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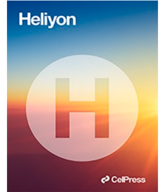

Research article

Creating a novel genetic diversity of *Trichoderma afroharzianum* by γ-radiation for xylanase-cellulase production

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

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

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ABSTRACT

Creating novel sources of a microbial strain using induced mutation can increase enzyme production for industrial use. According to this, we have developed a mutant strain of *Trichoderma afroharzianum* by Co^{60} gamma irradiation. *Trichoderma* mutants were isolated from an optimum dose of 250 Gy. The qualitative and quantitative screening were used for evaluating their enzyme production and the DNA barcoding method was used to identify the best *Trichoderma* mutant isolates. The highest cellulase (exo-glucanase, endoglucanase, β-glucosidase, and total cellulase) and xylanase activities were observed in superior mutant isolates of *Trichoderma afroharzianum* NAS107-M44 and *Trichoderma afroharzianum* NAS107-M82, which is approximately 1.6–2.5 times higher than its parent strain, respectively. The electrophoretic pattern of proteins showed that the exo-glucanase I, endo-glucanase III, and the xylanase I enzymes hydrolyzed the corn bran, synergistically. Overall, gamma irradiation-induced mutation could be an expedient technique to access such superior mutants for the bioconversion of corn bran wastes.

1. Introduction

Xylanases and cellulases are the major enzymes in the global enzyme market and are widely used in different biotechnological processes such as the food industry [1,2]. The utilization of xylanase and cellulase enzymes has enhanced in recent decades owing to their potential effectiveness in bakery and bread preparation. These enzymes improve the behavior of gluten and the viscoelastic properties and elasticity of the dough, making the dough softer and increasing the water absorption of the gluten network, which can increase the efficiency of the dough and increase the volume of bread [3,4].

Filamentous fungi such as *Penicillium* [5], *Aspergillus* [6], *Phanerochaete* [7], and *Trichoderma* [8] are cell factories for the high production of different lignocellulose degrading enzymes, and almost greatest significant industrialized source of enzymes belong to this category of organisms. Also, different species of *Trichoderma* fungi produce diverse extracellular enzymes including endo-xylanases (XYN) I, II, III, and IV, β-1,4-xylosidases I, and II, exo–glucanases or cellobiohydrolases (CBH) I and II, endo-glucanases I, II, III, IV, and V and β-1,4-D-glucosidases I, and II (BGL, EC 3.2.1.21), β-mannase, β-mannosidase, α-L-arabinofuranosidase, α-galactosidase, Acetylene xylan esterases, laccases (benzendiol: oxygen oxidoreductases), polygalacturonase, pectin lyase, and pectin esterase, that randomly biodegrade the glycosidic bonds of plant cell wall carbohydrates by different mechanisms of action [9]. Due to the wide variety of producing enzymes, it has become one of the important sources of enzyme production in the industry.

* Corresponding author.
E-mail address: haskari97@ag.iut.ac.ir (H. Askari).

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Plant residues from agriculture products, and the food industry have become a health and environmental hazard [10] and their biodegradation requires the use of an efficient and active organism with significant potential for enzyme production such as *Trichoderma* fungus. One of the ways to achieve this, is wide screening among a large population of wild-types in nature, which is very difficult and time-consuming. The solution to achieve this efficient biodiversity is genetic manipulations or mutation in wild-type organisms to create mutants that can be useful in industry.

Scientists can use genetic manipulation and exposure to physical mutagens UV, X, and gamma radiation and chemical mutagens such as ethyl methane sulfonate as suitable tools to improve the effectiveness of *Trichoderma* strains [11,12]. Numerous studies have shown that gamma radiation can affect the genetic diversity of filamentous fungi and cause positive induction [13–15] or negative [16] mutation in the performance of specific genes. Mutagenicity increases the productivity of industrial fermentations [17]. Mutagenicity is also used to change the ratio of metabolites produced in the fermentation medium to a more favorable distribution, to clarify the pathways of secondary metabolism, to obtain new compounds, and for other functions.

The utilization of agricultural waste as a cheap carbon source of fermentation can significantly decrease hydrolytic enzyme production costs, because these include about half of the total production costs [18]. Furthermore, due to the cellulase and xylanase synergistic interactions, *Trichoderma* strains that secrete both of these enzymes together, have great value industrially. Also, the extraction and purification processing will be more beneficial from the industry's point of view. This would save fermentation media, energy, the maintenance cost of fermenters, and human resources. The production of xylanase and cellulase enzymes in filamentous fungi (such as *Trichoderma*) is much higher than that of actinomycetes, bacteria, and yeasts that secrete the enzyme directly without any side effects and without the need to break down cell walls hemicellulosic material in the environment.

Bilal or corn is a crop that according to the Food and Drug Administration (FAO) in 2019 a world production of about 1137.72 million tons cultivated worldwide [19]. Corn bran and corn fiber are two of the major by-products produced by corn milling, which contains high amounts of hemicellulose, including 67.5% arabinoxylan, 22.5% cellulose, and 2.4% protein [20]. The purified corn bran contains high amounts of hemicellulose, which can be used as the finest substrate for induction of enzyme production, and it is possible to create added value from this by-product that currently has a low commercial value. The presence of high concentrations of hemicellulose in the outer surface of corn bran resulted in the physical barrier that limits the accessibility of the endo-glucanase enzymes to the cellulose chain. Also, due to the structural complexity of hemicellulosic corn bran, its efficient enzymatic depolymerization necessitates the synergistic actions of xylanase and cellulase enzymes.

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 corn bran using superior gamma radiated mutants of *Trichoderma.*

Therefore, the best wild-type *Trichoderma* strain was screened, identified, and then treated with altered doses of gamma irradiation to examine the possible improvement of xylanase, and cellulase production by gamma radiation-induced mutation.

2. Materials and methods

2.1. Preparation and purification of Trichoderma fungi

A total of 24 *Trichoderma* isolates which were previously obtained from the cultures collection of the nuclear agriculture school, in Alborz, Iran, were investigated in this study. These fungi were isolated from soil and plant debris containing *Trichoderma* fungi from forest areas around Lefour Dam, Javaram forests, and Seyed Kola region in Savadkuh, Mazandaran province of Iran in April 2019 and 2020. The initial identification of fungal isolates was based on morphological characteristics and the sequence of the ITS-rDNA region. The accession number of all isolates (T1- T24: 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) are available at the National Center for Biotechnology Information (NCBI) GenBank. The purified fungi are maintained in the stock cultures on Potato dextrose agar (PDA, Merck, Germany) medium containing 100 mg/L of chloramphenicol in our laboratory culture collection at 4 °C for later use, and the superior *Trichoderma* isolates and its mutants were registered in the microbial collection of the Iranian Biological Resource Center (IBRC).

2.2. Qualitative screening of the 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): KH_2PO_4, 2.0; Bactopeptone, 1.0; $(NH_4)_2SO_4$, 1.4; Urea, 0.3; $CaCl_2.6H_2O$, 0.4; $MgSO_4$. $7H_2O$, 0.3; $FeSO_4.7H_2O$, 0.005; $MnSO_4$, 0.0016; $ZnSO_4$, 0.0014; $CoCl_2.6H_2O$, 0.002 and 0.2 mL/L, Tween 80. This medium was supplemented with the addition of 17.5 g/L of agar, 0.1% (v/v) Triton X-100, 4 g/L of sorbose, and 0.5% w/v of arabinoxylan (obtained from psyllium husk) as a substrate. The medium was adjusted to pH 5.3. Three-day cultures of each wild-type isolate were prepared on water agar medium at 25 °C and a mycelial plug disc with a diameter of 8 mm was cut from the fresh thread of each fungus using a perforated cork and cultured on the center of Mendel's mineral salts solution medium. Plates were incubated at 28 °C for 3 days followed by the function of the secreted enzyme on the xylan by 18 h at 45 °C stored. The surface of the Petri dishes was flooded with 0.4% Congo red dye and washed with 1 M NaCl after 10 min. Acetic acid (5%) was then flooded to the surface of the Petri dishes for better visualization of the clear areas was obtained [21]. The best xylanase-producing isolates were screened by calculating hydrolysis capacity, which is the ratio between the clearing zone diameter of hydrolysis surrounding the

colonies and the colony zone diameter.

2.3. Gamma radiation-induced mutation of Trichoderma

Seven-day-old PDA culture of the best *Trichoderma* enzyme-producing isolate was washed with sterile saline solution (containing 9 g/L NaCl in distilled water) and washed spore suspensions (1×10^8 spore/mL) were irradiated (0, 100, 250, 500, 750, and 1000 Gy) using a gamma cobalt 60 source in Gammacell 220 radiator (MDS Nordion, Ottawa, Canada) at a dose rate of 4.5 kGy per hour, located in the Atomic Energy Organization, Tehran, Iran. The absorbed dose of gamma radiation was calculated using a Red-Perspex dosimeter (Harwell Dosimeters, UK). After the radiation operation, the spore suspension solutions were serial dilution and re-cultured on a PDA medium supplemented with 50 ppm chloramphenicol and incubated at 25 °C. The population of survival spores was counted and the criterion of the optimum dose of gamma irradiation for inducing mutation based on approximately 90–99 % (or 1–2 log cycle) reduction of spore germination on PDA medium was selected. The selected colonies from an optimum dose of gamma irradiation were sub-cultured three times in Malt yeast glucose agar media (MYG, containing, g/L: Malt extract, 5; yeast extract, 2.5; glucose, 10; agar, 20) to stabilize the changes caused by the induced mutation.

2.4. The screening of enzyme-producing superior mutant isolates

2.4.1. Qualitative screening of the best mutant isolates

Three-day cultures of each mutant isolate were prepared on water agar medium at 25 °C and qualitative screening of xylanase producer superior mutant isolates similar to the method described above was performed. The best xylanase-producing mutant isolates were screened based on the ratio between the hydrolysis capacity of mutant isolate (HCM), and the hydrolysis capacity of wild-type strain (HCW).

2.4.2. Quantitative screening of the best mutant isolates

The best *Trichoderma* mutant isolates were cultured on MYG agar media for seven days in 28 °C. One milliliter-washed spore suspension (1×10^7 spore's/mL saline solution) was used as inoculum of the 50 ml of TCM in 250 ml Erlenmeyer flasks (pH 4.8, containing 0.3% w/v glucose as a carbon source) and then incubated at 28 °C for 24 h under agitation rate of 150 rpm. The seed cultures underwent centrifugation (4500×*g*, 5 min), and the washed mycelium was subsequently transferred to 50 ml of *Trichoderma* fermentation medium (TFM), which consisted of TCM (at a pH of 5.3) supplemented with 0.5% w/v corn bran powder as a substrate. The growing conditions were described above previously, and triplicate flasks were harvested after 72 h of incubation at 28 °C. The supernatant obtained from the centrifugation of TFM (4500×*g*, 7 min, and 4 °C) was used for the extracellular protein content (μg/mL) and enzyme (cellulase and xylanase) activity assay [22].

2.5. The secreted protein concentration and enzyme potency tests

The secreted protein content (μg/mL) in the TFM supernatants of different best mutant isolates was estimated by the Bradford dye-binding method and measurement of reagent color changes at 595 nm by UV/Vis spectrophotometer (Jenway, USA) [23]. The bovine serum albumin (BSA) was used as a standard (0–500 μg/mL) for the protein content calculation. Also, cellulase and xylanase activity (U/mL) were determined by the dinitrosalicylic acid (DNS) method [22,24] by UV/Vis spectrophotometer (Jenway, USA) at 540 nm. The amount of enzyme that liberates 1 μmol of glucose or xylose per hour in a standard assay was defined as a unit (U) of cellulase or xylanase activity, respectively. Avicel, carboxymethyl cellulose (CMC), cellobiose, filter paper Whatman no. 1 (strip with size 1 × 6 cm or 50 mg), and xylan were used as a substrate, for exo-glucanase (avicelase), endo-glucanase (CMCase), β-glucosidase (cellobiase), total cellulase (Filter paperase or FPase), and Xylanase, respectively. Pure Glucose or xylose was used as a standard.

2.6. The molecular weight determination and electrophoretic pattern of proteins

The cold acetone precipitated protein samples (~300 μg) were dissolved in 100 μl double-distilled water, and an equal volume of sample buffer (100 μl) was added that confined 65 mM Tris–HCl, pH 6.8, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, and 0.2% (w/v) bromophenol blue. Before electrophoresis, the loading samples were boiled for 5 min. The electrophoretic pattern of the proteins and the molecular weight (Mw) were investigated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with a 5% (stacking gel) and 12.5% (separating gel) polyacrylamide gel [25] at constant amperage of 25 mA, and then proteins were stained with Coomassie Brilliant Blue R-250. Estimation of the Mw of protein bands was performed using Gel-Pro Analyzer 6.0 densitometry software (Media Cybernetics, Inc.) [8]. The Mw range of the protein marker (Sinaclon Company, Iran) was 11–245 kDa (kDa) [22,25].

2.7. Genotypic identification of the best Trichoderma strain and its mutant isolates

2.7.1. Production of fungal biomass

Trichoderma cultures (wild-type strain and its mutant isolates) were grown at a temperature of 28 °C in potato dextrose broth for 5–7 days at an agitation rate of 150 rpm. The washed mycelium using 25 mM of EDTA was lyophilized and stored at −70 °C for later use.

2.7.2. DNA extraction, sequence alignment, and molecular phylogeny

100 mg of lyophilized mycelium of each isolate was placed in sterile porcelain mortars and ground using liquid nitrogen, and mycelium powders were used for total genomic DNA extraction [13]. The quantity and quality of DNA obtained were examined by spectrophotometry.

The internal transcribed spacers (ITS-rDNA region) and translation elongation factor 1-α encoding gene (TEF-1α) were amplified using primer pairs ITS-1 (5′-CGTAGGTGAACCTGCGG-3′) and ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) [26] and EF1-728F primer (5′-CATCGAGAAGTTCGAGAAGG-3′), and EF1-986R primer (5′-TACTTGAAGGAACC CTTACC-3′) [27], respectively. The polymerase chain reaction (PCR) amplification was completed in a total reaction volume of 30 μL, inclosing 1X PCR buffer, 1.5 mM, $MgCl_2$, 1.25 unit Taq DNA polymerase (Sinaclon, Iran), 0.5 mM of each primer, 200 μM of each of the four deoxyribonucleotide triphosphates and 20 ng template DNA of each isolate. PCR amplification was carried out in a Bio-Rad thermocycler (USA) programmed for ITS-rDNA region and TEF-1α as follows: an initial denaturation for 5 min at 94 °C, followed by 35 cycles of denaturation at 93 °C for 45 s (for TEF-1α, 94 °C for 1 min), annealing at 57 °C for 30 s (for TEF-1α at 58 °C for 1 min), and extension at 72 °C for 1.5 min (for TEF-1α, 72 °C for 50 s), and the amplification was completed with one cycle of final extension at 72 °C for 5 min. Finally, PCR amplicons were separated by gel electrophoresis with 1.2 % (w/v) agarose in Tris/Borate/EDTA (or TBE) buffer containing Ethidium bromide (10 mg/ml). The electrophoresis was executed at a constant voltage of 90 V for 1.5 h in a TBE buffer solution with a concentration of 1x. The DNA sequences were obtained from Bio Magic Gen Co., China. Chromas 2.6.6 software was used to prune the last 15 bases that were rated below 10 in quality score. The ITS and TEF-1α sequences were submitted to BLAST interface analysis in the National Center for Biotechnology Information (NCBI) GenBank (http://blast.ncbi.nlm.gov/). Most of the sequences analyzed here alongside our sequences were obtained by searching BLAST. DNA sequence data of ITS and TEF were aligned using Clustal X 1.83 [28] with the default parameters, then the molecular analysis was carried out using consensus sequences obtained from BioEdit version 7.2.5 [29]. Phylogenetic analysis was performed with sequences of ITS and TEF-1α in Molecular Evolutionary Genetics Analysis (MEGA) software (ver. 10.05.1) [30]. The Neighbor-Joining method was used for inference of the evolutionary history [31]. The optimal phylogeny tree with the sum of branch length = 0.64017426 was tested by bootstrap analysis in 1000 replications and the percentage of replicating trees is shown next to the branches [32]. The computation of evolutionary distances was executed by employing the Maximum Composite Likelihood method [33].

2.8. Statistical analysis

All experiments have been done with three independent replications for each species. Differences in mean values of viable spore's population after gamma radiation, extracellular protein concentration, and qualitative, and quantitative xylanase activities were analyzed using a completely randomized design (CRD) and results were investigated by 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.

3. Results

The purified corn bran contains high amounts of hemicellulose [20], which can be used as the finest substrate for induction of xylanase-cellulase enzyme production by *Trichoderma* fungi. Pure cultures (24 *Trichoderma* isolates) were prepared by the single spore

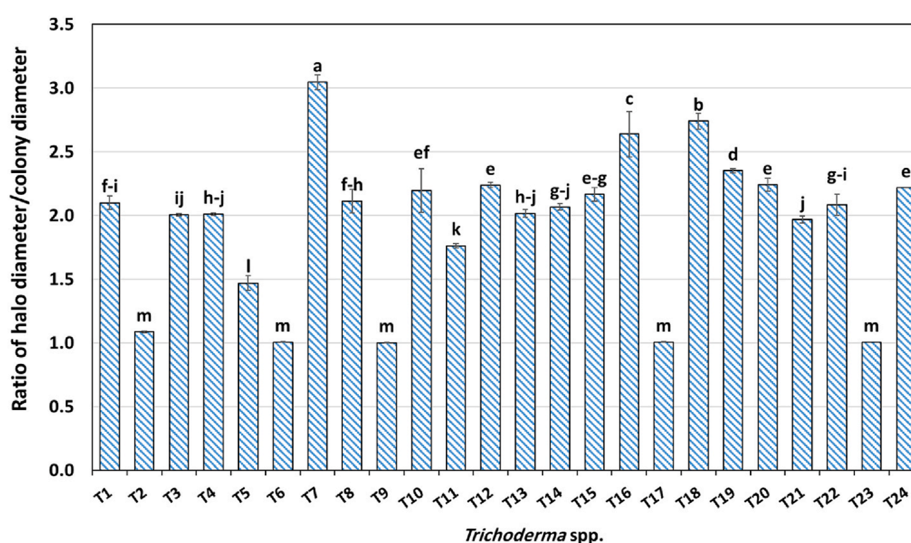

Fig. 1. Qualitative evaluation of different *Trichoderma* isolates for their xylanase activity using plate screening methods. Synthetic solid medium stained with the dye Congo red (0.4% w/v) after incubating at 28 °C for 3 days followed by 18 h at 50 °C. Different letters in each column indicate a statistically significant difference between different *Trichoderma* isolates.

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 °C.

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 [34], has been suggested to act as a physical barrier that limits the accessibility of the cellulase enzymes to the cellulose [35]. Thus, 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 the depolymerization of cellulose by cellulases within the fiber.

3.1. Qualitative screening of the best xylanase-producer strain

Fig. 1 shows the qualitative evaluation of xylanase enzyme production in our 24 different isolates of *Trichoderma* by the plate screening method. All the results of hydrolysis capacity in different isolates had 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.

3.2. Gamma radiation-induced mutation

The isolate code of T7 was selected as the best enzyme-producing species and a suspension of its fresh spore was exposed to different doses of gamma radiation (0–1000 Gy). The radiation rate on the used device was 4.5 k Gy/h. With an increased radiation dose, the fungal viability significantly decreased and the results are shown in Fig. 2. High doses of gamma radiation cause more DNA damage and then lead to fungal death. 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 [36,37]. The dose of 250 Gy was designated as an optimum dose of gamma irradiation-induced mutation that resulted in approximately 90–99 % (or 1–2 log cycle) reduction of spore sprouting on the PDA medium.

3.3. Screening of the best xylanase-cellulase producing mutant isolates

3.3.1. Qualitative screening of mutant isolates

The selected colonies from an optimum dose of gamma radiation (250 Gy) were subcultured three times on an MYG agar medium and were investigated after 7 d of incubation at 28 °C for screening of xylanase producer isolates. 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. 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 isolates, 17 mutant isolates were selected based on the ratio between the hydrolysis capacity of the mutant isolate (HCM) and the hydrolysis capacity of the wild-type strain (HCW) of more than 1.25 (Table 1). Also, M17 and M79 isolates were selected as mutants with hydrolysis capacity similar to the wild-type strain (control) and weaker than the control, respectively. Table 1 displays the results of

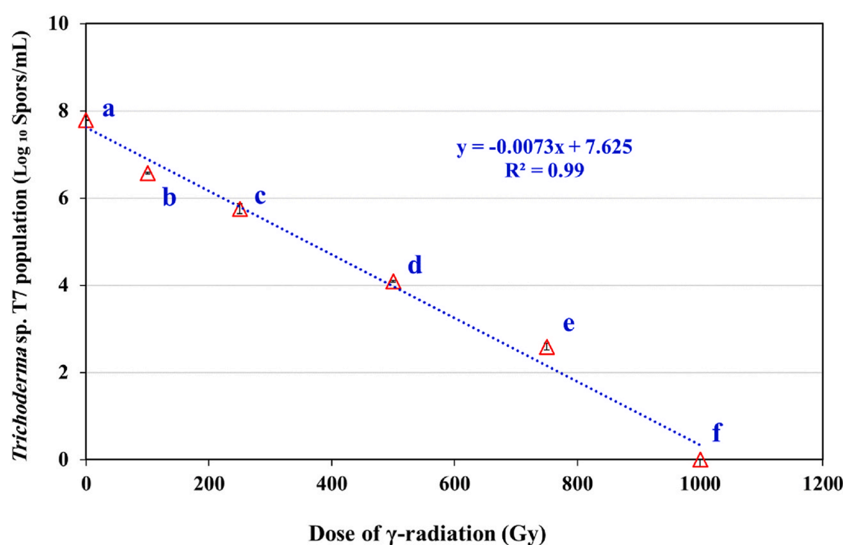

Fig. 2. Effect of gamma radiation doses on the viable count of *Trichoderma* sp. T7. Different letters in each symbol indicate a statistically significant difference between different doses of gamma-radiation.

comparing the mean hydrolysis capacity of mutant isolates (19 mutants) with the wild-type strain. All results had a statistically significant difference at the level $p < 0.05$. The highest hydrolysis capacity in qualitative screening tests was observed in mutant isolates of M56 (Fig. 3-b), M114, M89, M17, M62, and M44, respectively. Fig. 3(a–d) shows a comparison of the diameter of the xylan hydrolysis halo in the wild-type isolate and some of the superior mutant isolates. These results suggested that xylanase enzymes or cellulase enzymes affecting 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 can show a better comparison of the ability of mutant isolates to hydrolyze corn bran.

3.3.2. Quantitative screening of mutant isolates

The extracellular protein concentration was measured using the Bradford method in TFM and the results are shown in Table 2. All mutant isolates had significant differences ($p < 0.05$) in extracellular protein production. The changes in extracellular protein concentration varied from 188 to 274 μg/mL. The most extracellular protein concentration was observed in the TFM supernatant of M87 and other mutant isolates showed lower concentrations than the wild-type isolate.

The results of enzyme activity are revealed in Table 2, and all isolates had a statistically significant difference ($p < 0.05$). The Highest xylanase activity was observed in the M82 mutant isolate (12.40 ± 0.49 U/mL), which was approximately 2.5 times higher than the wild-type, and other mutant strains. Also, the results of cellulase activity are shown as international units (U), 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) was observed in mutant isolates of M44, M51, and M49, 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 M44 was approximately 3.79 times higher than the wild-type strain. Exo-glucanases or cellobiohydrolase (I and II) cleave the accessible reducing and non-reducing corn bran cellulose chain ends to liberate cellobiose and some glucose molecules [38]. Also, the highest EG activity was observed in mutant isolates of M44, M49, and M72, respectively. EGs can randomly hydrolyze internal glycosidic bonds in cellulosic cell walls of corn bran, especially in the amorphous regions of the cellulose chain. EG activity in the mutant isolate of M44 was approximately 1.63 times higher than the wild-type strain. Also, the mutant isolates of M76, M80, M69, M17, and M10 showed high β-glucosidase activity in comparison 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 corn bran synergistically. Synergism between EGs and exo-glucanases is the foremost broadly examined sort of synergy and is among the foremost quantitatively imperative for the hydrolysis of crystalline cellulose. The highest FPase or total cellulase activity (U/mL) was illustrated in mutant isolates of M72, M86, M44, M10, and M11, respectively. However, the mutant isolate of M44 could be the best superior mutant of cellulase producer due to that can produce a cellulase enzyme with higher activity in lower extracellular protein amounts, and a high level of total cellulase activity in this mutant isolate could be due to higher exo-glucanase activity and EG activity compared to other mutant isolates and the synergistic action of these enzymes on the cellulose chain.

3.4. The electrophoretic pattern of proteins

The variation and molecular weight distribution of enzyme-rich proteins in different superior mutants are shown in Fig. 4 (a, b).

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

Strains	Hydrolysis capacity	Ratio $HC_{Mutant}/HC_{Wild\text{-}type}$
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	$4.42^{e} \pm 0.02$	$1.32^{f\text{-}h} \pm 0.00$
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	$5.00^{b} \pm 0.05$	$1.50^{b} \pm 0.02$

[a]Different letters in each column indicate a statistically significant difference between different isolates.

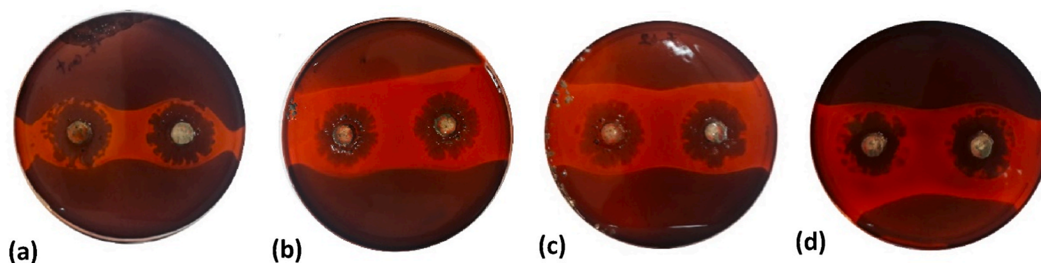

Fig. 3. Plates showing zone of hydrolysis of crude xylanase enzyme: *Trichoderma* sp. T7 (a) and (b) M56; (c) M82 and (d) M114.

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 corn bran as a substrate after 72 h fermentation at 28 °C and 150 rpm.

Strains	Protein (μg/ml)	Enzyme activity (U/ml)				
		Xylanase	Exo-glucanase	Endo-glucanase	β-glucosidase	Total cellulase
W. T.[a]	264.88 ± 9.60^{b}[b]	4.83 ± 0.17^{h}	1.96 ± 0.03^{j}	1.54 ± 0.08^{j}	0.47 ± 0.01^{j}	6.66 ± 0.25^{l}
M 10	242.73 ± 4.47^{c}	10.25 ± 0.94^{bc}	3.52 ± 0.38^{ef}	2.02 ± 0.15^{cd}	1.13 ± 0.03^{d}	10.66 ± 1.52^{bc}
M 11	200.96 ± 8.45^{ef}	$10.03 \pm 0.46^{b\text{-}d}$	3.67 ± 0.08^{de}	$2.00 \pm 0.16^{c\text{-}e}$	0.55 ± 0.02^{i}	10.32 ± 0.29^{cd}
M 17	243.71 ± 7.08^{c}	4.73 ± 0.82^{h}	3.16 ± 0.02^{gh}	$1.92 \pm 0.06^{d\text{-}h}$	1.19 ± 0.03^{c}	$7.97 \pm 1.19^{i\text{-}k}$
M 44	162.92 ± 4.09^{i}	9.91 ± 0.56^{cd}	7.44 ± 0.13^{a}	2.51 ± 0.03^{a}	0.68 ± 0.03^{h}	10.68 ± 0.06^{bc}
M49	202.73 ± 7.16^{ef}	8.66 ± 0.96^{e}	3.98 ± 0.15^{c}	2.04 ± 0.10^{c}	1.00 ± 0.03^{e}	$9.64 \pm 0.40^{d\text{-}g}$
M 51	222.53 ± 5.40^{d}	9.33 ± 0.23^{d}	3.00 ± 0.06^{h}	2.22 ± 0.04^{b}	0.68 ± 0.02^{h}	$9.47 \pm 0.14^{d\text{-}g}$
M 56	142.53 ± 1.05^{i}	7.96 ± 0.34^{ef}	2.94 ± 0.07^{h}	$1.92 \pm 0.11^{d\text{-}g}$	0.43 ± 0.00^{j}	$7.82 \pm 0.76^{i\text{-}k}$
M 62	226.65 ± 6.20^{d}	8.61 ± 0.78^{e}	3.52 ± 0.16^{ef}	$1.82 \pm 0.11^{g\text{-}i}$	1.14 ± 0.02^{d}	$8.38 \pm 0.96^{h\text{-}j}$
M 69	$196.16 \pm 8.21^{f\text{-}h}$	7.72 ± 0.96^{f}	3.33 ± 0.18^{fg}	$1.98 \pm 0.08^{c\text{-}e}$	1.24 ± 0.04^{b}	$9.21 \pm 0.54^{e\text{-}h}$
M 72	238.80 ± 2.70^{c}	10.63 ± 0.29^{b}	5.03 ± 0.17^{b}	2.03 ± 0.03^{c}	0.45 ± 0.00^{j}	11.86 ± 0.07^{a}
M 73	188.61 ± 9.78^{h}	9.35 ± 0.08^{d}	4.00 ± 0.09^{c}	$1.89 \pm 0.01^{e\text{-}h}$	0.80 ± 0.03^{g}	$8.74 \pm 0.15^{g\text{-}i}$
M 76	191.35 ± 2.10^{gh}	8.16 ± 0.30^{ef}	2.99 ± 0.14^{h}	1.76 ± 0.05^{i}	1.41 ± 0.06^{a}	7.76 ± 0.57^{jk}
M 79	206.45 ± 4.25^{e}	1.68 ± 0.16^{i}	1.81 ± 0.52^{j}	$1.94 \pm 0.04^{c\text{-}f}$	0.90 ± 0.02^{f}	2.73 ± 1.15^{m}
M 80	154.49 ± 6.14^{i}	6.90 ± 0.81^{g}	2.97 ± 0.07^{h}	$1.84 \pm 0.03^{f\text{-}i}$	1.26 ± 0.07^{b}	7.24 ± 0.13^{kl}
M 82	$198.31 \pm 4.96^{e\text{-}g}$	12.40 ± 0.49^{a}	3.91 ± 0.33^{cd}	$1.85 \pm 0.07^{f\text{-}i}$	0.43 ± 0.00^{j}	$8.95 \pm 0.15^{f\text{-}h}$
M 86	226.16 ± 4.73^{d}	9.54 ± 0.25^{cd}	3.49 ± 0.06^{ef}	1.81 ± 0.07^{hi}	0.99 ± 0.03^{e}	11.38 ± 1.37^{ab}
M 87	274.10 ± 3.50^{a}	10.69 ± 0.44^{b}	3.68 ± 0.05^{de}	$1.85 \pm 0.11^{f\text{-}i}$	0.87 ± 0.02^{f}	$9.79 \pm 0.10^{c\text{-}f}$
M 89	228.31 ± 10.25^{d}	9.54 ± 0.35^{cd}	3.26 ± 0.08^{fg}	$1.84 \pm 0.06^{f\text{-}i}$	0.65 ± 0.01^{h}	$10.10 \pm 0.29^{c\text{-}e}$
M 114	144.88 ± 18.72^{i}	7.62 ± 0.45^{f}	2.62 ± 0.36^{i}	$1.91 \pm 0.06^{e\text{-}h}$	0.43 ± 0.00^{j}	$8.39 \pm 1.02^{h\text{-}j}$

[a] Wild-type.
[b] Different letters in each column indicate a statistically significant difference between different mutant isolates.

The presence of diverse protein groups within the Mw extend of 11–135 kDa (kDa), indicates the diversity of enzymes with various Mw, which can hydrolyze corn bran cell wall compounds with different mechanisms. The proteins that weigh 17, 21, 32, and 43 kDa belong to a group of enzymes called xylanase (Xyl I, II, III, and IV, respectively) [39–43]. According to the densitometry results, the most Xyl I concentration (18 kDa) was secreted in the TFM supernatant of M11, M51, M49, M56, M62, M114, M72, M76, M69, M87, and M82 mutant isolates, respectively. However, the higher xylanase activity in M82 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 [44,45]. The results showed that the Cel12A protein band with a weight of 26 kDa was highest in the M82 lane when looking at the optical density analysis, 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. *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) [46,47], and five endo-glucanases, Cel5A (EG II, Mw 48 kDa) [48], Cel7B (EG I, Mw 50–55 kDa) [49], Cel12A (EG III, Mw 25 kDa) [50,51], Cel45A (EG V, Mw 23 kDa) [52], and Cel61A (EG IV, Mw 34 kDa; EC 3.2.1.4) [53].

Most *Trichoderma* samples (excluding mutant M79) had two enzyme bands called CBH I and CBH II. The highest optical density of CBH protein bands (CBH I and II) was observed in mutant isolates M44, M86, M87, M82, M11, M73, and M89, respectively. The highest secreted proteins of CBH II (62 kDa) and Cel5A (EG II, 48 kDa) were observed in the mutant isolate M44, as confirmed by the results obtained from exo-glucanase and endo-glucanase enzyme activity, respectively. Also, the Cel 3A protein band (which weighs 73 kDa and is also called BGL I) was present in all the profiles of *Trichoderma*, except for one called M76. The highest Cel3A (BGL I) protein concentration was secreted in the TFM supernatant of M51, M62, M49, M56, M82, M62, and M86 mutant isolates, respectively. But Cel 1A (BGL II) weighted 110 kDa and was observed in the protein profile of all the mutant isolates.

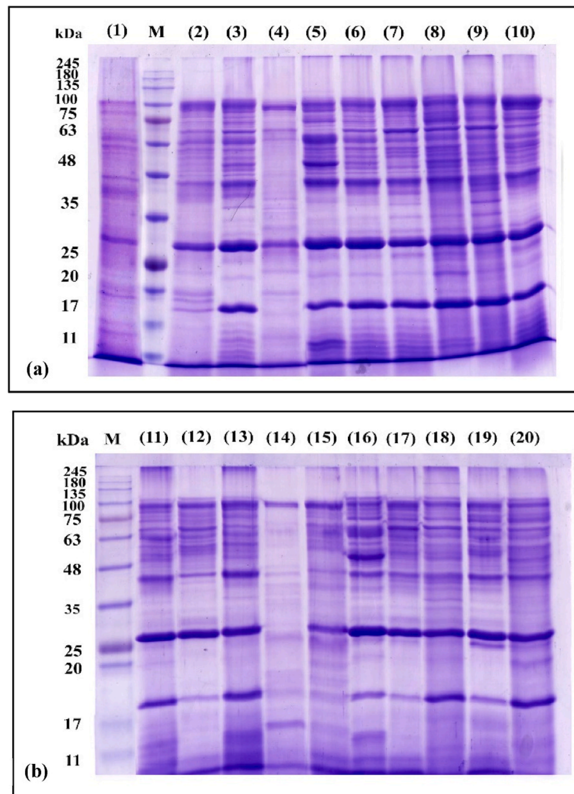

Fig. 4. Protein profile analysis of extracellular protein secreted in the supernatant of the TFM: (a:1) wild-type strain T7, (a: 2–10 & b:11–20) mutant isolates M10, M11, M17, M44, M49, M51, M56, M62, M72, M73, M76, M79, M80, M82, M86, M87, M89 and M114, respectively during fermentation time 72 h and pH of fermentation medium 5.5. (M) Protein molecular weight marker in the range of KDa 11–245.

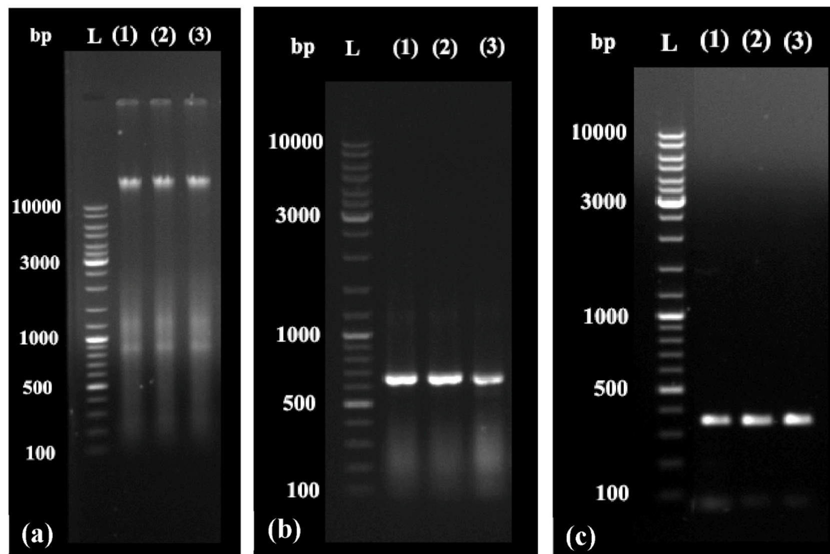

Fig. 5. Agarose gel electrophoresis of DNA extraction (a), PCR products amplification of the fungal isolates: The internal transcribed spacer (ITS) regions of the ribosomal DNA (b), and the translation elongation factor 1-α encoding gene (TEF1-α) (c); L: Molecular weight marker (100 bp-10 kb); (1, 2 and 3): DNA or PCR product of the *Trichoderma* sp. T7 and its mutant isolates NAS107-M44, and NAS107-M82, respectively.

3.5. Identification of the best Trichoderma strain and its mutant isolates

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 [54].

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. The quantity and quality of extracted DNA were examined by spectrophotometry and agarose gel electrophoresis (Fig. 5-a).

After the extraction operation, PCR amplification 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 isolates and sequenced for Trichoderma identification with approximately 650 and 350 bp, respectively (Fig. 5-b, 5-c). Sequence analysis of the ITS-rDNA region and TEF-1α is widely used for the precise reorganization of Trichoderma species [55]. The forward and reverse

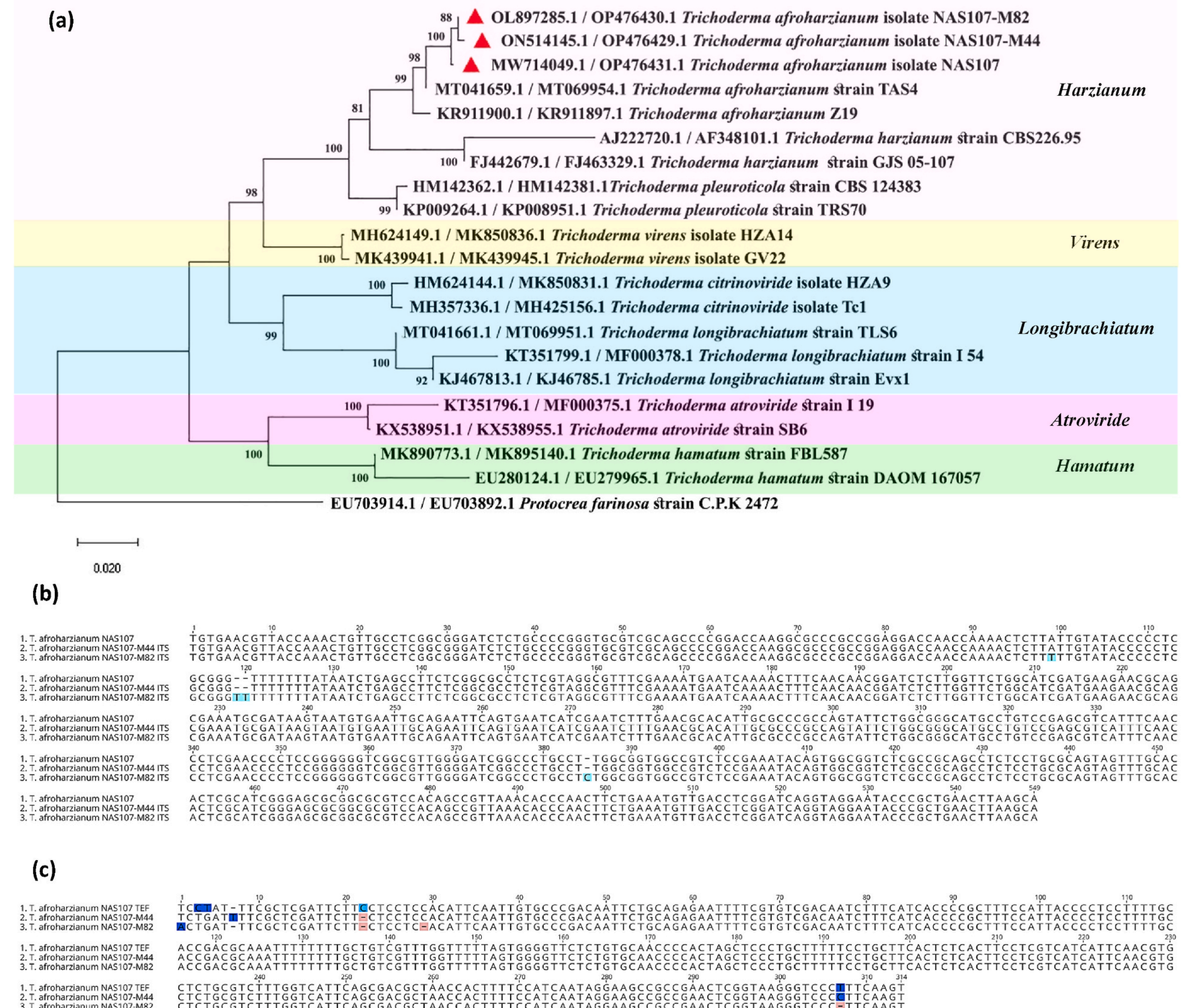

Fig. 6. Sequence and phylogenetic analysis of *T. afroharzianum* NAS107 (or T7 isolation code) and its superior mutants. (a) Maximum likelihood dendrogram based on the internal transcribed spacer (ITS) and the translation elongation factor 1-α encoding gene (TEF1-α) regions gene nucleotide sequences. Bootstrap values (in percentages) are based on 1000 replicates for each bootstrap value using the MEGA 10.0 software. Symbols in red triangles phylogeny represent our isolates. IBRC-M No.: The microbial collection code of the Iranian Biological Resource Center. Multiple sequence alignment of *T. afroharzianum* NAS107 (or T7) and its superior mutants ((b): ITS-rDNA region, and (c): TEF1-α region) showing conserved sequences, and genetic variations with "highlights". The multiple sequence alignment was performed using the ClustalX 1.83 program.

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.

Using bioinformatics tools, *T. afroharzianum* NAS107 was identified for *Trichoderma* sp. T7 and its mutant isolates and the phylogenetic relationship of our *Trichoderma* and other isolates are shown in Fig. 6(a–c). 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, 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 isolate of *Trichoderma* T7 isolate and its mutant isolates (M44 and M82) were registered in the microbial collection of Iranian Biological Resources Center (IBRC) with accession numbers IBRC-M 30594 (*T. afroharzianum* NAS107), IBRC-M 30595 (*T. afroharzianum* NAS107-M44), and IBRC-M 30594 (*T. afroharzianum* NAS107-M82), respectively.

4. Discussion

Trichoderma is one of the most well-known cellular filamentous fungi, which a complex of extracellular enzymes for the degradation of corn bran waste, easily was produced. Xylanase-cellulase complexes from a variety of organisms with different characteristics are used industrially in many applications, such as in the food and baking industry. We evaluated different isolates of *Trichoderma* for their hydrolysis potential of arabinoxylan compounds and corn bran through quantitative and qualitative screening and among them, *Trichoderma* isolate 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 in the GenBank database at the NCBI. Using genotypic identification, the T7 wild type strain and its mutant isolates were identified as *T. afroharzianum* NAS107.

Mutation or improvement of microbial strains is the cornerstone of any commercial fermentation of enzyme process. In most cases, it influences the overall economics of enzyme production because it allows an organism to carry out a biotechnological process more efficiently. For instance, a large number of the high-secreting mutants offer suitable strains for specific industrial objectives [56], such as the overproduction of antibiotics [57,58], and enhanced production of cellulase enzymes [59–61], lipases [62], citric acid [63] and bioethanol [64,65].

Several mutants of the best wild-type isolate (*T. afroharzianum* NAS107 or T7) were generated using gamma radiation-induced mutation to increase xylanase-cellulase production. 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 destroys the DNA structure of microorganisms and could lead to deactivation [66], while sometimes incomplete inhibition by less cellular alteration leads to the induced mutation of cells [67]. A high dose of gamma irradiation can affect directly the DNA of *Trichoderma*, and can inhibit growth or reproduction [68]. When ionizing beams interact with water particles in *Trichoderma* cells, it can hurt the DNA and influence how the living being works because of free radicals. Moreover, other factors such as the number or density of spores, can also affect the sensitivity or tolerance of *Trichoderma* to gamma radiation [56,57].

Our results showed that, statistically significant changes in xylanase-cellulase production of *T. afroharzianum* NAS107 (or T7 isolate) 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 [69].

We found that the gamma radiation mutants had higher levels of xylanase and cellulase activity compared to the normal strain. The highest extracellular enzyme activity was observed in mutant isolates of *T. afroharzianum* NAS107-M82, and *T. afroharzianum* NAS107-M44, in which their xylanase and cellulase activities were approximately 2.5 and 1.6 times higher than wild-type strain, respectively. In these mutant isolates, exo-glucanase (CBH I, cell 7A), endo-glucanase (EG III, Cel 12A), and xylanase (Xyl I) hydrolyzed the corn 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 [39,41]. Xylanase III (Mw of 32 kDa and pI 9.1) belongs to 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) was observed at pH 6.0–6.5 and 3.5–4.0, respectively [70]. 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 I, with high specific activity, and optimal temperature and pH of 45 °C and 5, respectively.

The entire group of enzymes that break down the cellulose structure of corn bran is called the cellulase system. This system includes CBHes, EGes, and cellobiases, which work synergically to break down the corn 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, corn bran cellulose crystallinity plays a critical role in its enzymatic hydrolysis. The presence of amorphous and crystalline regions in corn bran cellulose structure induces the *T. afroharzianum* NAS107 (or T7 isolate) 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-M44, 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-M44 and *T. afroharzianum* NAS107-M82 have both enzyme bonds of CBH I and CBH II. The high amount of exo-glucanase activity in *T. afroharzianum* NAS107-M44 was due to the high secretion of cellobiohydrolases. The activity of CBH I and CBH II results in a gradual decrease in the degree of polymerization (DP) of corn bran cellulose and produces cellobiose. The high production of CBH II and Cel5A (EG II) proteins in this mutant isolate, resulted in a maximum synergy of total cellulase. Comparing cellulase activity in different mutant isolates, especially *T. afroharzianum* NAS 107-M44, showed that optimal corn bran degradation is usually obtained with a large amount of exo-enzyme and a minor amount of endo-enzyme [71].

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 corn 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-M44 is due to the presence of CBH and EG enzymes and their cooperative behavior or synergism of them.

The results of enzyme activity indicated that the corn bran has a good substrate for xylanase-cellulase production by *T. afroharzianum* NAS107-M44 and NAS107-M82. Also, another study has reported that corn bran is a good substrate for the production of Acetyl xylan esterase enzymes by filamentous fungi [72].

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 can create helpful mutations in fungi, and using this method may lead to improved industrial fungi varieties. According to our study, two mutant isolates of *T. afroharzianum* NAS 107-M82 or *T. afroharzianum* NAS107-M44 could be a successful extracellular enzyme producer candidate for the bioconversion of corn bran wastes into xylanase-cellulase enzymes. Therefore, there could be improved strains by gamma radiation-induced mutation as a biological agent for xylanase-cellulase enzyme production. This method can also be used in other fungi to create new types of mutants with various uses.

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Data availability statement

Data will be made available on request.

Additional information

Genomes in this study are released in the Science Data Bank NCBI (https://www.ncbi.nlm.nih.gov/nuccore/) with GenBank accession numbers shown in material and methods.

CRediT authorship contribution statement

Hamed Askari: Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Investigation, Data curation. **Sabihe Soleimanian-Zad:** Project administration, Methodology. **Mahdi Kadivar:** Project administration, Methodology. **Samira Shahbazi:** Project administration, Methodology, Investigation.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Hamed Askari reports administrative support, article publishing charges, equipment, drugs, or supplies, statistical analysis, and writing assistance were provided by College of Agriculture, Isfahan University of Technology, Iran. Sabihe Soleimanian-zad reports equipment, drugs, or supplies and statistical analysis were provided by College of Agriculture, Isfahan University of Technology, Iran. Mahdi Kadivar reports equipment, drugs, or supplies and statistical analysis were provided by College of Agriculture, Isfahan University of Technology, Iran. Samira Shahbazi reports equipment, drugs, or supplies and statistical analysis were provided by Nuclear Science and Technology Research Institute, Atomic Energy Organization of Iran (AEOI), Iran. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e28349.

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