RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2025.087

ScienceAsia 51 (5): 2025: ID 2025087: **1–10**

High-yield *Trichoderma asperellum* laccase from microwave-LiCl composite mutagenesis and optimization

Lixia Zhu[a,*], Yige Ma[a], Wei Tan[a], Wenjing Ma[a], Jutian Chen[a], Lili Li[b]

[a] College of Life Science and Agronomy, Zhoukou Normal University, Zhoukou 466001 China
[b] Key Laboratory of Plant Genetics and Molecular Breeding, Zhoukou 466001 China

*Corresponding author, e-mail: justin2118@163.com

Received 5 May 2025, Accepted 26 Sep 2025
Available online 31 Oct 2025

ABSTRACT: The original strain of *Trichoderma asperellum* was subjected to mutagenesis using microwave irradiation and LiCl, followed by screening for high-yield laccase mutants via the guaiacol method. Optimal fermentation conditions were identified through single-factor and orthogonal experiments. The results indicated that a high-yield mutant strain, MP1-1, was obtained after 15 s of microwave mutagenesis and LiCl treatment at a concentration of 0.9 g/l, achieving a lethality rate of 90%. This mutant exhibited a 70.8% increase in laccase activity compared to the original strain. The optimized fermentation conditions were determined as follows: glucose as the carbon source, yeast extract as the nitrogen source, pH 6.0, Cu^{2+} concentration of 0.09 mmol/l, inoculum volume of 1%, and culture temperature of 28 °C. Under these conditions, the laccase activity reached 54.51 U/l. After a 14-day treatment, the weight loss rate of polyethylene treated with the mutant strain MP1-1 was 0.80%. Scanning electron microscopy revealed significant surface erosion, characterized by pits and holes on the polyethylene, along with the introduction of active functional groups such as carbonyl and ether bonds. The combination of microwave-LiCl compound mutagenesis and optimized fermentation conditions significantly enhanced the laccase production capacity of *T. asperellum*, providing a robust foundation for its industrial application.

KEYWORDS: *T. asperellum*, laccase, mutagenesis, optimization

INTRODUCTION

With the global accumulation of approximately 6 billion tons of plastic waste and only 9% being recycled [1], the environmental persistence of nondegradable plastics like polyethylene (PE) has become a pressing concern [2]. Moreover, microplastics have contaminated the environment including water, soil, and organisms such as *Anentome Helena* [3]. Biodegradation has emerged as a preferred solution due to its ecofriendly nature [4, 5], with fungal enzymes playing a crucial role in breaking down recalcitrant polymers [6]. Among these enzymes, laccases (EC. 1.10.3.2) belong to the family of multi-copper oxidases that catalyze the oxidation of phenolic compounds, utilizing molecular oxygen as a co-substrate and producing water and by-products in the reaction [7]. Laccases are widely distributed in plants, insects, bacteria, and fungi, and they exhibit remarkable catalytic promiscuity, enabling the oxidation of a broad range of phenolic and non-phenolic substrates. This unique property also enables them to play a role in the degradation of industrial dyes and plastics [8]. However, a significant limitation is that typical fungal hosts produce relatively low enzyme yields, insufficient for industrial-scale applications [8]. Thus, developing efficient strategies to enhance laccase production has become a critical research focus.

Fungi are recognized as promising sources of laccases due to their robust enzymatic systems, genetic versatility, and ease of cultivation [9]. Among fungal species, *T. asperellum* has garnered attention for its ability to secrete laccases and its adaptability to various fermentation conditions [10, 11]. In particular, the *T. asperellum* has demonstrated inherent laccase-producing potential in preliminary studies, making it an ideal chassis for further optimization [12]. However, wild-type *T. asperellum* strains typically exhibit low laccase productivity, limiting their practical utility. To overcome this constraint, mutagenesis and fermentation optimization have emerged as effective approaches to improve enzymatic yields.

Mutagenesis techniques, particularly physico-chemical composite mutagenesis, have been widely used to induce genetic variability and screen for high-yield mutants. For instance, ultraviolet (UV) radiation at 260 nanometers induces the formation of pyrimidine dimers, particularly thymine-thymine cyclobutyl dimers, which often result in mutations during DNA replication [13]. UV and microwave radiation have proven effective in improving laccase production strains [14, 15]. Similarly, random chemical mutagenesis has been successfully applied to enhance mycelial cell and spore productivity in *Fusarium incarnatum* strain LD-3 [16]. Physical mutagens can induce DNA damage and mutations by altering molecular vibrations, while chemical mutagens like lithium chloride (LiCl) enhance mutation efficiency by interfering with DNA replication [17, 18]. Compared to single mutagenesis methods, composite mutagenesis (e.g., microwave combined with LiCl) often exhibits synergistic effects, leading to higher mutation rates and more

stable heritable traits [18]. This strategy has been successfully applied to enhance enzyme production in various fungi, highlighting its potential for improving laccase yields in *T. asperellum* [14, 15].

In addition to mutagenesis, optimizing fermentation conditions is crucial for maximizing laccase production [19]. Factors such as carbon/nitrogen sources, pH, temperature, and metal ion concentrations significantly influence fungal growth and enzymatic secretion [19, 20]. For laccase production, specific inducers (e.g., copper ions) and nutrient ratios can upregulate laccase gene expression, further boosting yields [21]. Thus, integrating mutagenesis with systematic optimization of fermentation parameters offers a comprehensive approach to develop high-performance laccase-producing strains.

In view of this, the present study aimed to develop a high-yield laccase-producing strain through microwave-LiCl composite mutagenesis of *T. asperellum* MP1. A high-throughput screening method was employed to isolate positive mutants. Subsequently, the fermentation conditions were optimized using single factor experiments and an $L_9(3^4)$ orthogonal array design to establish an efficient laccase production system. The findings of this study will provide a cost-effective approach for enhanced laccase production, with potential applications in environmental pollutant (such as PE) degradation.

MATERIALS AND METHODS

Microorganisms and cultivation

The *T. asperellum* strain MP1 was isolated from soil samples and is currently preserved in the culture collection of Zhoukou Normal University, China, at −80 °C in an ultra-low temperature freezer. To propagate of *T. asperellum* MP1 mycelium, the strain was cultivated on potato dextrose agar (PDA) solid medium. The PDA medium, consisting of potatoes (200 g/l), glucose (20 g/l, Solarbio, Beijing, China), and agar (15 g/l, Solarbio), was sterilized at 121 °C for 20 min. The strain was then incubated at 28 °C for 15 days. The cultured strains were sampled by punching a hole in the center of the PDA solid plate medium using a 5 mm diameter hole punch and were placed in a centrifuge tube containing 10 ml of 0.05% Tween-80 sterile water. Spore suspensions were prepared by shaking and mixing the contents of the test tube with a scroll shaker, and the number of spores per unit area was calculated using a hemocytometer. The spores were eluted with an aqueous solution and prepared into a 1×10^6 spores/ml spore suspension, which was stored in a refrigerator at 4 °C for subsequent experimental applications.

Additionally, a PDA medium supplemented with guaiacol (Solarbio) was prepared, comprising 200 g of potatoes, 20 g of glucose, 0.4 ml of guaiacol, 20 g of agar, and 1,000 ml of distilled water. The medium was adjusted to its natural pH and then sterilized at 121 °C for 20 min. Subsequently, the strain MP1 was inoculated onto the guaiacol-PDA medium in triplicate and incubated at 28 °C for 5 days. The colony diameter and the diameter of the discoloration halo were measured to preliminarily assess the laccase-producing capability of the strain. Furthermore, to evaluate laccase activity, the strains were inoculated into PDA liquid medium and cultured at 28 °C with agitation, followed by centrifugation at 150 rpm for 5 days. This liquid culture method provided a dynamic environment conducive to enzyme production and allowed for the quantification of laccase activity in the supernatant.

The growth medium contained potatoes (200 g/l) and glucose (20 g/l). The basic fermentation medium was composed of potatoes (200 g/l), glucose (2.5 g/l), yeast extract (5 g/l), peptone (10 g/l), $MgSO_4 \cdot 7\,H_2O$ (1.5 g/l), KH_2PO_4 (1.5 g/l), $CaCl_2 \cdot 2\,H_2O$ (0.1 g/l), and Tween 80 (1 g/l, Solarbio).

Mutagenesis and screening of high-yield strains

The strain MP1 was subjected to mutagenesis using both microwave and LiCl treatments. For microwave mutagenesis, the spore suspension of MP1 (1×10^6 spores/ml) was aliquoted into a 75-mm-diameter petri dish at a volume ratio of 1:50 and placed on a magnetic stirrer inside a microwave oven (model MM720C-P00C00, 700 W, 2450 MHz; Midea, Foshan, China). The suspensions were irradiated for durations of 5, 10, 15, and 20 s. A control group, which was not subjected to microwave irradiation, was also prepared, and each treatment was replicated three times. After irradiation, the spore suspensions were serially diluted 10-fold, and 0.1 ml aliquots were plated onto PDA medium. These plates were inverted and incubated at 28 °C for 5 days to allow colony formation, and the colonies were subsequently counted to determine the lethality rate. The optimal irradiation time was identified based on both lethality and positive mutation rates.

For LiCl mutagenesis (Solarbio), 1 ml of the spore suspension was added to the PDB supplemented with LiCl at concentrations of 0.1, 0.3, 0.5, 0.7, and 0.9 g/l. A control without LiCl was also included to monitor spore germination under standard conditions. All cultures were maintained at 28 °C with agitation at 150 rpm for 5 days, after which the spore germination rates were assessed to calculate the lethality rate and identify the optimal LiCl concentration for mutagenesis.

For compound mutagenesis, 1 ml of the microwave-irradiated spore suspension was added to liquid PDA containing different concentrations of LiCl. This mixture was then cultured at 28 °C with shaking at 150 rpm for 3 days. A control using untreated spore suspension was also included to evaluate the lethality rate resulting from the combined mutagenic treatments.

The lethality rates of strain MP1 after microwave,

LiCl, and compound mutagenesis treatments were calculated as follows to determine the optimal dosages:

$$\text{Lethality rate (\%)} = \frac{(\text{U}-\text{T})}{\text{U}} \times 100, \qquad (1)$$

where U is the total number of colonies/spore generations of the sample without mutagenesis treatment, and T is the total number of colonies/spore generations of the sample after different mutagenesis treatments.

Genetic stability of mutant strains

The highly virulent mutant strains, along with the original strain MP1, were prepared in a spore suspension with a concentration of 1×10^6 spores/ml. Using a pipette gun, 1 ml of the spore suspension was inoculated onto guaiacol-PDA medium and cultured at 28 °C for 5 days. The colony diameter and diameter of discoloration circle were measured and compared. Strains exhibiting faster mycelial growth and strong discoloration ability were selected and subsequently inoculated into PDB medium. These strains were incubated at 28 °C and then centrifuged at 150 rpm to assess laccase activity. The superior strains were identified by subculturing for five generations, during which laccase activity was measured at each passage. Each experimental group was performed in triplicate. Laccase activity was determined using the method described by Umar and Ahmed [22]. The enzyme activity was monitored for 1 to 3 min. Briefly, the incubation mixture was centrifuged at $10\,000 \times g$ for 10 min at 4 °C to obtain a cell-free supernatant. A mixture containing 1 ml of supernatant, 1 ml of guaiacol, and 3 ml of sodium acetate buffer (pH 6.7) was prepared and incubated at 25 °C for 10 min. The reaction mixture was then transferred to a glass cuvette, and the absorbance was measured at 470 nm using a spectrophotometer. Enzyme activity was expressed as International Units (IU), where 1 IU is the amount of enzyme required to oxidize 1 μmol of guaiacol per min. Laccase activity was calculated using the following formula:

$$\text{Laccase activity (U/l)} = \frac{\Delta\text{Abs470} \times V_t}{(\varepsilon \times 1 \times V_s)}, \qquad (2)$$

where $\varepsilon = 740$ M/cm extinction coefficient of guaiacol, V_t = total volume of reaction mixture (ml), V_s = volume of the sample (ml), and 1 = length of cuvette (1 cm).

Optimization of fermentation conditions

Optimization experiments were conducted to obtain the highest laccase activity from the mutant strain. To identify the optimal incubation time for laccase production, experimental cultures were incubated for five days, and laccase activity was measured at 24 h intervals. The effects of various carbon sources (glucose, lactose, sucrose, and soluble starch) were evaluated at a concentration of 2.4 g/l, as reported by Gou et al [21]. The seed culture was inoculated into the fermentation medium at a 2% inoculum volume, incubated at 28 °C, and shaken at 150 rpm for five days, after which laccase activity was assessed.

Additionally, different nitrogen sources, including ammonium sulfate, peptone, yeast extract, and beef extract, were tested at a concentration of 20 mmol/l (which was identified in preliminary experiment). The effect of pH on laccase activity was examined across a range of pH values (4.0, 4.5, 5.0, 5.5, and 6.0). The impact of Cu^{2+} on laccase production was examined at concentrations of 0, 0.02, 0.04, 0.06, 0.09, 0.12, 0.15, and 0.20 mmol/l. Furthermore, the effects of inoculum volume (0.5, 1, 2, 2.5, and 3 ml of spore suspension) and incubation temperature (10, 20, 25, 28, 35, and 40 °C) were systematically evaluated to determine their influence on laccase production.

Based on the above single-factor experiment, appropriate carbon sources and nitrogen sources were determined. An $L_9(3^4)$ orthogonal experiment was designed with four factors and three levels to evaluate the effects of these factors on laccase activity and to identify fermentation conditions to maximize enzyme production. The four key factors (Cu^{2+} concentration, inoculum volume, initial pH, and incubation temperature) were selected as independent variables.

Degradation of PE by selected mutant strains

This experiment was conducted in glass test tube batch reactors to evaluate the degradation efficiency of the crude laccase solution derived from original strain MP1 and mutant strains MP1-1 on PE (Solarbio) before and after optimizing laccase production. The crude enzyme solutions were prepared by culturing both strains under their optimized conditions for 72 h (logarithmic phase), followed by centrifugation ($10\,000 \times g$, 10 min, 4 °C) to remove mycelia from the fermentation broth. The supernatants were collected, and their laccase activities were quantified prior to use. The degradation assays were performed in glass test tube batch reactors. Each reaction contained 50 mg of PE film immersed in 5 ml of crude enzyme solution. A blank group without any crude laccase solution obtained from strains was included for comparison. All treatments were incubated in triplicate at 28 °C under shaking at 150 rpm for two weeks. After incubation, PE degradation was assessed through weight loss measurement, surface morphological examination using a Hitachi SU3500 scanning electron microscope (Hitachi, Tokyo, Japan), and functional group analysis using a Thermo Scientific Nicolet iS50 Fourier-transform infrared spectroscopy (FTIR) spectrometer (Thermo Fisher Scientific, Massachusetts, USA).

Statistical analysis

Statistical analysis was performed using SPSS software (Version 19.0, IBM-SPSS, Armonk, NY, USA). One-way analysis of variance (ANOVA), combined with

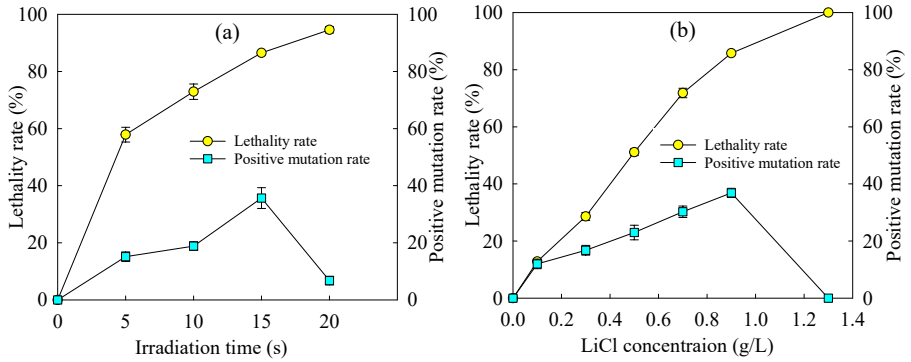

Fig. 1 Lethality rate and positive mutation rate of strain MP1 under microwave treatment (a) and LiCl-induced mutation (b).

Duncan's multiple range test, was employed to determine the significance between means at $p < 0.05$. The figures were created using SigmaPlot 15.0 (Systat Software Inc., London, UK).

RESULTS AND DISCUSSION

Identification of laccase-producing strains

By evaluating both the diameter of the halo zone and the quantitative laccase activity, strains with superior enzyme production can be efficiently identified. In solid-state fermentation systems, strain MP1 exhibited a colony diameter of 20 mm and a chromogenic zone diameter of 45 mm, indicating substantial laccase secretion. Parallel liquid fermentation experiments revealed corresponding extracellular laccase activity of 5.83 U/l, confirming its robust enzymatic potential. Based on these results, strain MP1 is a suitable candidate as the parental strain for further mutagenesis studies aimed at enhancing laccase production.

Determination of microwave mutagenesis time and LiCl concentration

Generally, a lethality rate between 85% and 90% is associated with a relatively high positive mutation rate. As shown in Fig. 1, the mortality rate increased with the increasing duration of irradiation. Specifically, microwave irradiation for 20 s resulted in a lethality rate of 94.60%. The positive mutation rate of the MP1 strain showed a continuous increase from 0 to 15 s, followed by a decline at 20 s. Based on the combined analysis of lethality and positive mutation rates, the optimal microwave irradiation time for MP1 strain mutagenesis was determined to be 15 s (Fig. 1a).

The relationship between LiCl mutagenesis, strain lethality, and positive mutation rate is shown in Fig. 1b. The lethality rate increased progressively with higher LiCl concentrations, reaching 100% at 1.30 g/l. The positive mutation rate generally increased with LiCl concentrations up to 0.9 g/l, where the lethality rate was 85.80% and the positive mutation rate peaked at 36.84%. Therefore, the optimal LiCl concentration for mutagenesis was identified as 0.9 g/l.

Determination of laccase activity of composed mutagenesis strain

Mutation breeding stands out as a simple, rapid, selective, and versatile approach and remains the most widely used and successful technique in strain cultivation [14]. However, prolonged exposure to a single mutagenic agent can lead to a "fatigue effect", reducing the strain's sensitivity to the agent and diminishing its mutagenic efficacy. Compound mutagenesis, which involves multiple mutagenic agents, has been shown to yield superior mutagenic effects [23]. Therefore, following combined microwave and LiCl mutagenesis, the treated spore suspension was serially diluted, plated, and incubated. Mycelial growth patterns were systematically recorded, and lethality rates were calculated. Using this combined mutagenesis approach, 20 strains exhibiting high laccase production were successfully isolated. Ten candidates exhibiting the largest chromogenic halos (indicative of laccase-mediated guaiacol oxidation) were selected for fermentation analysis. Among these, mutant MP1-1 exhibited the most pronounced phenotypic improvements, with colony diameter and chromogenic halo size increasing by 75.00% and 44.44%, respectively, compared to the parental strain MP1 (Table S1). Four mutants outperformed MP1 in laccase production, with MP1-1 showing the highest activity-1.22 times greater than that of the original strain. This was consistent with findings from Feng et al [24], who developed a high kojic acid-producing mutant strain, AR-47, using a combination of microwave mutagenesis, UV irradiation, heat-LiCl treatment, and atmospheric and room temperature plasma (ARTP). Similarly, UV-microwave compound mutagenesis has been shown to enhance spore production and virulence in *Beauveria bassiana* [25]. These results highlight the effectiveness of the combined mutagenesis strategy in enhancing laccase production.

To determine the optimal culture duration, strain MP1-1 was cultured for a 9-day period, with laccase activity measured every two days. As shown in Fig. 2, the highest laccase activity was observed on day 5, reaching a level 1.22 times higher than that of the orig-

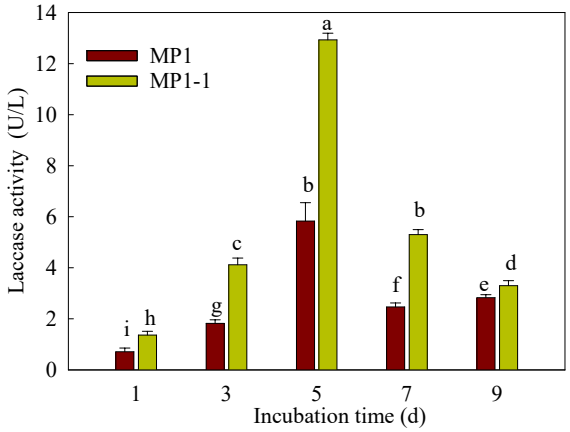

Fig. 2 Enzyme activity dynamics of the mutated strain MP1-1 and the parental strain MP1.

Table 1 Laccase activity of strain MP1-1.

Generation	Laccase activity (U/l)
1	12.86[a]
2	12.95[a]
3	13.16[a]
4	12.85[a]
5	13.05[a]

Different lowercase letters in the same column indicate significant difference at $p < 0.05$.

inal strain MP1. Therefore, a 5-day culture period was established as the standard for subsequent experiments using MP1-1. However, Velásquez-Quintero et al [26] reported maximum laccase activity in the white-rot fungi *Pleurotus ostreatus* after seven days of fermentation, while *Aspergillus flavus* achieved peak activity on the 12th day of incubation [27]. These variations highlight the species-specific nature of laccase production and the influence of experimental conditions on enzyme activity.

Stability determination of microwave-LiCl mutated strain

To evaluate the genetic stability of the microwave-LiCl mutated strain MP1-1, it was subjected to five consecutive subcultures, with laccase activity measured after 5 days of shake-flask fermentation for each generation. As shown in Table 1, the laccase activity of strain MP1-1 remained consistent across generations, ranging from 12.85 to 13.16 U/l, with no significant variation observed between generations. These results demonstrate that MP1-1 exhibits robust genetic stability, making it a promising candidate for further research and applications.

Optimization of fermentation of strain MP1-1

To maximize laccase production by strain MP1-1, this study emphasizes the critical role of optimizing physicochemical parameters, including temperature, pH, fungal biomass, substrate concentration, and culture media composition, in enhancing laccase production efficiency. As shown in Fig. 3a, strain MP1-1 demonstrated effective laccase production when cultivated with various carbon sources, including lactose, glucose, sucrose, and soluble starch. Among these, glucose emerged as the most favorable carbon source, producing the highest laccase activity of 14.37 U/l. This finding aligns with those of Othman et al [28], who reported that *Trichoderma harzianum* S7113 also exhibited the highest laccase activity when glucose was used as the carbon source. In contrast, the lowest laccase production was observed with soluble starch, a polysaccharide, likely due to increased medium viscosity, which restricts nutrient and oxygen availability, impedes cell propagation, and reduces metabolic rates [28]. The corresponding laccase activities of MP1-1 in media containing sucrose, lactose, and soluble starch were 8.65, 7.86, and 6.34 U/l, respectively, confirming the superior efficacy of glucose in promoting laccase production.

Nitrogen sources play a vital role in microbial physiology by influencing metabolic processes. The investigation into nitrogen sources revealed that yeast extract significantly enhanced laccase production, achieving an activity of 38.31 U/l, notably higher than that obtained with peptone (29.64 U/l), ammonium nitrate (23.88 U/l), and ammonium sulfate (18.06 U/l). This observation is consistent with the findings of Velásquez-Quintero et al [26], who reported that yeast extract is a suitable inducer of ligninolytic enzymes such as laccase. Therefore, yeast extract was identified as the optimal nitrogen source for laccase production by strain MP1-1.

Laccase, a member of the multicopper protein family, exhibits activity and yield that are influenced by Cu^{2+} concentration. Copper sulfate concentrations ranging from 0.0 to 0.2 mmol/l were tested to determine the optimal concentration for maximal laccase synthesis. The highest laccase production was achieved at 0.06 mmol/l, as copper serves as an inducer at appropriate concentrations but becomes inhibitory at higher levels. Excessive copper concentrations can be detrimental to fungal growth and enzymatic activity [11, 28].

The production medium was adjusted to initial pH ranging from 4.0 to 6.0 to assess their impact on laccase production. The results revealed that pH profoundly affects enzyme production, with the highest laccase activity observed at an initial pH of 5.5. This is likely due to pH-dependent effects on enzyme structure and stability [29]. These findings align with previous studies indicating that slightly acidic pH values are op-

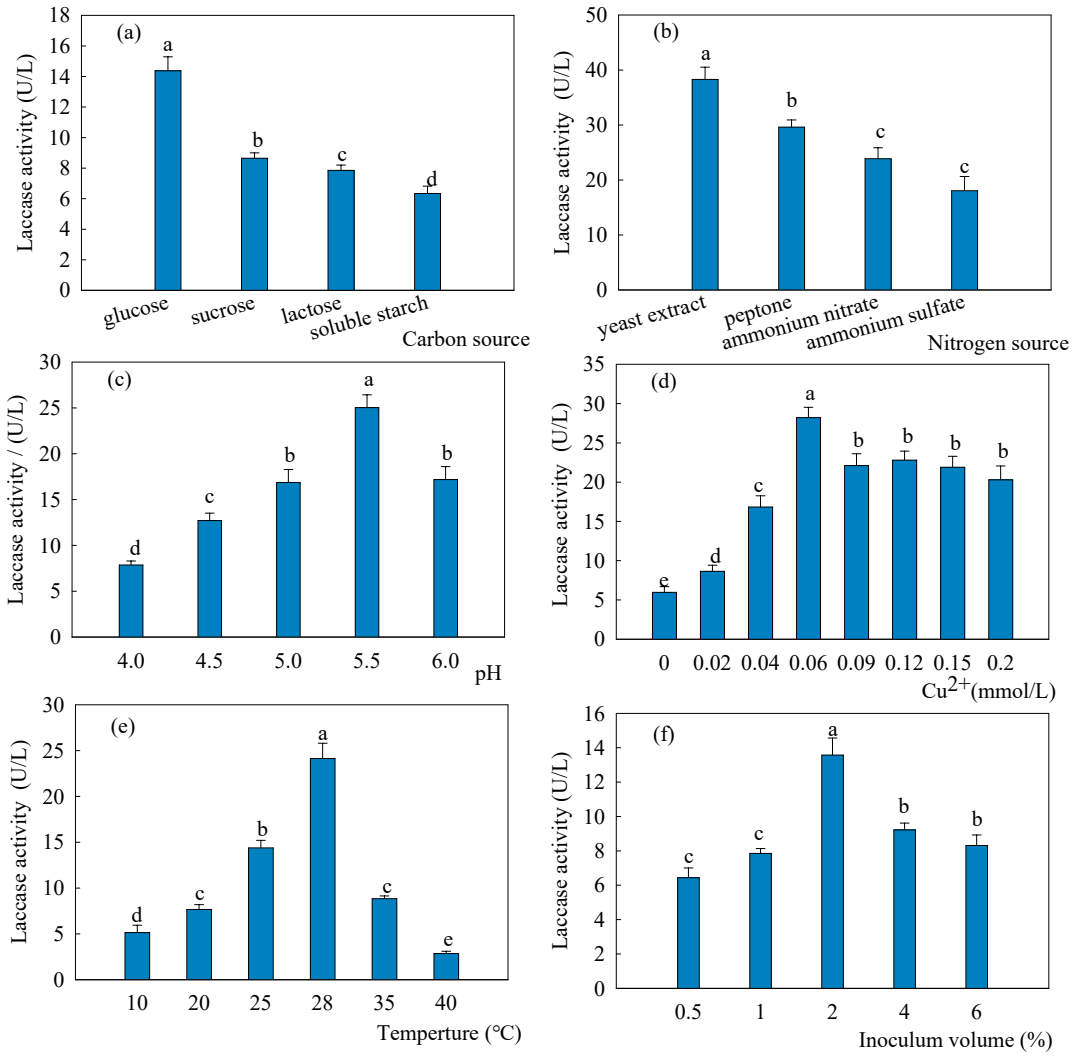

Fig. 3 Effects of carbon source (a), nitrogen source (b), Cu^{2+} (c), initial pH (d), temperature (e), and inoculum volume (f) on laccase activity of strain MP1-1.

timal for the growth and enzyme biosynthesis of most fungal cultures, while lower pH values hinder fungal growth and delay laccase biosynthesis [28]. Optimal laccase production was also dependent on pH and temperature conditions. The maximum yield of 25.04 U/l was attained at pH 5.5, with no further increase at higher pH levels. The impact of incubation temperature was investigated by cultivating strain MP1-1 at various temperatures. Temperature optimization studies indicated that 28 °C was optimal, yielding 24.14 U/l of laccase, whereas temperatures deviating from this optimum negatively affected the strain's metabolic activity and laccase production. This result is consistent with the findings of Othman et al [28], who reported that elevated temperatures reduce ligninolytic enzyme production, likely due to alterations in cell membrane structure and function, as well as increased protein catabolism [12].

Furthermore, the inoculate volume was found to be a critical factor influencing laccase activity. As the inoculum volume increased, the laccase activity of the strain initially increased and then decreased. An inoculum volume of 2% was optimal, yielding the highest laccase activity of 13.58 U/l. Deviations from this volume led to reduced enzyme production, highlighting the importance of precise inoculum control for maximizing laccase synthesis.

Orthogonal experiment

To investigate the relationships between the components of medium and optimize their concentrations for laccase production by strain MP1-1, an orthogonal matrix method was employed. Based on preliminary studies, an $L_9(3^4)$ orthogonal array was selected,

Table 2 Results of orthogonal experiments.

Number	(A) Cu^{2+} (mmol/l)	(B) pH	(C) Temperature (°C)	(D) Inoculum volume (%)	Laccase activity (U/l)
1	0.03	5	25	1	9.03[g]
2	0.03	5.5	28	2	35.42[b]
3	0.03	6	35	4	19.44[d]
4	0.06	5	28	4	15.63[f]
5	0.06	5.5	35	1	14.58[f]
6	0.06	6	25	2	18.4d[e]
7	0.09	5	35	2	17.36[e]
8	0.09	5.5	25	4	30.56[c]
9	0.09	6	28	1	54.51[a]
K1	63.89	42.01	57.99	78.13	
K2	48.61	80.56	105.56	71.18	
K3	102.43	92.36	51.39	65.63	
k1	21.30	14.00	19.33	26.04	
k2	16.20	26.85	35.19	23.73	
k3	34.14	30.79	17.13	21.88	
R	12.85	3.94	18.06	2.31	

Different lower letters indicate significant difference at $p < 0.05$. K1, K2, and K3 indicate the sum of the results at the corresponding level of 1, 2, and 3 for each factor. k1, k2, and k3 indicate the average of the results at the corresponding level of 1, 2, and 3 for each factor. R indicates the extreme difference of each factor.

Table 3 Analysis of variance of orthogonal experiments.

Source of variation	Sum of square	Degree of freedom	Mean square	F-value	p-value
Model	4750.28	8	593.79	4029.71	0.000
(A) Cu^{2+}	1538.47	2	769.24	5220.41	0.000
(B) pH	1386.56	2	693.28	4704.93	0.000
(C) Temperature	1746.80	2	873.40	5927.33	0.000
(D) inoculum volume	78.45	2	39.22	266.19	0.000

which incorporated four variables (pH, temperature, inoculum volume, and Cu^{2+}) at three tested levels. The variables optimized in this study included pH, temperature, inoculum volume, and Cu^{2+} concentration. The tested levels for each variable are summarized in Table 2.

Analysis of the results revealed that the order of factors influencing laccase activity, ranked by the magnitude of the range (R), was as follows: temperature (C) > Cu^{2+} concentration (A) > pH (B) > inoculum volume (D). The optimal combination for maximizing laccase production by strain MP1-1 was determined to be C2A3B3D1, which corresponded to a temperature of 28 °C, a Cu^{2+} concentration of 0.09 mmol/l, a pH of 6.0, and an inoculum volume of 1%. Temperature exhibited the most significant impact on enhancing laccase activity. This temperature-dependent dominance may be attributed to the mesophilic nature of *T. asperellum* (optimal growth at 25–30 °C). However, this finding contrasts with the results reported by Zhuo et al [30], who identified Cu^{2+} as the most critical factor for stimulating laccase production in *Pleurotus ostreatus* HAUCC 162. Such discrepancy likely stems from interspecies variation in copper homeostasis. In their study, laccase activity reached 522.7 U/l after adding 4 mmol/l Cu^{2+} and incubating for 10 days, compared to only 135.2 U/l in control samples.

Under these optimized conditions, the laccase activity of strain MP1-1 reached 54.51 U/l, representing a 3.21-fold increase compared to pre-optimization levels. This significant enhancement in laccase production underscores the suitability of the selected culture conditions for maximizing enzyme yield. The results clearly demonstrate the effectiveness of the orthogonal matrix method in identifying the optimal parameters for laccase production. However, the laccase activity of MP1-1 was lower than that reported by Zhuo et al [30]; this might be attributed to organic acids secreted during its growth, which induced a self-toxic effect and resulted in reduced growth vitality [2].

The variance analysis of the regression model (Table 3) indicated that the F-value of the model was 4029.714, with a corresponding p-value of < 0.001. This suggested that the model was statistically significant and well-suited for predicting the effects of Cu^{2+} concentration, temperature, pH, and inoculum volume on laccase activity. The determination coefficient (R^2) of the model was 0.999 (adjusted (R^2 = 0.999), demonstrating a strong correlation between the predicted values and the experimental values. According to F-values, Cu^{2+} concentration, temperature, pH, and inoculum volume were all found to signifi-

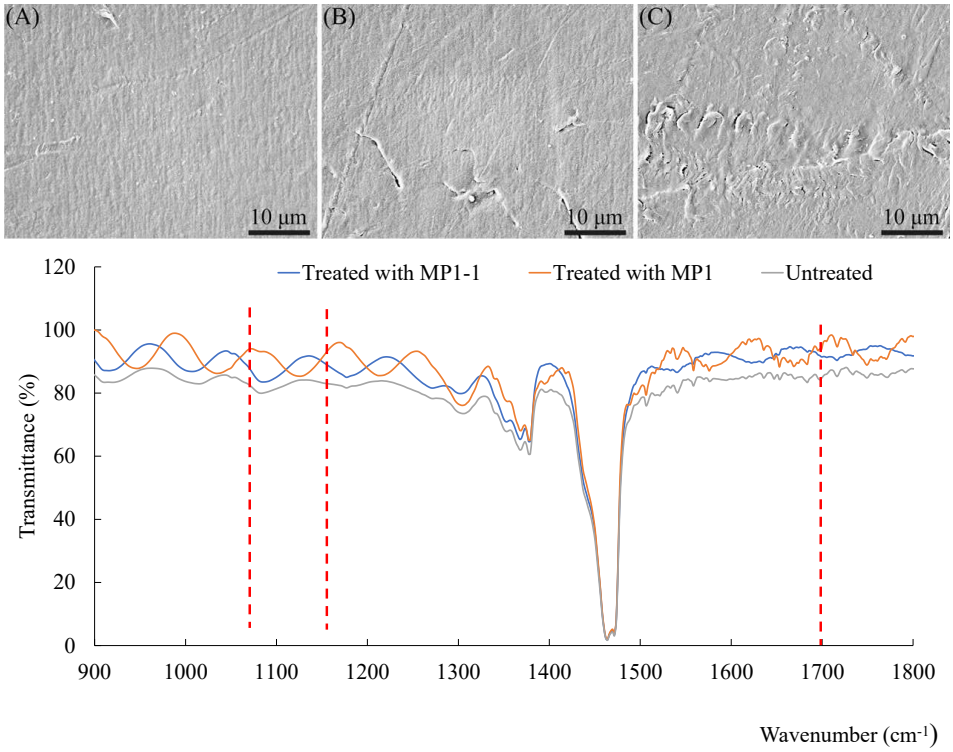

Fig. 4 Surface microscopic characterization (a) and functional group changes (b) in PE. A, CK; B, inoculated with the strain MP1; C, inoculated with the strain MP1-1.

cantly influence the laccase activity of strain MP1-1. Furthermore, the relative importance of these factors, ranked in descending order of their impact, was as follows: temperature (C) > Cu^{2+} concentration (A) > pH (B) > inoculum volume (D). This ranking aligns with the previously discussed findings, confirming the consistency and reliability of the results.

Effect of strain MP1-1 on PE degradation

The PE-degrading capability of the mutant strain MP1-1 was evaluated by incubating PE films with crude cell lysate, given the pivotal role of laccase in PE degradation. After a two-week incubation period, the weight-loss rate of PE films treated with optimized strain MP1-1 reached 0.77% (Table S2), representing a 10-fold increase compared to the abiotic control, a 1.85-fold enhancement over the treatment with strain MP1, and a 97.44% improvement relative to the pre-optimized MP1-1 culture. These findings underscore the significantly elevated laccase activity of mutagenized strain MP1-1, suggesting its potential for PE degradation. Similarly, Taghavi et al [31] observed a 5.5% weight loss of PE obtained within 100 days, which is longer than our 14-day period; when normalized to a 14-day timeframe, their estimated rate (≈ 0.77%) is comparable to our results. However, PE degradation efficiency varies across strains and experimental conditions. Ruan et al [32] demonstrated that *Trichoderma harzianum* could result in a weight loss rate of 3.39% after 30 days, while another study demonstrated that the degradation efficiency of PE-MPs by the mutagenic bacterium XZ-60S was increased by 53.65% using atmospheric and room temperature plasma mutagenesis after 50 days [33]. The observed variations in degradation efficiency may stem from strain-specific metabolic capabilities and mutagenesis-induced enzymatic enhancements, reinforcing the importance of optimized microbial selection for plastic bioremediation. Additionally, laccase plays a vital role in PE degradation. Therefore, the genetically engineered strains developed through genetic recombination technology can greatly enhance the ability of *Trichoderma* to degrade PE.

Morphological and structural alterations in PE films were further characterized using scanning electron microscope (SEM) and FTIR. SEM imaging revealed that untreated PE films exhibited a smooth, uniform surface, whereas films exposed to strain MP1-1 displayed pronounced erosion, pitting, and increased surface roughness (Fig. 4a), indicative of enzymatic degradation. Notably, the extent of surface deterioration was more pronounced in films treated with the optimized MP1-1 strain than with the parental MP1 strain, corroborating its enhanced biodegradative capacity. These findings align with those of Zhang et al [4], who emphasized that fungal treatment

could penetrate PE and highlighted the importance of fungal adhesion to PE for effective biodegradation.

FTIR spectral analysis provided further evidence of chemical modifications induced by enzymatic activity. Untreated PE films exhibited no notable absorption peaks in 1020–1275 cm^{-1} (C—O—C ether bonds) or 1550–1610 cm^{-1}/∼1716 cm^{-1} (carbonyl/carboxyl vibrations) (Fig. 4b). In contrast, MP1-treated films exhibited weak peaks in these regions (e.g., 1716 cm^{-1}, Transmittance = 90.37%), while MP1-1-treated films showed significantly stronger peaks (1716 cm^{-1}, Transmittance = 94.67%), indicating increased introduction of polar functional groups (—C=O carbonyl bonds; C—O—C ether bonds). These modifications are typical of oxidative cleavage of PE's C—C backbones, a process mediated by laccase [1, 4]. It is believed that formation of hydrolytic bonds in plastic structures is a crucial step in the biodegradation process, which would simplify the biodegradation process by allowing the microbial enzymes to attack these functional groups. Without formation of these functional groups, there would be no interaction between microbial enzymes and plastic samples [31]. These spectral shifts are consistent with laccase-mediated PE depolymerization, as previously documented by Esmaeili et al [34], who observed similar C=C bond formation (700–900 cm^1) following fungal treatment.

Collectively, these results demonstrate that optimized MP1-1 enhances PE degradation through elevated laccase activity, which promotes surface erosion, oxidative cleavage, and functional group introduction in PE. Its performance under controlled conditions highlights its potential as a candidate for PE bioremediation, particularly when combined with strategies to extend incubation time or target microplastic fractions.

CONCLUSION

This study demonstrates that microwave-LiCl mutagenesis effectively enhanced the laccase production capacity of *T. asperellum* MP1-1, achieving a 1.22-fold increase in activity (54.51 U/l) under optimized fermentation conditions. The mutant strain exhibited promising PE degradation potential, with a 0.77% weight loss after 14 days of treatment, accompanied by characteristic surface erosion and the oxidative introduction of carbonyl and ether bonds into the PE structure. These findings confirm the critical role of laccase-mediated oxidation in fungal PE degradation and position MP1-1 as a viable candidate for further development in plastic waste bioremediation. Future research should focus on strain improvement and process scaling to advance toward practical applications.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at https://dx.doi.org/10.2306/scienceasia1513-1874.2025.087.

Acknowledgements: This work was supported by the National Natural Science Foundation of China (32201414); Open Fund of the State Key Laboratory of Wheat and Corn Crops Co established by the Ministry and the Province (SKL2023KF08); Projects of the Joint Fund of Henan Province's Science and Technology Research and Development Program (Industrial Category) (225101610054, 245101610081); the Innovation Training Program for College Students in Henan Province (202510478027); 2025 Henan Provincial Higher Education Institutions Young Backbone Teachers Training Plan.

REFERENCES

1. Tao XY, Ouyang HR, Zhou AF, Wang DY, Matlock H, Morgan JS, Ren AT, Mu DS, et al (2023) Polyethylene degradation by a *Rhodococcous* strain isolated from naturally weathered plastic waste enrichment. *Environ Sci Technol* **57**, 13901–13911.
2. Chamas A, Moon H, Zheng JJ, Qiu Y, Tabassum T, Jang JH, Abu-Omar M, Scott SL, et al (2020) Degradation rates of plastics in the environment. *ACS Sustainable Chem Eng* **8**, 3494–3511.
3. Sooksawat T, Wattanakornsiri A, Kohkaew R, Page LM, Tongnunui S (2023) Microplastic accumulation in local dominant shellfish from the Khwae Noi Basin in Western Thailand and its environmental factors. *ScienceAsia* **49**, 445–453.
4. Zhang Y, Pedersen JN, Eser BE, Guo Z (2022) Biodegradation of polyethylene and polystyrene: from microbial deterioration to enzyme discovery. *Biotechnol Adv* **60**, 107991.
5. Ali SS, Elsamahy T, Al-Tohamy R, Zhu DC, Mahmoud YAG, Koutra E, Metwally MA, Kornaros M, et al (2021) Plastic wastes biodegradation: mechanisms, challenges and future prospects. *Sci Total Environ* **780**, 146590.
6. Srikanth M, Sandeep TSRS, Sucharitha K, Godi S (2022) Biodegradation of plastic polymers by fungi: A brief review. *Bioresour Bioprocess* **9**, 42.
7. Zhang AL, Hou YH, Wang YT, Wang QF, Shan XJ, Liu JA (2023) Highly efficient low-temperature biodegradation of polyethylene microplastics by using cold-active laccase cell-surface display system. *Bioresour Technol* **382**, 129164.
8. Thongkred P, Lotrakul P, Prasongsuk S, Imai T, Punnapayak H (2011) Oxidation of polycyclic aromatic hydrocarbons by a tropical isolate of *Pycnoporus coccineus* and its laccase. *ScienceAsia* **37**, 225–233.
9. Khalid N, Asgher M, Qamar SA (2020) Evolving trend of boletus versicolor ibl-04 by chemical mutagenesis to over-produce laccase: process optimization 3-step purification and characterization. *Ind Crops Prod* **155**, 112771.
10. Umar A (2021) Screening and evaluation of laccase produced by different *Trichoderma* species along with their phylogenetic relationship. *Arch Microbiol* **203**, 4319–4327.
11. Salem MM, Mohamed TM, Shaban AM, Mahmoud YAG, Eid MA, El-Zawawy NA (2024) Optimization purification and characterization of laccase from a new endophytic *Trichoderma harzianum* AUMC14897 isolated from opuntia Ficus-Indica and its applications in dye decolorization and wastewater treatment. *Microb Cell Fact* **23**, 266.
12. Fu K, Fan L, Luo X (2023) Identification of laccase genes in *Trichoderma asperellum* Ts93. *Biotechnol Lett* **45**,

479–487.
13. Zhao LH, Ma QQ, Nie F, Chen W, Sun HJ (2018) Increasing laccase activity of white rot fungi by mutagenesis and treating papermaking wastewater. *IOP Conf Ser Earth Environ Sci* **191**, 012053.
14. Deepa T, Gangwane AK, Sayyed RZ, Sapna Enshasy HAE, Zaidel DNA (2020) UV induced mutagenesis elevates the production of laccase in *Enterobacter cloacae*. *J Sci Ind Res India* **79**, 442–448.
15. Aljammas HA, Yazji S, Azizieh A (2022) Enhancement of protease production from *Rhizomucor miehei* by mutagenesis with ethyl methanesulfonate ultraviolet and microwaves: A preliminary study. *Bioresour Technol Rep* **20**, 101287.
16. Chhaya U, Gupte A (2019) Studies on a better laccase-producing mutant of *Fusarium incarnatum* LD-3 under solid substrate tray fermentation. *3 Biotech* **9**, 100.
17. Kang LZ, Fei H, Lin JF, Guo LQ, Bai WF (2013) Breeding of new high-temperature-tolerant strains of *Flammulina velutipes*. *Sci Hortic* **151**, 97–102.
18. Dong YT, Miao RY, Feng RC, Wang T, Yan JJ, Zhao X, Han X, Gan Y, et al (2022) Edible and medicinal fungi breeding techniques a review: Current status and future prospects. *Curr Res Food Sci* **5**, 2070–2080.
19. Chauhan PS, Goradia B, Jha B (2018) Optimization and up scaling of ionic liquid tolerant and thermo-alkali stable laccase from a marine *Staphylococcus arlettae* S1-20 using tea waste. *J Taiwan Inst Chem Eng* **86**, 1–8.
20. Suman SK, Malhotra M, Khichi SS, Ghosh S, Lata S (2021) Optimization and kinetic modeling of *Trametes maxima* IIPLC-32 laccase and application in recalcitrant dye decolorization. *New J Chem* **45**, 2110–2121.
21. Gou ZC, Lu MJ, Cui XY, Wang XQ, Jiang MY, Wang YS, Wang ZQ, Yu XX, et al (2022) Enhanced laccase production by mutagenized *Myrothecium verrucaria* using corn stover as a carbon source and its potential in the degradation of 2-chlorophen. *Bioprocess Biosyst Eng* **45**, 1581–1593.
22. Umar A, Ahmed S (2022) Optimization purification and characterization of laccase from *Ganoderma leucocontextum* along with its phylogenetic relationship. *Sci Rep* **12**, 2416.
23. Wei LL, Mao YR, Liu H, Ke CZ, Liu XL, Li SB (2022) Effect of an inorganic nitrogen source $(NH_4)_2SO_4$ on the production of welan gum from *Sphingomonas* sp. mutant obtained through UV-ARTP compound mutagenesis. *Int J Biol Macromol* **210**, 630–638.
24. Feng WM, Liang JR, Wang BB, Chen JH (2019) Improvement of kojic acid production in *Aspergillus oryzae* AR-47 mutant strain by combined mutagenesis. *Bioproc Biosyst Eng* **42**, 753–761.
25. Ni XP, Li HJ, Xia YD, Lin Y, Wang CT, Li C, Liu JA, Zhou GY (2023) Breeding of highly virulent *Beauveria bassiana* strains for biological control of the leaf-eating pests of *Dalbergia odorifera*. *Forests* **14**, 316.
26. Velásquez-Quintero C, Merino-Restrepo A, Hormaza-Anaguano A (2022) Production extraction and quantification of laccase obtained from an optimized solid-state fermentation of corncob with white-rot fungi. *J Cleaner Prod* **370**, 133598.
27. Kumar R, Kaur J, Jain S, Kumar A (2016) Optimization of laccase production from *Aspergillus flavus* by design of experiment technique: partial purification and characterization. *J Genet Eng Biotechnol* **14**, 125–131.
28. Othman AM, Mahmoud M, Abdelraof M, Karim GSAA, Elsayed AM (2021) Enhancement of laccase production from a newly isolated *Trichoderma harzianum* S7113 using submerged fermentation: Optimization of production medium via central composite design and its application for hydroquinone degradation. *Int J Biol Macromol* **192**, 219–231.
29. Wu YR, Nian DL (2014) Production optimization and molecular structure characterization of a newly isolated novel laccase from *Fusarium solani* MAS2 an anthracene-degrading fungus. *Int Biodeterior Biodegrad* **86**, 382–389.
30. Zhuo R, Yuan P, Yang Y, Zhang S, Ma FY, Zhang XY (2017) Induction of laccase by metal ions and aromatic compounds in *Pleurotus ostreatus* HAUCC 162 and decolorization of different synthetic dyes by the extracellular laccase. *Biochem Eng J* **117**, 62–72.
31. Taghavi N, Singhal N, Zhuang W, Baroutian S (2021) Degradation of plastic waste using stimulated and naturally occurring microbial strains. *Chemosphere* **263**, 127975.
32. Ruan Y, Hu F, Zhao J, Li YH, Ling J, Cao J, Zhang L (2023) Degradation of polyethylene particles by *Trichoderma harzianum* and recombinant laccase cloned from the strain. *J Appl Polym Sci* **140**, e54599.
33. Xiong Z, Chen X, Zou Z, Peng L, Zou L, Liu B, Li Q (2025) Improving efficiency of bacterial degradation of polyethylene microplastics using atmospheric and room temperature plasma mutagenesis. *Bioresour Technol* **418**, 131930.
34. Esmaeili A, Pourbabaee AA, Alikhani HA, Shabani F, Esmaeili E (2013) Biodegradation of low-density polyethylene (LDPE) by mixed culture of *Lysinibacillus xylanilyticus* and *Aspergillus niger* in soil. *PLoS One* **8**, e71720.

Appendix A. Supplementary data

Table S1 Screening of dominant laccase-producing strains.

Strain	Colony diameter (mm)	Discoloration circle (mm)
MP1	20^{d}	45^{c}
MP1–1	35^{a}	65^{a}
MP1–2	33^{b}	60^{b}
MP1–3	30^{c}	58^{b}
MP1–4	30^{c}	59^{b}

Different lowercase letters in the same column indicate significant difference at $p < 0.05$.

Table S2 Weight loss of PE films incubated with different strains.

Strain	Weight loss (%)
Abiotic control	0.07^{d}
MP1	0.27^{c}
MP1–1 (before optimization of propagation process)	0.39^{b}
MP1–1 (after optimization of propagation process)	0.77^{a}

Different lowercase letters in the same column indicate significant difference at $p < 0.05$.