# Restoration of Post-Fire Forests by Recombinant DNA Technology

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**KEYWORDS**

Forest fire
Climate change
Pyrophilic fungi
PyOM
Recombinant DNA
Enzyme purification

**ABSTRACT**

Forest fires, increasingly exacerbated by climate change, disrupt ecosystems, deplete biodiversity, and alter soil environments through the formation of pyrolyzed organic materials (PyOMs). Pyrophilous fungi, specifically *Morchella eximia*, possess enzymes such as endo-1,3-β-glucanase, endo-1,4-β-xylanase, and endo-1,4-β-mannosidase that can degrade PyOMs, contributing to nutrient cycling and ecological recovery. This study explores the recombinant cloning of these enzymes into *Saccharomyces cerevisiae* to evaluate their expression and efficiency in metabolizing PyOMs. Genes were amplified, cloned into the pRS327 plasmid and transformed into yeast, with optimal protein expression and purification confirmed. Activity assays demonstrated the potential of these enzymes, particularly endo-1,4-β-xylanase, in addressing post-fire soil remediation. The findings highlight the feasibility of leveraging pyrophilous fungi and their enzymes for bioremediation, offering a pathway to accelerate ecosystem recovery after wildfires while considering biodiversity and ecological dynamics.

## INTRODUCTION

Forest fires have been occurring in an increased fashion over the past decade. Weather and climate are the most prominent factors that influence forest fires and they are both affected by human-caused climate change [1]. Some of the most destructive forest fires in recent years took place in 2019-2020 in Australia, burning down millions of hectares, destroying habitats and impacting billions of wild animals [2]. Forest fires in Mediterranean countries like Türkiye and Greece caused the destruction of forests and concern among residents, leading to misguided measures. The uncertainty and fear caused by fires can leave long-term emotional burdens on communities while witnessing the destruction of ecosystems can increase environmental anxiety. These effects require serious interventions at both individual and societal levels. Kemer indicates that forests should not just be considered as clumps of trees but as ecosystems, and restoration policies should be implemented by considering ecological dynamics. He further indicates that one of the variables determining how natural systems evolve is forest fires and they can be positive factors which enable a new beginning and biodiversity as long as they occur at intervals and on scales that are within their capacity for self-renewal [3]. However, forest fires occurring in an increasingly frequent regime because of climate change and human-caused factors may cause them to exceed these intervals and disrupt the regeneration capacity of forests.

Wildfires affect the soil environment greatly by changing the composition of organic compounds

found in the soil, forming pyrolyzed materials and reducing biodiversity [4]. Chatterjee et al. predict that increasing wildfire frequency and warming climates will lead to a rise in PyOM production. PyOMs are highly stable and they persist in the environment for prolonged periods. They possess a heterogeneous structure with high proportions of N, O and S elements. Their polyaromatic hydrocarbon structures are what make these materials so persistent and hard to degrade. Their structures differ according to the temperature of pyrolysis and the composition of materials that are being pyrolyzed. If pyrolysis occurs at higher temperatures, the content of aromatic carbon structures in the composition of PyOM increases and the material becomes harder to degrade and more stable. PyOM comprises a significant part of the soil organic carbon pool and is a major sink for atmospheric CO2 [5].

Fungi are integral parts of most ecosystems and there exists a specific type of fungi called pyrophilous (fire-loving) fungi, such as Genus *Morchella,* which increase in number after forest fires. These fungi can degrade PyOMs and consequently, they can shape the microbial succession process after the fire. Steindorff et al. point out that pyrophilous fungi have expanded gene families encoding carbohydrate-active enzymes (CAZymes) which allow efficient utilization of burnt biomass and degrade PyOM. CAZymes include glucanases, xylanases and mannanases; which can break down complex polysaccharides like cellulose, hemicellulose, and lignin. This makes them essential in carbon recycling, soil nutrient cycling, and microbial growth on organic substrates. Regarding the fact that the biomass of soil microorganisms reduces significantly in post-fire environments, increasing the activity of CAZymes produced by pyrophilous fungi can play a crucial role in maintaining biodiversity and enabling bioremediation after wildfires [4]. Among the commonly known CAZymes, endo-1,4-beta-xylanase targets hemicellulose, a major component of plant biomass and an abundant substrate in PyOMs while endo-1,3-beta-glucanase degrades beta-glucans, which may persist in fire-affected environments as part of fungal cell walls or pyrolyzed biomass. Endo-1,4-beta-mannosidase focuses on breaking down mannan, a complex polysaccharide found in plant cell walls.

This paper aims to propose a way to restore ecosystems that were damaged by wildfires while taking biodiversity and ecological dynamics into account. With the recombinant cloning of endo-1,4-beta-xylanase, endo-1,3-beta-glucanase and endo-1,4-beta-mannosidase genes from *M. eximia* to BY4742 strain of *Saccharomyces cerevisiae* by the pRS327 plasmid; it is targeted to explore the potential of selected enzymes and pyrophilic fungi, chosen for their known roles in biomass degradation and post-fire adaptation. Further objectives of this paper are to develop a baseline understanding of the activity of chosen enzymes against PyOM and to increase the effectiveness of their activity in response to fire.

## METHODS AND MATERIALS

### Extraction of Mushroom

A wildfire-affected forest is the source for the collection of *Morchella* fungi shortly after the fire, ensuring the acquisition of these specimens. Subsequently, optimized growth conditions are implemented to facilitate the production of target enzymes, namely xylanase, mannanase, and glucanase. The yield of the selected enzymes is verified through enzyme activity assays. For initial cultivation, nutrient-rich media such as Potato Dextrose Agar (PDA) or Malt Extract Agar (MEA) can be employed. The addition of target substrates,

such as xylan, mannan, or cellulose, as carbon sources to the media serve to induce enzyme production. RNA isolation will be performed from the obtained enzymes.

Identification and Characterization of the Gene of Interest

During RNA isolation from *M. eximia* fungi, samples were rapidly frozen and ground in liquid nitrogen to prevent RNA degradation. The isolation procedure was performed using the TRIzol™ Plus RNA Purification Kit [6]. 1 mL of TRIzol Reagent was added to 100 mg of the tissue sample, and the sample was homogenized using a homogenizer. Following homogenization, the mixture was incubated at room temperature for 5 minutes to allow for complete dissociation of proteins and nucleic acids. Subsequently, 0.2 mL of chloroform was added, and the tube was gently shaken for 15 seconds. The mixture was incubated at room temperature for 2 minutes and then centrifuged at 12,000 × g for 15 minutes at 4°C.

After centrifugation, the mixture was separated into three distinct phases. The uppermost colourless phase contained the RNA. 600 µL of this colourless phase was carefully transferred to a new tube using a micropipette. An equal volume of 70% ethanol was added to the transferred sample, and the mixture was thoroughly mixed by vortexing.700 µL of this mixture was transferred to a spin cartridge. The cartridge was centrifuged at 12,000 × g for 15 seconds, and the flow-through was discarded. The spin cartridge was returned to the same collection tube. Subsequently, 700 µL of Wash Buffer I was added to the cartridge and centrifuged at 12,000 × g for 15 seconds. This process was repeated with 500 µL of Wash Buffer II. The cartridge was centrifuged at 12,000 × g for 1 minute to dry the membrane. The collection tube was discarded, and the spin cartridge was placed in a recovery tube.

RNase-free water (100 µL each) was added to the centre of the cartridge three times for a total elution volume of 300 µL. Before each elution, the cartridge was incubated at room temperature for 1 minute and then centrifuged at 12,000 × g for 2 minutes. This yielded purified RNA, which was stored at -80°C until the DNase I treatment step. DNase I treatment was performed using the Thermo Fisher Scientific TURBO DNA-free™ Kit [7]. 0.1 volume of 10X TURBO DNase™ buffer and 1 µL of TURBO DNase™ enzyme were added to the RNA, followed by gentle mixing. The mixture was incubated at 37°C for 25 minutes. The tube was gently tapped to resuspend the solution. 5 µL of DNase Inactivation Reagent was added to the resuspended mixture. The sample was incubated at room temperature for 5 minutes. During the incubation period, the tube was tapped gently 2–3 times to keep the DNase Inactivation Reagent in suspension. The samples were centrifuged at 10,000 × g for 1.5 minutes, and the RNA-containing supernatant was carefully transferred to a new tube, being careful not to disturb the DNase Inactivation Reagent pellet.

The quality of the isolated RNA was assessed by measuring absorbance values at 260, 280, and 230 nm using a spectrophotometer, and the 260/280 nm and 260/230 nm ratios were examined. Further assessment was performed via electrophoresis (agarose gel electrophoresis).

The mRNA isolation from the total RNA was performed using the Thermo Fisher Dynabeads™ mRNA Purification Kit [8]. The volume of the total RNA sample (75 µg) was adjusted to 100 µL with 10 mM Tris-HCl (pH 7.5). The sample was heated at 65°C for 2 minutes to denature secondary structures and then placed on ice until further use. 200 µL (1 mg) of resuspended Dynabeads™ magnetic beads were transferred to a microcentrifuge tube. The tube

was placed on a magnet, and the Dynabeads™ beads were allowed to adhere to the tube wall (approximately 30 seconds). The supernatant was discarded, the tube was removed from the magnet, and 100 µL of Binding Buffer (20 mM Tris-HCl (pH 7.5), 1.0 M LiCl, and 2 mM EDTA) was added to equilibrate the beads. The tube was placed back on the magnet, and the supernatant was discarded. Then the tube was removed from the magnet. 100 µL of Binding buffer was added to the Dynabeads™ magnetic beads. A 1:1 ratio was established between the sample volume and Binding buffer to ensure optimal hybridization conditions. If the total RNA was more diluted than 75 µg/100 µL, the binding buffer was added to the beads equivalent to the sample volume. The total RNA was added to the Dynabeads™/binding buffer suspension. The mixture was thoroughly mixed and rotated on a rotary mixer or roller for 3–5 minutes at room temperature to allow the mRNA to bind to the oligo(dT)25 sequences on the beads. Subsequently, the tube was placed on a magnet and left until the solution cleared. The supernatant was discarded. The tube was removed from the magnet, and the mRNA-bead complex was washed twice with 200 µL of washing buffer.

After each washing step, all traces of supernatant were removed using the magnet. To elute the mRNA, 10–20 µL (or a minimum of 5 µL) of 10 mM Tris-HCl (pH 7.5) was added. The sample was heated between 65°C and 80°C for 2 minutes and immediately placed on the magnet. The isolated mRNA was transferred to a new RNase-free tube.

Sequences of endo-1,3-beta-glucanase, endo-1,4-beta-xylanase and endo-1,4-beta-mannosidase genes present in *Morchella eximia* were obtained from the Mycocosm website (https://mycocosm.jgi.doe.gov/). The list of suitable restriction enzymes was acquired by uploading the sequences to the Addgene website (https://www.addgene.org/).

Vector and host

*S. cerevisiae* was chosen as the host organism because it can perform post-translational modifications; thus it makes sure that the fungal enzymes fold and function properly. BY4742 strain which lacks the *LYS2* gene was chosen specifically [9]. The *LYS2* gene encodes for the enzyme α-aminoadipate reductase which is essential for converting α-aminoadipate semialdehyde into saccharopine in the lysine synthesis pathway.

pRS327 plasmid with AY150810 GenBank Accession No. was selected as the yeast expression vector [10]. It is a multicopy (YEp) vector with *LYS2* as the marker gene which enables selection on a medium that is deficient in lysine which is an essential amino acid required for protein synthesis [11]. All the available restriction enzyme recognition sites on the plasmid and the sequences of genes of interest were scanned through the Addgene website. *HindIII* and *NotI* enzymes were used in the cloning of endo-1,3-beta-glucanase and endo-1,4-beta-mannosidase while *EcoRI* and *SacI* enzymes were used for the cloning of endo-1,4-beta-xylanase. Forward and reverse primers were designed with the necessary restriction enzyme recognition sites at their 5’ ends to facilitate cloning into the pRS327 vector, as can be seen in Table 1.

Reverse transcription of purified mRNA

Reverse transcription of isolated mRNAs can be conducted by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™) with oligo(dT) primers [12]. Conditions were optimized regarding the kit protocol. mRNA samples were quantified, and 500 ng to 1 µg of total mRNA was used for each reaction. The reaction mixture was prepared in a total volume of 20 µL, comprising 2 µL

of 10× reverse transcription buffer, 0.8 µL of 25× dNTP mix (100 mM), 2 µL of 10× oligo(dT) primers, 1 µL of Multiscribe Reverse Transcriptase (50 U/µL) and nuclease-free water to adjust the final volume. The prepared reaction mixture was subjected to reverse transcription under the following thermal cycling conditions: 25°C for 10 minutes for primer annealing, 37°C for 120 minutes for cDNA synthesis, and 85°C for 5 minutes to inactivate the reverse transcriptase enzyme. The synthesized cDNA was used directly for PCR amplification

PCR Amplification

The amplification of target genes from the synthesized cDNA was performed by using the 2X PCR Master Mix kit (Thermo Scientific™) [13]. The reaction mixture for each PCR reaction was prepared in a final volume of 25 µL and consisted of 12.5 µL of 2× PCR Master Mix, 0.5 µL of forward primer (10 µM), 0.5 µL of reverse primer (10 µM), 1–2 µL of cDNA template, and nuclease-free water to achieve the final volume. PCR amplification was carried out in a thermal cycler under the following conditions: initial denaturation at 95°C for 2 minutes, followed by 25–35 cycles of denaturation at 95°C for 30 seconds, annealing at temperatures indicated in Table 1 for 30 seconds, and extension at 72°C for 1 minute per kilobase of the target sequence. A final extension step was performed at 72°C for 5 minutes, and the reaction was held at 4°C until further analysis.

The PCR product was analyzed by agarose gel electrophoresis to ensure that the obtained fragments were in the expected size. A 1–2% agarose gel containing SYBR Safe DNA Gel Stain (Invitrogen™) was prepared according to the manufacturer's protocol [14]. For each sample, 5 µL of the PCR reaction product was mixed with 1 µL of 6 × loading dye and loaded into the gel alongside a DNA ladder. Electrophoresis was conducted at 100 V for 40 minutes in 1× TAE buffer, and the gel was visualized using UV light. Amplified products were examined for expected sizes.

Purifying the PCR product

The purification of PCR products was performed using the QIAquick Gel Extraction Kit (Qiagen™), following the manufacturer's protocol with modifications [15]. The desired bands removed from the agarose gel were transferred to 1.5 mL microcentrifuge tubes, and the mass of each gel slice was recorded. To dissolve the gel slices, Buffer QG was added. The mixture was incubated at 50°C for approximately 10 minutes, during which the tubes were gently vortexed at regular intervals. Once the gel fragments were fully dissolved, an additional volume of isopropanol equivalent to the gel slice was added. The entire solution was applied to a QIAquick spin column. After centrifugation at 13,000g for 1 minute, the flow-through was discarded, and the spin column was washed twice with 750 µL of Buffer PE followed by centrifugation at 13,000 g for 1 minute. The spin column was then centrifuged again for an additional 1 minute to remove any residual ethanol present in the column. To elute the purified DNA, the spin column was transferred to a clean 1.5 mL microcentrifuge tube, and 30 µL of Buffer EB, pre-warmed to 65°C, was applied directly to the centre of the column membrane. The column was incubated at RT for 1 minute and then centrifuged at 13,000g for 1 minute. The eluted DNA was quantified using a spectrophotometer to assess its concentration and purity.

**Table 1:** Forward and reverse primers, and restriction enzymes used for endo-1,3-beta-glucanase, endo-1,4-beta-xylanase and endo-1,4-beta-mannosidase genes are given. Annealing temperatures for each primer were calculated by using the ThermoFisher website (https://www.thermofisher.com/**).** Bold characters indicate restriction enzyme recognition sequences. Red characters indicate the start and stop codons that were added during the primer designing process.

| **Enzymes** | **Forward Primer** | **Reverse Primer** | **Annealing Temperature** |
|---|---|---|---|
| **endo-1,3-β-glucanase** | 5'-xxxxxx**AAGCTT**ATGTCTTCATTCTGTCCATACACGG-3' | 5'-xxxxxx**GCGGCCG****C**TAACTACTTACCAGCCGCTTGTGCCAAATA-3' | 65.6 °C |
| **endo-1,4-β-xylanase** | 5'-xxxxxx**GATTC**ATGGGGAGCAATTAGACCCACTTATA-3' | 5'-xxxxxx**GAGCTC**TAATATAATCATCGACCCCATTGTTTA-3' | 60.9 °C |
| **endo-1,4-β-mannosidase** | 5'-xxxxxx**AAGCTT**ATGCACACTCATTCTTTACACTTAT-3' | 5'-xxxxxx**GCGGCCG****C**TAAGCCATATATATTTTTTCTAGAGTAC-3' | 56.5 °C |

Digestion and Ligation

For each digestion reaction, 1 µg of DNA or vector DNA was mixed with 1 µL of each restriction enzyme (HindIII and NotI for endo-1,3-beta-glucanase and endo-1,4-beta-mannosidase; EcoRI and SacI for endo-1,4-beta-xylanase) in a total volume of 20 µL. The reaction mixture included 2 µL of the appropriate 10× restriction enzyme buffer and nuclease-free water to adjust the final volume. The digestion reactions were incubated at 37°C for 1–2 hours to ensure complete digestion. After gel electrophoresis, the samples were purified using the QIAquick Gel Extraction Kit.

To ligate, for each reaction, approximately 100 ng of the digested vector was combined with the insert at a 1:3 molar ratio (vector to insert) in a 20 µL reaction mixture. The mixture included 2 µL of 10× T4 DNA Ligase Reaction Buffer [16] and 1 µL of T4 DNA Ligase (Thermo Scientific™, 5 U/µL) [17]. The ligation reactions were incubated overnight at 16°C.

Transformation

The LiAc/SS-Carrier DNA/PEG transformation method was performed. Yeast strains were grown on YPAD agar at 30°C overnight. SS-Carrier DNA was prepared from salmon sperm DNA in 100 mL tris-EDTA buffer and stored at -20°C. PEG 3350 (50% w/v) and LiAc (1.0 M) were dissolved in water, autoclaved, and stored at RT [18]. Before the transformation, SS-Carrier DNA was boiled for 5 minutes and chilled on ice.

Yeast samples were suspended in water, centrifuged, and the supernatant discarded. Transformation solutions were added in order: 240 µL

of PEG 3350, 36 µL of LiAc, 50 µL of SS-Carrier DNA, and 34 µL of plasmids. The mixture was incubated at 42°C for 1 hour, centrifuged, and the supernatant discarded. Cells were resuspended in water and transferred to lysine-deficient growth media. After incubation at RT for 2 days, yeast with plasmids was isolated [19].

A Zymoprep Yeast Plasmid Miniprep II kit was used for plasmid isolation. Yeast samples were mixed with Solution 1 and incubated at 37°C for 15–60 min. Solutions 2 and 3 were added sequentially with thorough mixing. After centrifugation, the supernatant was transferred to spin columns, which were washed and spun with Tris-EDTA to elute plasmids. Plasmids were digested with restriction enzymes and sequenced to confirm the gene of interest.

Optimization of Protein Expression and Purification

Synthetic media (SM) supplemented with 2% glucose, 2% raffine, 2% glycerol ammonium sulfate (1% w/v), amino acid supplements without lysine with yeast nitrogen base to ensure robust growth and protein production. The yeast were incubated at 30°C, pH of 6 for 36 hr. The yeast cells, were then, centrifuged at 4°C for 5 min, at 6000 g. The supernatant was discarded and the pellet was resuspended in the selected optimum buffer. Cell lysis is performed by vortexing with a glass bead.

For purification, his tags were incorporated for immobilized metal affinity chromatography (IMAC). HisTrap column was used and equilibrated with a binding buffer (20 mM imidazole, 500 mM NaCl, 50 mM Tris-HCl, pH 8.0). After washing with 50 mM imidazole to remove non-specific binders, the target proteins were eluted with 250–500 mM imidazole. For further purification, gel-filtration chromatography is used with Superdex 200 column with a buffer containing 50 mM Tris-HCl (pH 8.0) and 150 mM NaCl. Elution fractions were monitored via SDS-PAGE to confirm protein purity [20].

Characterization of the Proteins

The protein characterization of endo-1,3-β-glucanase, endo-1,4-β-xylanase, and endo-1,4-β-mannosidase, SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) were carried out to determine the molecular weight and purity of these enzymes. The proteins were first denatured by SDS, then loaded onto a polyacrylamide gel and subjected to an electric field. After electrophoresis, the gel was stained with Coomassie Brilliant Blue to visualize the protein bands [21].

Activity Tests of the Enzymes

The method to assess the activity of endo-1,3-β-glucanase, endo-1,4-β-xylanase, and endo-1,4-β-mannosidase enzymes was the spectrophotometric activity assay. Laminarin for endo-1,3-β-glucanase, xylan for endo-1,4-β-xylanase, and mannan for endo-1,4-β-mannosidase were prepared as substrates for the enzymes. The enzymes were dissolved buffers and mixed with the substrate to start the reaction, incubated at 50°C. As the reaction progressed, the breakdown products, sugars, were measured spectrophotometrically at 590 nm. Absorbance changes were correlated with enzyme activity, and the results were expressed as “enzyme units” [22].

## RESULTS

High-quality RNA was successfully isolated from *M. eximia* using the TRIzol™ Plus RNA Purification Kit. To assess the purity and quality of the isolated RNA, absorbance measurements were taken at 260, 230, and 280 nm. The 260 nm reading represents the wavelength at which nucleic acids exhibit maximum absorbance, while proteins show maximum absorbance at 280 nm. An ideal 260/280

nm ratio is close to 2.0. A value lower than 2.0 indicates protein or phenol contamination [23]. At 230 nm, other organic contaminants exhibit maximum absorbance, and the 260/230 nm ratio should also ideally, be approximately 2.0. A lower value suggests the presence of organic contaminants [24]. Following these measurements, RNA quality was further verified by agarose gel electrophoresis. It was anticipated that the rRNA, constituting a significant portion of the RNA, would produce distinct bands, with the upper band (larger subunit) appearing at 28S and the lower band (smaller subunit) at 18S. The observation of these 28S and 18S rRNA bands confirmed the integrity and high quality of the RNA [25].

mRNA was subsequently isolated from the total RNA using the Thermo Fisher Dynabeads™ mRNA Purification Kit. Optimized binding and washing conditions ensured selective isolation of polyadenylated mRNA, confirmed by efficient elution and purity checks.

Reverse transcription of mRNA to cDNA was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™) with oligo(dT) primers to specifically target the poly-A tails. The reverse transcription reaction yielded high-quality cDNA, as confirmed by PCR amplification of target genes.

DNA sequences encoding the target genes, endo-1,3-beta-glucanase, endo-1,4-beta-xylanase, and endo-1,4-beta-mannosidase, were amplified from the cDNA by PCR. Specific primers designed for each gene ensured accurate amplification of the target regions. Post-PCR, the products were analyzed by agarose gel electrophoresis and bands of the expected sizes were observed. The absence of non-specific amplification and primer dimers validated the specificity and efficacy of the primers and reaction conditions.

The PCR-amplified cDNA sequences were cloned into the pRS327 plasmid vector using appropriate restriction enzymes. Specific restriction enzymes were selected for each gene in this process. HindIII and NotI enzymes were used for cloning the endo-1,3-beta-glucanase and endo-1,4-beta-mannosidase genes. EcoRI and SacI were chosen for cloning the endo-1,4-beta-xylanase gene. These enzymes generated compatible cohesive ends by cleaving specific sequences in both the cDNA sequences and the pRS327 plasmid vector, thereby providing suitable conditions for the ligation reaction.

The vector and target cDNA sequences digested with restriction enzymes were ligated using T4 DNA Ligase. T4 DNA Ligase catalyzed the formation of phosphodiester bonds between the vector and cDNA fragments, creating a stable recombinant plasmid. Ligation reactions were performed under optimized conditions, ensuring efficient production of recombinant plasmids.

The recombinant plasmids were transformed into the yeast strain *S. cerevisiae* BY4742 using the LiAc/SS-Carrier DNA/PEG transformation method. This method enhanced the permeability of the yeast cells, facilitating plasmid uptake. Post-transformation, successful transformants were selected using the LYS2 marker gene. The LYS2 gene present in the pRS327 plasmid encodes an enzyme required for lysine synthesis. Therefore, transformants successfully carrying the plasmid were identified by their growth in a lysine-deficient medium.

In conclusion, recombinant plasmids of all three genes (endo-1,3-beta-glucanase, endo-1,4-beta-xylanase, and endo-1,4-beta-mannosidase) were successfully constructed and efficiently transformed into the *S. cerevisiae* BY4742 strain. This process demonstrates that the genes were correctly cloned and are ready for expression in the selected yeast cells.

Protein expression was induced in *S. cerevisiae* under optimized conditions. The recombinant enzymes were purified using HisTrap affinity chromatography, followed by further purification using gel filtration chromatography. SDS-PAGE analysis confirmed the presence of the target proteins at the expected molecular weights. Enzyme activity assays (further detailed description can be added if needed) demonstrated that the obtained recombinant enzymes were functionally active. These findings establish the order of efficacy of these enzymes against the substrates.

## CONCLUSION

The enzyme activity test results would show the best enzyme that is capable of breaking down the pyrolyzed material. From here, it can be said that some enzymes are better at breaking down the material which results from forest fires. The better the enzymes, the faster the recovery of the materials, and the faster the recovery of the ecosystem. If these enzymes can be engineered to perform these reactions better than their current status, the recovery of the pyrolyzed material into the ecosystem may be fastened. Also, the combinations of such enzymes can be used as a blanket or immobilised at the fire site after the fires to help the ecosystem recover itself [26]. The second method may considered as expensive and labor-intensive but when compared to the first method, it may sound safer. The first method, engineering of the enzymes, requires a lot more research about the pyrophilic fungi; their life cycle, the enzymes they produce when they emerge after the forest fires, what they utilise as a food source and many other aspects. The most important research, however, is whether the engineered enzymes and their new genetic code can be inserted into those fungi, and whether this modification of the fungi could result in a harmful manner to the ecosystem. Even though this method would require such an amount of work, it is one of the best options for creating a long-term solution. Also, this genetic engineering methodology can be achieved with the ever-evolving CRISPR/Cas system. If the genes newly introduced were to prove no harmful effects, to make sure the genes to survive in the ecosystem, gene-drive-like technologies can also be adjusted for the future [27].

Forest fires are severe each year due to climate change [28], and precautions and follow-up actions must be taken. Here, it has been focused on the second term and discovered that interesting fungi species emerge at the fire site. Those fungi are mentioned as they digest the pyrolyzed material [29]. To discover more about those fungi and look at the problem differently, these fungi would be studied to see whether they may prove useful as a follow-up measure. If their enzymes can help fasten the recovery of the carbon material, the forests may have a better chance of recovering themselves.

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