xylanase production in 24 strains of *Trichoderma* by plate screening method. The highest hydrolysis capacity was observed in the T7 strain, which was selected for gamma radiation-induced mutation ($p<0.05$).

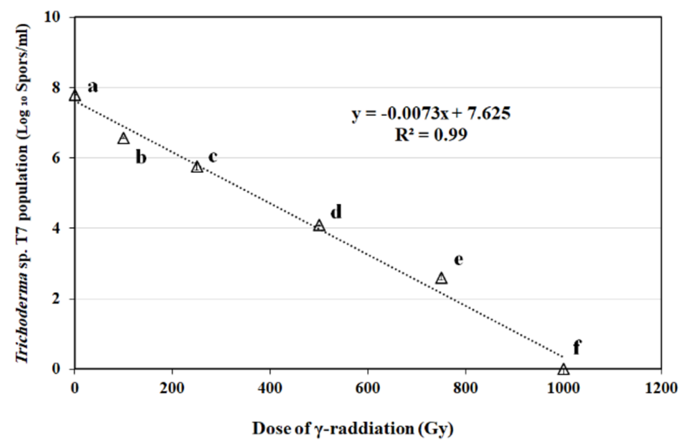

Figure 3: Effect of gamma radiation doses on the viable count of *T7* (*T. afroharzianum*).

3.2 Gamma radiation-induced mutation

The washed spore supsentsions of *T7* strain were exposed to different doses of gamma radiation (0-1000 Gy). As shown in Fig. 3, the fungal viability decreased significantly with an increased radiation dose. According to the results, the dose of 250 Gy resulted in 1.825 log cycle reduction of spore germination on the PDA medium. Therefore, this dose was chosen as the optimal dose of gamma radiation-induced mutation.

3.3 Screening of the best xylanase-cellulase-producing mutants

3.3.1 Qualitative screening of mutants

The selected colonies from the optimum dose of gamma radiation (250 Gy) were sub-cultured three times on an MYG agar medium for screening of xylanase producing mutants. 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. 118 pure cultures were selected for further investigations regarding the activity of xylanase.

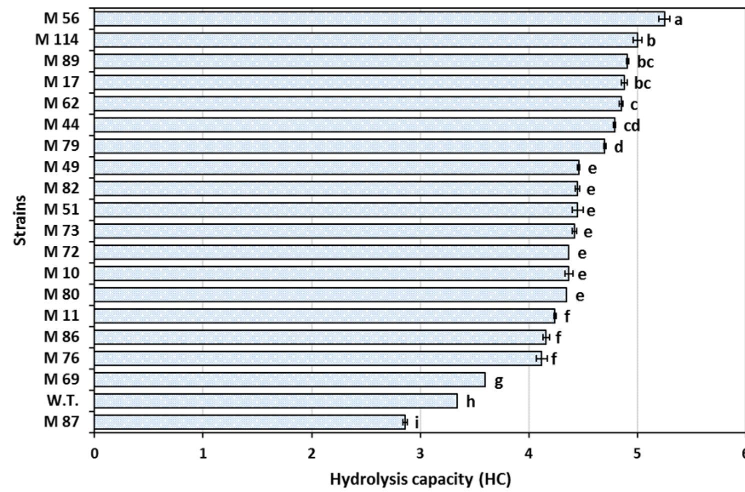

Figure 4: Maximum hydrolytic capacity (HC) value of *Trichoderma* xylanase producing mutants.

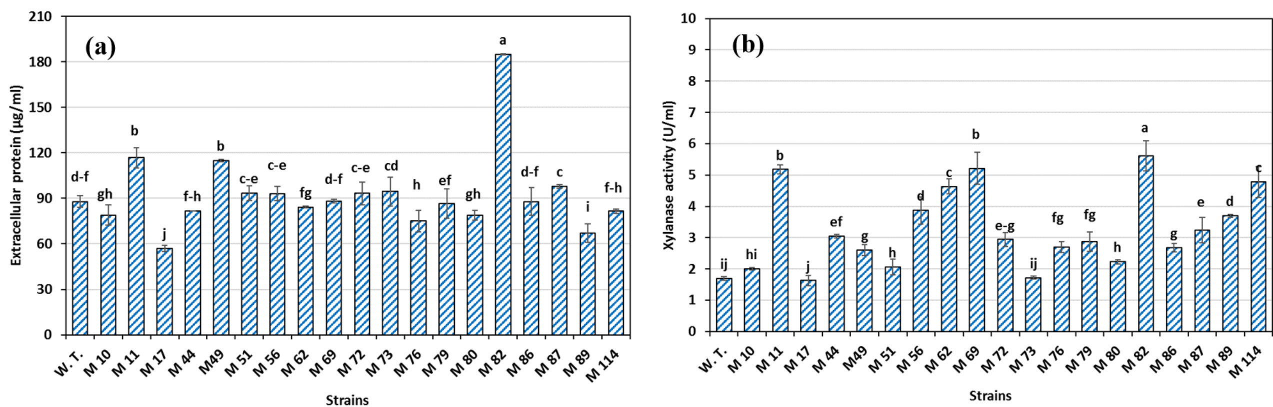

Figure 5: Extracellular protein concentration (μg.mL^{-1}) (a), and xylanase activity (U.mL^{-1}) (b) of different mutant strains of *Trichoderma* in the supernatant of the TFM containing wheat bran as a substrate after 72 h fermentation at 28 °C and 150 rpm.

Figure 4 displays the results of comparing the mean hydrolysis capacity of mutants (19 mutants) and the wild-type strain. Of the 118 isolates, 17 mutant isolates were selected based on the HC ratio (HCM/HCW) of more than 1.25. Also, M69 and M87 were selected as mutants with hydrolysis capacity approximately similar to the wild-type strain. The highest hydrolysis capacity was observed in M56, M114, M89, M17, M62, and M44 mutants ($p<0.05$).

3.4 Quantitative screening of mutant strains

The extracellular protein concentration was measured using the Bradford method in TFM (Fig. 5-a). The changes in extracellular protein concentration varied from 56.97 to 185 μg.mL^{-1}. The most extracellular protein concentration was observed in the TFM supernatant of M82 ($p<0.05$). The results of xylanase activity are revealed in Fig. 5-b. The highest xylanase activity was observed in the M82 mutant strain (5.61 ± 0.48 U.mL^{-1}), which was approximately 3.3 times higher than the wild-type ($p<0.05$).

The results of cellulase activity are shown as international units (U) (Fig. 6), in which one unit of cellulase (Exo-, endo-glucanase, β-glucosidase, and total cellulase) activity is definite as the amount of enzyme required to liberate 1 μmol of glucose per hour.

The highest exo-glucanase activity (U.mL^{-1}) was observed in M82, and M44 mutants. These results showed that these mutants, probably have a high ability to decompose crystalline regions of lignocellulosic materials. Exo-glucanase activity in M82 was approximately 1.78 times higher than the wild-type strain. Exo-glucanases or cellobiohydrolase (I and II) cleave the accessible reducing and non-reducing wheat bran cellulose chain ends to liberate cellobiose and some glucose molecules (Zhang and Lynd, 2004). Also, the highest endo-glucanase (EG) activity was observed in M82, and M69. 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 M82 was approximately 1.48 times higher than the wild-type strain. The mutants of M62, M73, M72, M86, and M82 showed high β-glucosidase activity compared to other mutants. Overall, the total cellulase system contains exo-glucanases, endo-glucanases, and β-D-glucosidases, all of which synergistically hydrolyze the crystalline cellulosic cell wall of wheat bran. The interaction between endoglucanases and exo-glucanases represents the most extensively studied form of synergy and

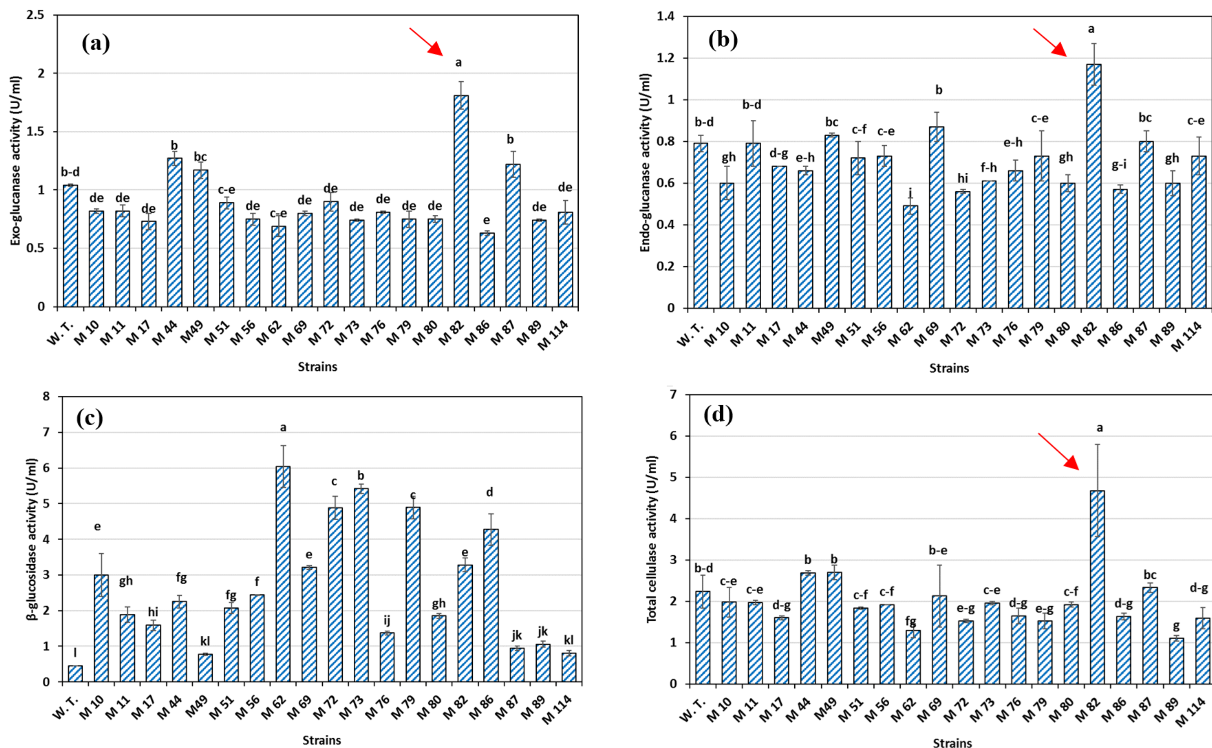

Figure 6: Cellulase enzyme activity (U.mL^{-1}) of different mutants of *Trichoderma* in the supernatant of the TFM containing wheat bran as a substrate after 72 h fermentation at 28 °C and 150 rpm. (a): Exo-glucanase activity, (b): Endo-glucanase activity, (c): β-glucosidase activity, and (d): Total cellulase activity.

is one of the most quantitatively significant factors in the hydrolysis of crystalline cellulose. The highest total cellulase activity (U.mL^{-1}) was illustrated in M82, M49, and M44 mutants, respectively. However, M82 could be the best superior cellulase producer mutant, because it produces both exo-glucanase and endo-glucanase enzymes with higher activity. A high level of total cellulase activity in this mutant could be due to higher exo-glucanase activity and EG activity compared to other mutants and the synergistic action of these enzymes on the cellulose chain.

3.5 The electrophoretic pattern of proteins

The variation and molecular weight distribution of enzyme-rich proteins of T7 the wild type strain and M82 the superior mutant are shown in Figs. 7-a and 7-b. The weight range between 11 and 135 kDa indicated the diversity of proteins with various molecular weights (Mw). According to references the proteins with molecular weights of 17, 21, 32, and 43 kDa are classified as xylanases, specifically designated as 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). In our densitometry results, Xyl I (17 kDa) and Xyl II (21 kDa), Xyl IV(43 kDa), and Cel 12A (EG III, 26 kDa) were secreted in the TFM supernatant of T. afroharzianum NAS107-M82 mutant. However, the higher xylanase activity in M82 (Fig. 5-b) may be due to the presence of the Cel12A enzyme, which, besides acting in the cellulose chain, has acted against beta-1,3-glucan and beta-1,4-xylan hydrocolloids (Hayn et al., 1993; Karlsson et al., 2002).

The molecular weights of cellulases derived from various *Trichoderma* species differ based on the particular enzyme and the strain in question. Research focused on the purification of cellulase from *Trichoderma* viride and *Trichoderma* reesei has indicated that the molecular weights of the enzymes vary between 23 and 87 kDa (Abdul-Hadi et al., 2016; Shafique and Shafique, 2011; Yasmin et al., 2013). *Trichoderma* species can produce 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, 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 of M82 (Fig. 7-a, lane 2 had two enzyme bands called CBH I (66 kDa) and CBH II (60.5 kDa). The highest optical density of CBH protein bands (CBH I and II) was observed in M82 (Fig. 7-b). Also, Cel12A (EG III, 32.37%), Xyl I (19.67%), Cel6A (CBH II, 9.76%), Cel5A (EG II, 9.72%), and Xyl IV (6.16%) were observed among the most secreted proteins from this mutant, as confirmed by the results obtained from exo-glucanase and endo-glucanase and xylanase activity (Fig. 8). The Cel 3A protein band (which weighs 73 kilodaltons and is also called BGL I) was present in the profiles of the M82 mutant of *Trichoderma*. But Cel 1A (BGL II) weighed 110 kDa and was observed in the protein profile of M82 mutants with a molecular weight of 107 kDa. These findings highlighted the diversity in cellulase molec-

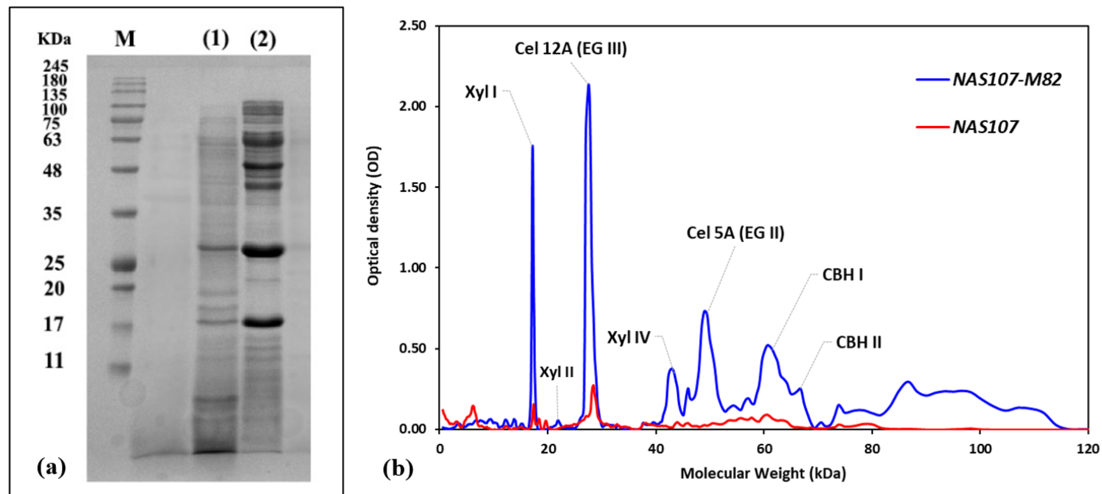

Figure 7: Protein profile analysis (a) and densitometric analysis (b) of extracellular protein secreted in the supernatant of the TFM: (1) wild-type strain *T. afroharzianum* NAS107, (2) the mutant of T. afroharzianum NAS107-M82 during fermentation time 72 h and pH of fermentation medium 5.5. (M) Protein molecular weight marker in the range of 11-245 KDa.

ular weights among *Trichoderma* afroharzianum NAS107-M82 mutant secretion.

3.6 Identification of the best Trichoderma strains; wild type, and mutants

Traditionally, morphological identification was used for *Trichoderma* phenotypic and taxonomic studies. Recent discoveries have identified novel species of *Trichoderma*; however, their morphological similarities pose significant challenges in distinguishing between them. Consequently, the examination of DNA sequences has facilitated the classification and comprehension of the evolutionary relationships among fungi. In this context, DNA extraction was conducted from the wild-type strain T7 as well as its superior mutants, M82. The quantity and quality of extracted DNA were examined by spectrophotometry and agarose gel electrophoresis (Fig. 9-a).

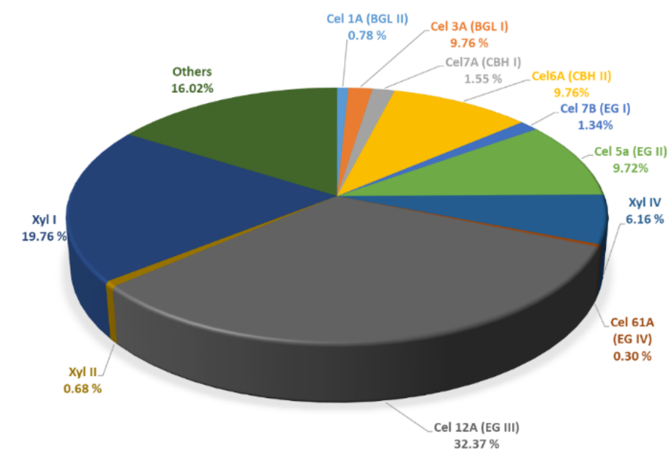

Figure 8: The abundance of secreted enzymes in the supernatant fermentation media of the *T.* afroharzianum NAS107-M82 mutant during fermentation time 72 h and pH 5.5.

PCR was performed for the amplification of the internal transcribed spacer (ITS) and the translation elongation factor 1-α encoding gene (TEF1-α) regions gene, which was successfully amplified in two strains and sequenced for *Trichoderma* identification with approximately 650 and 350 bp, respectively (Figs. 9-b and 9-c). Sequence analysis of the ITS-rDNA region and TEF-1α are widely used for the precise reorganization of *Trichoderma* species (Druzhinina et al., 2005). The forward and reverse sequences 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.

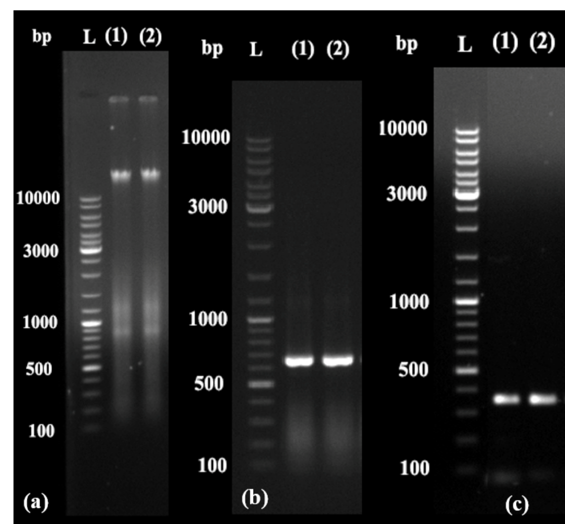

Figure 9: Agarose gel electrophoresis of DNA extraction (a), PCR products amplification of the fungal strains: The internal transcribed spacer (ITS) regions of the ribosomal DNA (b), and the translation elongation factor 1-α encoding gene (TEF1-α) (c); L: Molecular weight marker (100 bp-10 kb); (1, and 2): DNA or PCR product of the *Trichoderma* sp. T7 and its mutant NAS107-M82, respectively.

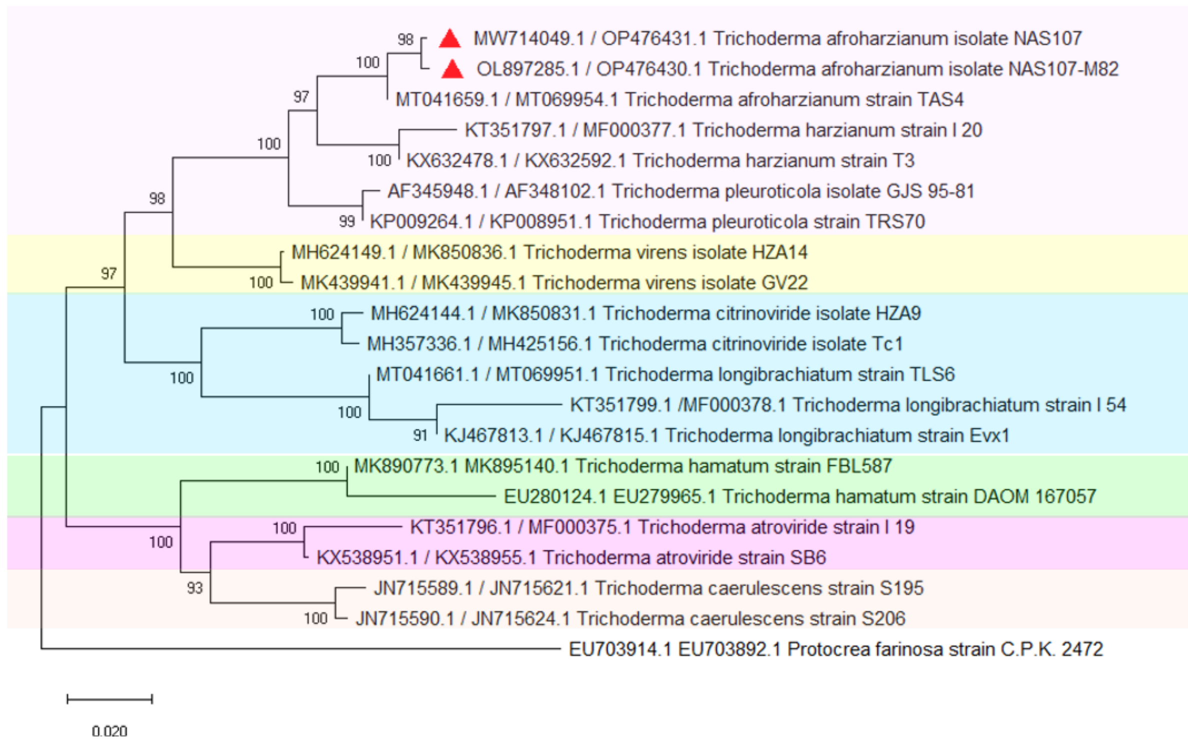

Figure 10: Phylogenetic relationship of *Trichoderma* afroharzianum NAS107 and its mutant of *T. afroharzianum* NAS107-M82 (symbols in red triangle) using the internal transcribed spacer (ITS) and the translation elongation factor 1-α encoding gene (TEF1-α) regions gene nucleotide sequence alignment using maximum likelihood method at 1000 replications for each bootstrap value using the MEGA 10.0.

Using bioinformatics tools, *T. afroharzianum* NAS107 was identified for *Trichoderma* sp. T7 and its mutant and the phylogenetic relationship of our *Trichoderma* and other strains are shown in Fig. 10. 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 strains selected are included in the phylogenetic clade Harzianum, and consequently, the results enable a broader insight into xylanase and cellulase enzymes produced by these *Trichoderma* species along with their phylogenetic relationship.

The superior strain of *Trichoderma* T7 strain and its mutant of M82 were registered in the microbial collection of Iran Biological Resources Center (IBRC) with accession numbers IBRC-M 30594 (*T. afroharzianum* NAS107), and IBRC-M 30594 (*T. afroharzianum* NAS107-M82).

4 Discussion

Trichoderma species are known for their ability to secrete various extracellular enzymes with industrial significance. These enzymes include cellulases, xylanases, proteases, chitinases, glucanases, and lipases, among others, which play crucial roles in biocontrol, bioethanol production, and fermentation processes (Al Tharf et al., 2024; Athukorala and Samaraweera, 2023; de Paula et al., 2019; Sharma and Salwan, 2019; Sun et al., 2021).

Xylanase-cellulase complexes have significant industrial applications, particularly in the degradation of lignocellulosic biomass for the production of liquid fuels and chemicals (Soni et al., 2020). These complexes play a crucial role in synergistically breaking down plant cell walls, with xylanase degrading hemicellulose and cellulase targeting cellulose, key components of lignocellulose (You et al., 2021). The use of xylanase and cellulase, along with pectinases, accounts for a substantial portion of the world enzyme market, highlighting their importance in various industries (Soni et al., 2020). Furthermore, the modification of xylanases to enhance their catalytic efficiency at lower temperatures has shown promising results, especially when used in combination with cellulase for the degradation of different types of lignocellulosic biomass, leading to increased sugar yields and improved process economics (You et al., 2021). Overall, xylanase-cellulase complexes offer a versatile and economically beneficial solution for biomass conversion in industrial settings.

We evaluated different strains of *Trichoderma* for their hydrolysis potential of arabinoxylan compounds and wheat bran through quantitative and qualitative screening and among them, the *Trichoderma* strain of T7 was selected as the best wild-type strain and was identified using ITS-rDNA and TEF1-α regions gene nucleotide sequence and its sequencing results were submitted the GenBank database at the NCBI. Using molecular identification, the T7 wild-type strain and its mutants were identified as *T. afroharzianum* NAS107.

Mutation plays a crucial role in the improvement

of microbial strains for various industrial applications (Steensels et al., 2014), such as antibiotics (Barreiro et al., 2012; Kardos and Demain, 2011), 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 producer mutants (Mobini-Dehkordi et al., 2008; Zhao et al., 2022).

Techniques like random mutagenesis, site-directed mutagenesis, and genetic engineering are employed to enhance the productivity and efficiency of microorganisms (Feldmane et al., 2024; Ghosh et al., 2023; Kosariya et al., 2023). These methods aim to create cost-effective strains with increased metabolic capacities and stress tolerance, addressing the limitations of naturally occurring strains (Feldmane et al., 2024; Kosariya et al., 2023).

In this paper, gamma radiation was used to induce mutations in the best xylanase producing wild-type strain (*T. afroharzianum* NAS107). Although there are numerous studies on the application of gamma radiation to induce 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 by a high dose of gamma irradiation, leading to growth or reproduction inhibition (Tauxe, 2001). When ionizing beams interact with water particles in *Trichoderma* cells, it can hurt the DNA and endanger the life of the cell. Moreover, other factors such as the number or density of spores, can also affect the sensitivity or tolerance of *Trichoderma* to gamma radiation (Bischof et al., 2016; Dillon et al., 2006; Ribeiro et al., 2013).

Our results showed that statistically significant changes in xylanase-cellulase production of *T. afroharzianum* NAS107 (or T7 strain) appear after irradiation with gamma 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 (Gherbawy, 1998).

The optimum dose of gamma radiation-induced mutation in *T. afroharzianum* species for enhancing enzyme production and biocontrol abilities has been extensively studied. Similar researches indicated that an optimal dose of 250 Gy of gamma radiation has been effective in inducing mutants with improved characteristics, such as enhanced extracellular cellulase production and antagonistic abilities against soilborne fungal pathogens (Abbasi et al., 2016; Askari et al., 2024; Bagheri et al., 2018; Ghasemi et al., 2019; Moradi et al., 2015). This dose has led to significant increases in cellulase activity, sporulation, colonization rates, and genetic differences in the mutants compared to the wild-type strains. The gamma-induced mutants have shown higher hydrolysis capacity, cellulase secretion, and biocontrol efficacy, making gamma radiation a valuable tool for improving the performance of *Trichoderma* species in various applications.

The highest extracellular enzyme activity was observed in mutants of *T. afroharzianum* NAS107-M82, in which their xylanase and cellulase activities were approximately 3.3 times higher than wild-type strains. In this mutant, 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 with pI 9.1) belongs to the Family 10 glycosyl hydrolase and was first identified in T. reesei PC-3-7. Xylanase I exhibits optimal activity at a pH range of 4.0 to 4.5, whereas xylanase II demonstrates peak performance within a pH range of 4.0 to 6.0. The optimum pH for xylanases III and IV (pI 7.0, 43 kDa) was observed at pH 6.0 to 6.5 and 3.5 to 4.0, respectively (Clarkson et al., 1999). According to the results of Fig. 5-b, the mutant of NAS107-M82 in this study could be the superior mutant that can produce xylanase, with high specific activity at optimal temperature and pH of 45 °C 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 strain) and its mutants; 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. In addition to substrate properties, fermentation conditions and potential mutants for enzyme production also affected the extent of synergy observed. In our study, the *T. afroharzianum* NAS107-M82, 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. 7), 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 enzymatic actions of CBH I and CBH II lead to a progressive reduction in the degree of polymerization (DP) of cellulose derived from wheat bran, resulting in the formation of cellobiose. The M82 mutant had the highest CBH II and Cel5A (EG II) protein production, leading to the maximum synergy of total cellulase. Comparing cellulase activity in different mutants, 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 leads 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 is randomly hydrolyzed and thereby free reduced and non-reduced chain ends for hydrolysis by CBH I and II are 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.

5 Conclusions

The research highlights the potential of gamma radiation to enhance the enzyme production capabilities of the fungus *T. afroharzianum*. The mutant strain *T. afroharzianum* NAS 107-M82 is particularly noted for its ability to produce xylanase and cellulase, enzymes essential for the bioconversion of wheat bran waste. This suggests that gamma radiation-induced mutations can lead to the development of superior fungal strains that serve as effective biological agents for enzyme production. Furthermore, the methodology employed in this research may be extended to other fungal species, opening avenues for creating new mutants with various industrial applications.

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Conflict of Interest

The authors declare no potential conflict of interest regarding the publication of this work.

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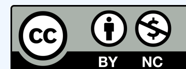

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