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Comprehensive Analysis of Catalytic Characteristics and Molecular Mechanisms in Mutant *Trametes versicolor* Strains with Enhanced Laccase Activities

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Abstract: The commercial potential of *Trametes versicolor* laccases in the degradation of various persistent contaminants is significant. Despite numerous attempts through rational metabolic engineering to enhance the properties of laccases, the outcomes have proven unsatisfactory for practical implementation. The present study successfully generated two novel mutants, namely, TA-04 and TA-15, derived from *Trametes versicolor* ATCC20869, utilizing atmospheric and room temperature plasma (ARTP). The laccase activities of TA-04 and TA-15 showed a significant increase to 136.507 ± 4.827 U/mg DCW and 153.804 ± 6.884 U/mg DCW, respectively, which were 1.201 and 1.354 times than that of the original strain. The laccase activities of the mutant strains TA-04 and TA-15 surpassed that of the original strain by 10.372% and 18.914%, respectively, at a higher pH level of five. Sequencing analysis of TA-04 and TA-15 revealed that several alternative amino acids within their active regions may enhance their catalytic characteristics under a higher temperature and pH condition. This study employed ARTP mutagenesis to propose two highly efficient microbial mutants derived from *Trametes versicolor* ATCC20869, exhibiting enhanced laccase activities. These mutants hold promising potential for the degradation of diverse environmental pollutants.

Keywords: laccase; *Trametes versicolor*; fermentation; catalytic characteristics; molecular mechanisms

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1. Introduction

Laccase (EC. 1.10.3.2), one of polyphenol oxidases, belongs to the family of copper blue proteins. It catalyzes the four electron reduction of molecular oxygen to water while simultaneously oxidizing diverse phenolic and non-phenolic compounds, as well as many environmental pollutants, without producing toxic by-products [1]. Laccase exhibits a broad spectrum of degradable substrates, encompassing dyes such as lignin, azo dyes, chlorophenol compounds, polycyclic aromatic hydrocarbons, and endocrine disruptors and can be applied in diverse fields including dye degradation, pesticide degradation, drug production, biological monitoring, and the textile industry. Although laccase enzymes with similar functions have been found in bacteria, fungi, plants, and animals, *Trametes versicolor* laccases are renowned for their exceptional biotransformation or biodegradation capabilities towards a broad range of both phenolic and non-phenolic lignin-related compounds, as well as highly recalcitrant environmental contaminants [2,3].

Nevertheless, the *Trametes versicolor* laccase poses challenges such as inadequate secretion and reduced enzyme activity [4,5]. Despite numerous attempts by scholars to enhance the catalytic properties of laccase through various methodologies in recent years, achieving satisfactory results has remained challenging. Yang et al. expressed laccase gene *Lac1* derived from *Basidiomycote Cerrena* sp. in *Pichia pastoris* X33, assessing

the levels of laccase activity at 6.3 U/mL [6]. Gu et al. cloned two novel laccase genes, *Lac3* and *Lac4*, from basidiomycete *Coprinus comatus* and efficiently expressed them in *Pichia pastoris* KM71H, achieving the required levels of laccase activity at 6.89 U/mL and 14.65 U/mL, respectively [7]. Stevens et al. obtained hyperthermophylic laccases by the means of targeted mutagenesis, wherein among the eight amino acid mutations, only the E170Y mutant exhibited a significant 23% increase in activity, while all other mutations had negligible effects [8]. Li et al. employed chemical reagents and ultraviolet radiation to induce mutations in *Penicillium* simplex, aiming to obtain laccase-producing strains with enhanced productivity. However, the resulting increase in the maximum laccase activity was merely 18% [9]. However, the spatial configuration of fungal laccase exhibits a remarkable degree of conservation, hence posing challenges in the acquisition of beneficial mutations using conventional approaches [10]. Considering the aforementioned phenomena, stochastic mutations can be employed for microbial breeding. The utilization of atmospheric pressure and room temperature plasma (ARTP) has emerged as a novel and efficient technique for inducing mutations in microorganisms, thereby facilitating microbial mutation breeding and the establishment of microbial mutation libraries. The method exhibits exceptional effectiveness in generating mutant strains while maintaining their genetic stability. Furthermore, it exemplifies easy operation, safety measures, stability assurance, and freedom from contamination [11,12].

The present study demonstrates the successful development of two mutant strains, TA-04 and TA-15, with significantly enhanced laccase production through the implementation of ARTP method. The unit mycelial laccase activities of TA-04 and TA-15 showed a significant increase to 136.507 ± 4.827 U/mg DCW and 153.804 ± 6.884 U/mg DCW, respectively, which were 1.201 and 1.354 times that of the original strain, respectively. By optimizing the fermentation conditions and improving the best parameters of laccase production, we successfully achieved a significant enhancement in the enzyme activities for both mutant laccase strains, with values reaching 1120.184 ± 15.024 U/mg DCW and 1297.773 ± 16.853 U/mg DCW. Concurrently, there has been a significant improvement in the performance of both mutant laccase enzymes concerning their resistance to alkali and high temperatures, as well as their capacity for dye degradation. The sequencing analysis of the mutated enzymes and homologous modeling facilitated the investigation of the molecular mechanism for the enhancement of laccase activity. This study offers a theoretical foundation for future research endeavors aimed at enhancing the activity of diverse laccase enzymes and broadening the scope of laccase application in environmental pollution control using ARTP mutagenesis.

2. Materials and Methods

2.1. Strains and Cultivation Techniques

The strain utilized in this study was *Trametes versicolor*, specially obtained from ATCC under the catalog number of 20869. After culturing the strain of *Trametes versicolor* on PDA medium at 28 °C for 14 days, 7 mm agar plugs were transferred into a conical flask containing 150 mL of PDB medium and incubated at 30 °C with continuous shaking at 150 r/min for 7 days [13].

The solid PDA medium consisted of the following components (g/L): potato, 200.00; KH_2PO_4, 3.00; $MgSO_4 \cdot 7H_2O$, 1.47; vitamin B1, 0.02; glucose, 20.00; and agar, 20.00. The PDB fermentation medium comprised the following components (g/L): potato, 212.50; KH_2PO_4, 3.00; $MgSO_4 \cdot 7H_2O$, 1.50; vitamin B1, 0.02; and glucose, 20.00. The carbon source was sterilized separately to prevent Maillard reactions and added immediately prior to inoculation [10,14]. The PDB medium refers to a PDA medium that does not contain agar. Guaiacol-PDA medium was prepared by adding guaiacol at a mass-to-volume ratio of 0.04% to the PDA medium.

2.2. Construction of a Mutagenesis Library Using the ARTP Method

The mutagenesis of *Trametes versicolor* spore suspensions was conducted using ARTP mutagenesis techniques. Following a 14-day incubation period in the PDA medium, the spores should be appropriately prepared by suspending them in sterile deionized water to achieve a concentration of approximately 1×10^6. Subsequently, a 10 µL aliquot of the culture was evenly distributed onto a sterile iron plate for exposure to ARTP-IIS (Wuxi TMAXTREE Biotechnology Co., Ltd., Wuxi, China) at an irradiation power of 120 W and a gas flow rate of 11 SLM. The distance between the exit of the plasma torch nozzle and the sample plate was 3 mm. After transferring slides containing ARTP-treated mycelium to EP tubes containing 1 mL of sterile water, elution was performed with agitation. Then, 100 µL of the dilution was inoculated onto the PDA medium and incubated at 28 °C to calculate the fatality rate [15,16]. The mutagenized strains were reinoculated on guaiacol-PDA medium and cultured at 30 °C for 72 h. Guaiacol serves as the characteristic substrate for laccase. During the strain's growth, laccase produced by the strain oxidizes guaiacol, resulting in a reddish-brown color change. The diameter of the discoloration ring corresponds to the activity of the laccase enzyme in the strain, exhibiting a positive correlation between larger diameters and higher enzymatic activity.

2.3. Assays for Laccase Enzyme Activity

The enzyme activity of laccase was assessed through spectrophotometric measurement of the oxidation reaction between 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonate (ABTS) and the enzyme, employing a colorimetric assay. The contents of the fermentation product were meticulously extracted, after which the dialysis bag was utilized to eliminate impurities. The resulting solution was subsequently transferred into a centrifuge tube, and centrifuged at 10,000 r/min for a duration of 10 min. The supernatant was thenceforth utilized for enzyme activity determination. The ABTS solution (800 µL) should be thoroughly mixed with the diluted enzyme solution (200 µL) to ensure complete homogeneity. The optical density (OD) value should be recorded at 30 s intervals throughout the 3 min reaction period, while ensuring continuous stirring. A UV spectrophotometer was utilized to quantify the absorbance at 420 nm. Laccase activity was calculated by measuring the change in absorbance at 420 nm using deionized water as a blank control. A unit of laccase activity (U) was defined as the quantity of laccase capable of oxidizing 1 µmol ABTS per minute under the assay conditions. All measurements were performed in triplicate [17,18]. Protein content was estimated using the Bradford method [19].

2.4. Comprehensive Analysis of Enzymatic Properties of Laccase

The temperature (ranging from 20 °C to 80 °C) was precisely adjusted in order to determine the thermal stability, and subsequent readings were recorded following a controlled temperature increase of 10 °C every 15 min [20,21]. The pH of the ABTS solution was adjusted to a range of 2.0–7.0 using both acetate and phosphate buffers, followed by measuring the laccase activity at a temperature of 40 °C [5]. The metal ions (Cu^{2+}, Ca^{2+}, Zn^{2+}, Mg^{2+}, K^+, Fe^{2+}, Na^+, Mn^{2+}) utilized in this study were present at concentrations of 2.5 and 5.0 mM, respectively. A 60-microliter volume of a metal ion solution was combined with an equivalent volume of an enzyme solution to assess its impact on the laccase enzyme activity [22,23]. The relative enzyme activity is considered to be 100% upon mixing the enzyme solution with deionized water in a 1:1 ratio. The mixture was incubated at 45 °C for a duration of 30 min prior to the analysis of laccase activity. Reactive Brilliant Blue X-BR, Congo Red, and acidic fuchsin were selected as representatives of anthraquinones, azoles, and triphenylmethane dyes, respectively, to investigate the degradation of the dyes by a fermented crude enzyme solution and purified laccase from the mutant strain. The concentration of dye was adjusted to 100 mg/L, while the enzyme activity equivalent was set at 6.5 U/L within the experimental system, and the temperature was maintained at 45 °C. The wavelengths for reaction indication include Reactive Brilliant Blue X-BR (λ_{max} = 603 nm), Congo Red (λ_{max} = 497 nm), and acidic fuchsin (λ_{max} = 545 nm). Reactions

were performed in triplicate for each experiment. Decolorization activity was calculated as the decolorization rate, expressed as $[(I - F)/I] \times 100\%$, where I represents the initial absorbance and F denotes the absorbance after the decolorization reaction [24–26].

2.5. Optimization of Fermentation Conditions Using Single-Factor and Box–Behnken Experiments

The enzymatic characterization revealed that both pH and copper ions exert a significant influence on the production of laccase. To validate this phenomenon, subsequent shake flask experiments were conducted. The initial pH of the PDB medium was adjusted within the range of 3.0 to 7.0 using HCl (1.0 mol/L) and NaOH (1.0 mol/L). Subsequently, mycelia were inoculated into these media and incubated at a temperature of 30 °C with the agitation speed set at 150 r/min for a duration of 7 days, aiming to investigate the impact of initial pH on laccase enzyme activity produced by *Trametes versicolor*. Copper sulfate was selected as the raw material for the provision of copper ions, with a concentration range of 0–3.0 mM. The optimal addition concentration of copper ions was determined by measuring the biomass of the bacterium in the growth stage and the enzyme activity of laccase. After determining the optimal concentration of copper ion addition, the appropriate timing for introducing copper ion was subsequently determined. The PDB medium was supplemented with a final concentration of 1.0–8.0 mM farnesol solution, which was subsequently sterilized. Then, the strains were introduced into the shake flask medium and incubated for 7 days (30 °C, 150 rpm). Regular intervals were employed to assess the bacterial biomass and enzyme-producing activity. After conducting one-way experiments to determine the optimal additions of medium pH, copper ions, and farnesol, Box–Behnken experiments were performed on the two mutant strains in order to achieve enhanced laccase yields [27,28].

2.6. Analysis of Homology Modeling

The original and mutant strains were cultured in liquid PDB medium for 5 days, followed by the collection of mycelia in centrifuge tubes. Subsequently, the mycelia were rapidly frozen using liquid nitrogen and dispatched to Nanjing Paisnano Genomics. The BioXM software was utilized to compare the sequences of laccases from wild-type and mutant strains, in order to identify mutated base sites and corresponding amino acid changes. The altered catalytic properties of the enzyme were analyzed through homology modeling using the Swiss-Model server http://swissmodel.expasy.org/ (accessed on 23 September 2023) and PyMOL molecular graphics system [29,30].

2.7. Statistical Analysis

To check for reproducibility, the experiment was repeated three times. Subsequently, a one-way analysis of variance (ANOVA) was executed using SPSS Statistics 26, followed by Tukey's Honestly Significant Difference (HSD) post hoc test to ascertain the statistical significance of the data ($p \leq 0.05$).

3. Results

3.1. ARTP Mutagenesis Enhances Laccase Activity in Trametes versicolor

The attainment of a high rate of colony lethality is crucial for the efficient mutation and screening of mutants. The spore mortality rate gradually increased as the ARTP treatment time was extended, with a gradient of 10 s. It reached 92.330% at 50 s and further rose to 97.640% at 60 s. Notably, by the end of the treatment period (90 s), there was virtually no survival observed among the original strain (Figure 1a). According to previous reports, a lethality rate of 90% is considered appropriate [31,32]. Therefore, in this experiment, an exposure time of 50 s was selected to achieve the desired lethality rate. After ARTP treatment for 50 s, the culture was diluted with sterile water and spread onto PDA agar plates supplemented with kanamycin at a concentration of 20 μg/mL and guaiacol at a concentration of 0.4 g/L [33]. The selection of 23 mutant strains, named TA-01 to TA-23, was based on the measurement of the color ring diameter surround-

ing the treated colonies. After being cultured for 7 days, these 23 strains were subsequently transferred to a shake flask fermentation medium for an additional 7 days, during which the activity of laccase was analyzed. The laccase activity of TA-04 and TA-15 was 136.507 ± 4.827 U/mg DCW and 153.804 ± 6.884 U/mg DCW, respectively, as shown in Figure 1b. These values exhibited a significant increase of 39.225% and 90.423%, respectively, compared to the original strain, surpassing the levels observed in other strains. Subsequently, we conducted a genetic stability assessment of these two high-yield mutant strains, namely, TA-04 and TA-15. The mutant strains were continuously passaged and cultured, while the initial generation of vigorously developing strains was maintained at a temperature of 4 °C as the control group. The results revealed that the mutant strain TA-04 displayed relative laccase activities of 100.000%, 96.033%, 98.347%, 99.160%, 98.305%, and 94.828% in the first to sixth generations, respectively. Similarly, the mutant strain TA-15 exhibited relative laccase activities of 100.000%, 98.647%, 100.588%, 97.661%, 98.802%, and 98.788% in the first to sixth generations, respectively. Therefore, it can be deduced that these two mutant strains exhibit a favorable genetic stability. Figure 1c displays the results of linear regression analyses, demonstrating the increased activity of *Trametes versicolor* ATCC20869, mutant TA-04, and mutant TA-15. These analyses visually highlight the superiority of the mutant strains over the wild type, further supporting the potential of these variants for biotechnological applications.

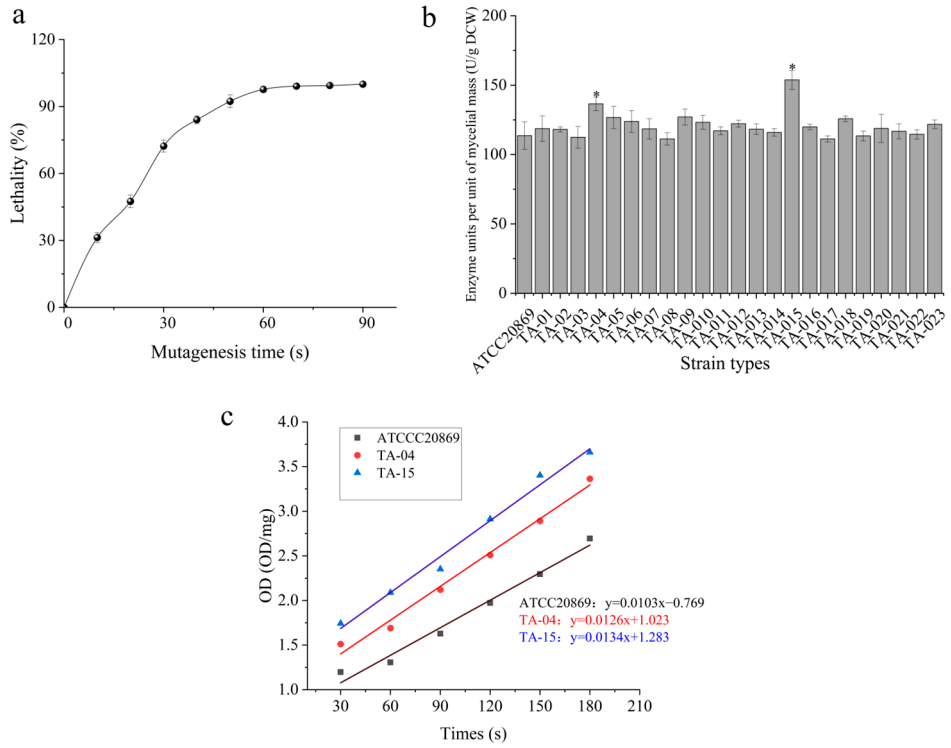

Figure 1. (**a**) Lethality rate of ARTP. (**b**) Laccase activity per unit mycelium of *Trametes versicolor* ATCC20869 and mutant strains. (**c**) Linear regression analysis of laccase from *Trametes versicolor* ATCC20869, mutant TA-04, and mutant TA-15. All experiments were conducted in triplicate, the values were expressed as mean ± SD, and statistical significance was indicated by * ($p \leq 0.05$).

3.2. Analysis of the Enzymological Properties of Mutant Laccases

The activities of laccase from the original strain *Trametes versicolor* ATCC20869, TA-04, and TA-15 were analyzed at a range of temperatures (20–80 °C) with increments of 10 °C per gradient. The relative activity of laccase experienced an increase, followed by a subsequent decrease, initially exhibiting an increase below 40 °C and subsequently demonstrating a decline beyond 50 °C, as shown in Figure 2a. Beyond 60 °C, the structural integrity of laccase may be compromised, leading to a rapid decline in its activity. However, TA-15

demonstrated a remarkable stability under high temperature conditions, indicating its significant potential for practical applications. In the meantime, it was observed that both the original strain and TA-04 exhibited an optimal activity temperature of 40 °C, while TA-15 displayed a heightened optimal activity temperature of approximately 50 °C [34].

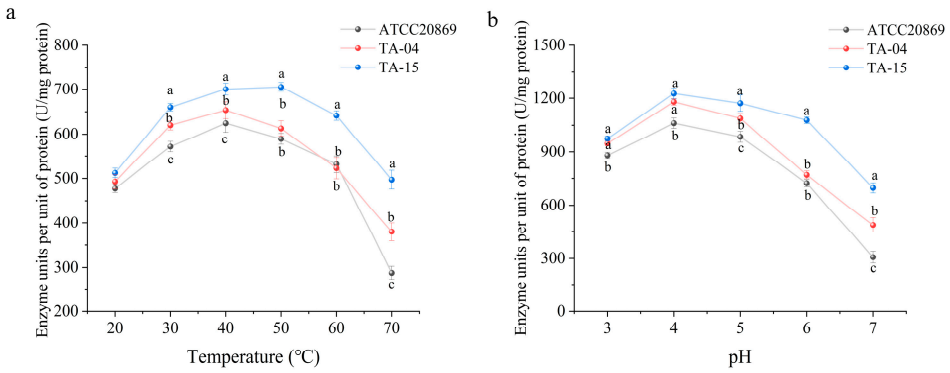

Figure 2. Effects of temperature (**a**) and pH (**b**) on unit protein laccase activity of *Trametes versicolor* ATCC20869, mutant TA-04, and mutant TA-15. All experiments were conducted three times, the values were expressed as mean ± SD, and statistical significance was indicated by different letters at different points ($p \leq 0.05$).

The activity of enzymes is influenced not only by reaction temperature but also by the ambient pH. Therefore, the present study aimed to investigate the impact of environmental pH (with each unit increase representing a gradient) on the mutant laccases in order to determine their optimal pH. The changes in laccases of the original strain and the two mutant strains were assessed across a range of pH values (from 3.0 to 7.0) [35,36]. Under acidic conditions, the laccase activity of per unit protein exhibited an increase and reached its maximum value at a pH of 4.0. Subsequently, as the pH increased to 5.0, there was a gradual decline in laccase activity of unit protein. However, the unit protein laccase activities of strains TA-04 and TA-15 surpassed that of the original strain by 10.372% and 18.914%, respectively, at a higher pH level of 5 (Figure 2b).

The catalytic activity of laccase is hypothesized to be influenced by metal ions due to its classification as a multicopper oxidase [37,38]. In this study, various metal ions including Cu^{2+}, Ca^{2+}, Zn^{2+}, Mg^{2+}, K^{+}, Fe^{2+}, Na^{+}, and Mn^{2+} were added to the reaction buffer. A 60 µL volume of metal ion solution was combined with an equivalent volume of enzyme solution to evaluate its impact on laccase activity. The relative enzyme activity is considered to be 100% when deionized water is substituted for the metal ion solution and mixed with the enzyme solution in a 1:1 ratio. The experimental results demonstrated that the laccase activity of all three strains could be enhanced to varying degrees by metal ions, including Mg^{2+}, Cu^{2+}, Zn^{2+}, Na^{+}, and Mn^{2+}. Notably, Cu^{2+} exhibited the most pronounced enhancement effect, with a 2.5 mM metal ion solution increasing the enzyme activity of the three groups of strains by approximately 15%, while a 5 mM metal ion solution increased the enzyme activity of the three groups of strains by more than 20%, which may be attributed to its presence in the catalytic activity center of laccase (Figure 3) [39].The inhibitory effect of Fe^{2+} on laccase activity might be due to its occupation at the active center during the reaction process, leading to structural alterations in laccase and hindering substrate binding [40].

Subsequently, the degradation efficiency of the original strain, TA-04, and TA-15 was assessed through experimental evaluation using commonly employed dyes including Reactive Brilliant Blue X-BR, Congo Red, and acidic fuchsin. The decolorization of Reactive Brilliant Blue X-BR by laccase exhibits its excellent efficacy, with the mutant strain TA-15 demonstrating an approximate 20% enhancement in decolorization rate compared to the original strain (Figure 4a). The laccase from *Trametes Pubescens*, as demonstrated by Zheng et al. [41], showed a decolorization rate of 54.24% towards Congo Red. Notably,

the original strain's laccase displayed a comparable decolorization rate, while the mutant strains TA-04 and TA-15 achieved even higher rates at 60.503% and 68.343%, respectively (Figure 4b). The degradation efficiency of three laccase strains was finally evaluated using acidic fuchsin, and their degradation patterns exhibited similarity. After 6 h, the decolorization rate reached approximately 80% (Figure 4c). The above findings suggest that the mutated laccase exhibits a superior efficacy in dye degradation compared to previous studies [24,26].

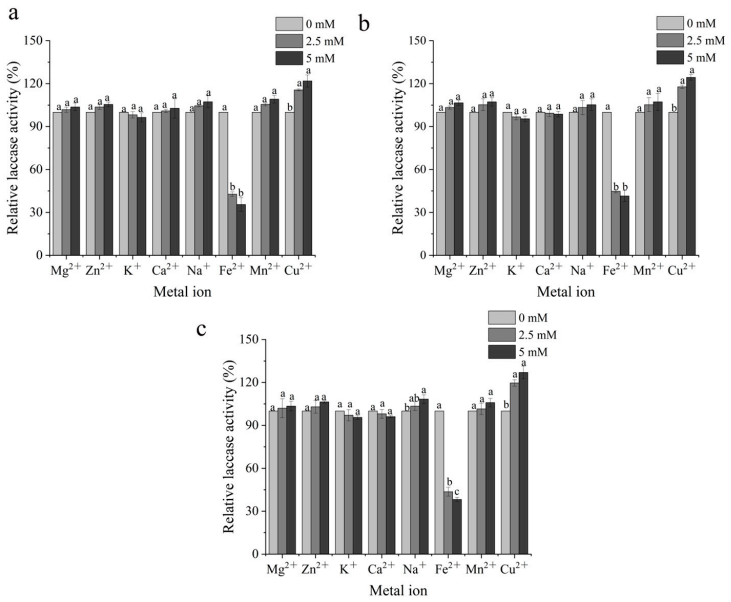

Figure 3. Effects of different concentrations of metal ions on laccase activity of *Trametes versicolor* ATCC20869 (**a**), TA-04 (**b**), and TA-15 (**c**). All experiments were conducted three times, the values were expressed as mean ± SD, and statistical significance was indicated by different letters at different points ($p \leq 0.05$).

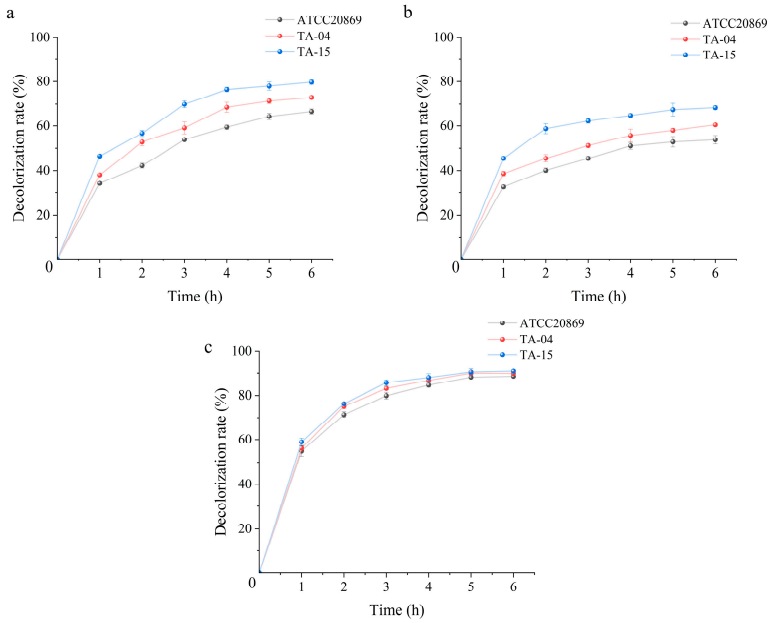

Figure 4. Degradation rate of different dyes, i.e., Reactive Brilliant Blue X-BR (**a**), Congo Red (**b**), and acidic fuchsin (**c**), over time by the *Trametes versicolor* ATCC20869, mutant strain TA-04, and mutant strain TA-15. All experiments were conducted three times, the values were expressed as mean ± SD, and statistical significance was indicated by different letters at different points ($p \leq 0.05$).

3.3. Optimization of Laccase Production Conditions through One-Factor Analysis

Previous studies have demonstrated the significant impact of copper ions and pH levels on enhancing the enzymatic activity of laccase. In this research endeavor, our objective is to further investigate the influence of copper ions and pH on the production process of laccase through one-way experiments. Additionally, based on previous research findings, farnesol has been identified as a quorum sensing molecule that exerts a significant influence on the mycelial morphology and biological characteristics of various fungi, thereby potentially impacting laccase production [42,43].

The experimental results demonstrated that all concentrations of farnesol (ranging from 1 mM to 4.0 mM) exhibited the ability to enhance the enzymatic activity of the laccases from the original strain, TA-04, and TA-15. Upon gradually increasing the farnesol concentration to 4.0 mM, the laccase activity (Figure 5) reached their maximum values, with the laccase enzyme activities reaching 179.35 ± 8.295 U/g DCW, 244.261 ± 5.413 U/g DCW, and 232.683 ± 7.042 U/g DCW, respectively; thereafter, a decline in laccase production was observed, indicating that higher concentrations of farnesol might induce cytotoxic effects leading to a reduced enzyme induction [42,44]. Under the optimal concentration of farnesol induction, the unit mycelial laccase activity of three strains increased by more than 1.5 times compared with the control group. The findings suggest that farnesol not only enhances intracellular laccase synthesis but also promotes fungous biomass production, thereby serving as a straightforward and efficient inducer.

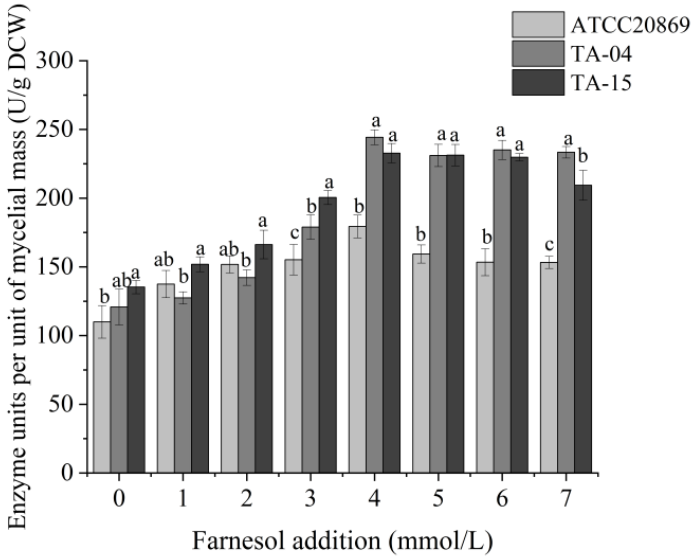

Figure 5. Effect of farnesol at different concentrations on laccase activity per unit of mycelium of *Trametes versicolor* ATCC20869, TA-04, and TA-15. All experiments were performed in triplicate, and the values represent mean ± SD. Statistical significance is denoted by different letters above or below bars at each time point ($p \leq 0.05$).

Based on the results obtained from the conducted experiments, it was observed that varying concentrations of copper ions (each at a gradient of 0.5 mM) exhibited diverse levels of enhancement in laccase synthesis. When the concentration of copper ions gradually increased to 2.0 mM, the laccase activity of the three strains reached the maximum value. However, upon surpassing this threshold concentration, a decline in the unit mycelial laccase activity (Figure 6) was observed, indicating that the high concentration of copper ions might exert an inhibitory effect on the growth of the mycelium [45].

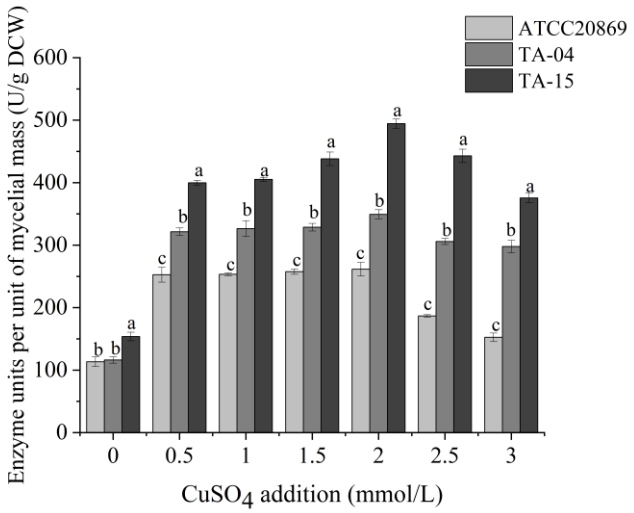

Figure 6. Effects of different concentrations of $CuSO_4$ on laccase activity per unit of mycelium of *Trametes versicolor* ATCC20869, TA-04, and TA-15. All experiments were performed in triplicate, and the values represent mean ± SD. Statistical significance is denoted by different letters above or below bars at each time point ($p \leq 0.05$).

pH levels can exert an influence on the active site of laccase or alter the substrate's degree of dissociation, thereby potentially impacting the enzymatic activity through structural modifications [46]. Upon reaching a pH level of 4.0, the laccase activity exhibited its peak values. However, surpassing this threshold led to a subsequent decline in the unit mycelial laccase activity (Figure 7) [46,47].

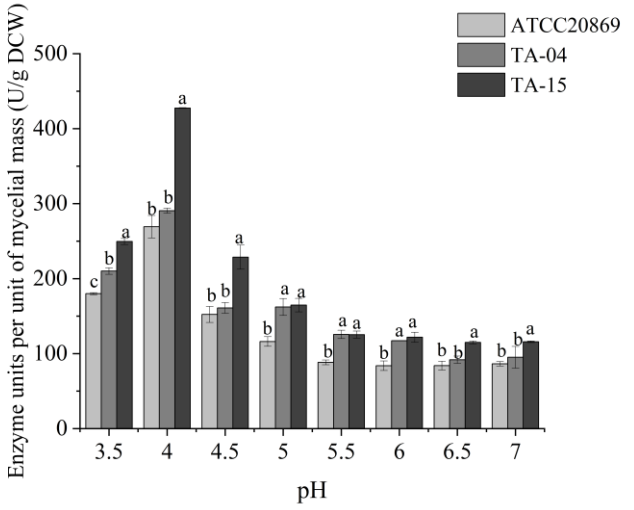

Figure 7. Effect of different pH on laccase activity per unit of mycelium of *Trametes versicolor* ATCC20869, TA-04, and TA-15. All experiments were performed in triplicate, and the values represent mean ± SD. Statistical significance is denoted by different letters above or below bars at each time point ($p \leq 0.05$).

3.4. Experimental Findings Based on the Box–Behnken Design

The Box–Behnken experimental results were analyzed using Design Expert software version 8.0.6, based on the Single-Factor experiments. Regression fitting was conducted to obtain the analysis of variance results of the quadratic regression model, as presented in Tables 1 and 2. The relevant data of the quadratic regression analysis were acquired [48,49]. The $p < 0.05$ of TA-04 mutant strain in Table 1 indicates that the model is significant. The lack of fit term of the model represents the probability that the predicted value of the model does not fit the actual value, $p > 0.05$. The lack of fit term is not significant;

thus, the model was chosen correctly. The correlation coefficients R-Squared = 0.9880 and Adj R-Squared = 0.9726 of the model indicate that the equation fits well. The $p < 0.001$ of TA-15 mutant strain is extremely significant, indicating that the regression model has a statistical significance. The mismatch term $p = 0.8174$, $p > 0.05$, and the mismatch term is not significant; thus, the model selection is correct. The correlation coefficients R-Squared = 0.9874 and Adj R-Squared = 0.9712 of the model indicate that the equation fits well. The ANOVA results of the quadratic regression model for *Trametes versicolor* ATCC20869 are presented in Table S1 simultaneously. In summary, the regression equation provides a suitable model for predicting and analyzing laccase production.

Table 1. TA-04 mutant strain's Response Surface Quadratic Model ANOVA results.

Source	Sum of Squares	df	Mean Square	F Value	*p*-Value Prob > F	
Model	1.290×10^6	9	1.433×10^5	64.20	<0.0001	significant
A-Farnesol	50,387.73	1	50,387.73	22.57	0.0021	
B-$CuSO_4$	70,855.31	1	70,855.31	31.74	0.0008	
C-pH	12,754.88	1	12,754.88	5.71	0.0481	
AB	54,779.69	1	54,779.69	24.54	0.0016	
AC	25,717.73	1	25,717.73	11.52	0.0115	
BC	1404.46	1	1404.46	0.63	0.4537	
A^2	3.916×10^5	1	3.916×10^5	175.41	<0.0001	
B^2	2.895×10^5	1	2.895×10^5	129.67	<0.0001	
C^2	2.806×10^5	1	2.806×10^5	125.71	<0.0001	
Residual	15,626.53	7	2232.36			
Lack of Fit	8755.18	3	2918.39	1.70	0.3040	not significant
Pure Error	6871.35	4	1717.84			

Notes: R-Squared = 0.9880 and Adj R-Squared = 0.9726.

Table 2. TA-15 mutant strain's Response Surface Quadratic Model ANOVA results.

Source	Sum of Squares	df	Mean Square	F Value	*p*-Value Prob > F	
Model	4.358×10^6	9	4.842×10^5	60.96	<0.0001	significant
A-Farnesol	1.782×10^5	1	1.782×10^5	22.44	0.0021	
B-$CuSO_4$	35,744.91	1	35,744.91	4.50	0.0716	
C-pH	3.241×10^5	1	3.241×10^5	40.81	0.0004	
AB	63,791.16	1	63,791.16	8.03	0.0253	
AC	4074.30	1	4074.30	0.51	0.4970	
BC	4276.23	1	4276.23	0.54	0.4869	
A^2	8.165×10^5	1	8.165×10^5	102.80	<0.0001	
B^2	1.524×10^6	1	1.524×10^6	191.91	<0.0001	
C^2	1.021×10^6	1	1.021×10^6	128.53	<0.0001	
Residual	55,595.16	7	7942.17			
Lack of Fit	10,526.47	3	3508.82	0.31	0.8174	not significant
Pure Error	45,068.68	4	11,267.17			

Notes: R-Squared = 0.9874 and Adj R-Squared = 0.9712.

Based on the analysis of response surface curves and contour plots, it is evident that the three factors under investigation exerted significant influences on the laccase enzyme activity of the two mutant strains, TA-04 and TA-15. Furthermore, the interaction between these factors was found to be statistically significant, thereby facilitating the identification of optimal fermentation conditions for maximizing enzyme production (Figures 8 and 9). Meanwhile, the response surface curve and contour plot analysis of *Trametes versicolor* ATCC20869 also demonstrated a significant influence of these three factors on the unit mycelial laccase activity of the original strain (Figure S1). The optimal

fermentation conditions for the mutant strains were determined through software analysis. It was inferred that TA-04 had the highest enzyme activity of 1173.96 U/mg DCW at 3.82 mM farnesol, 2.11 mM copper sulfate, and pH 3.95, while for TA-15, the optimal conditions were 4.59 mM farnesol, 2.00 mM copper sulfate, and pH 3.91, with an enzyme activity of 1287.21 U/mg DCW. After conducting the experiment three times under software-estimated conditions, the enzyme activity of TA-04 was 1120.184 ± 15.024 U/mg DCW, whereas the enzyme activity of TA-15 was 1297.773 ± 16.853 U/mg DCW. These values were found to be 9.875 and 11.441 times higher than before optimization, respectively, indicating a significant enhancement in laccase efficiency and providing substantial practical and theoretical guidance for large-scale laccase production. The experimental enzyme activity closely approximated the estimated value of the software, thereby validating the calculation model and demonstrating its practical significance.

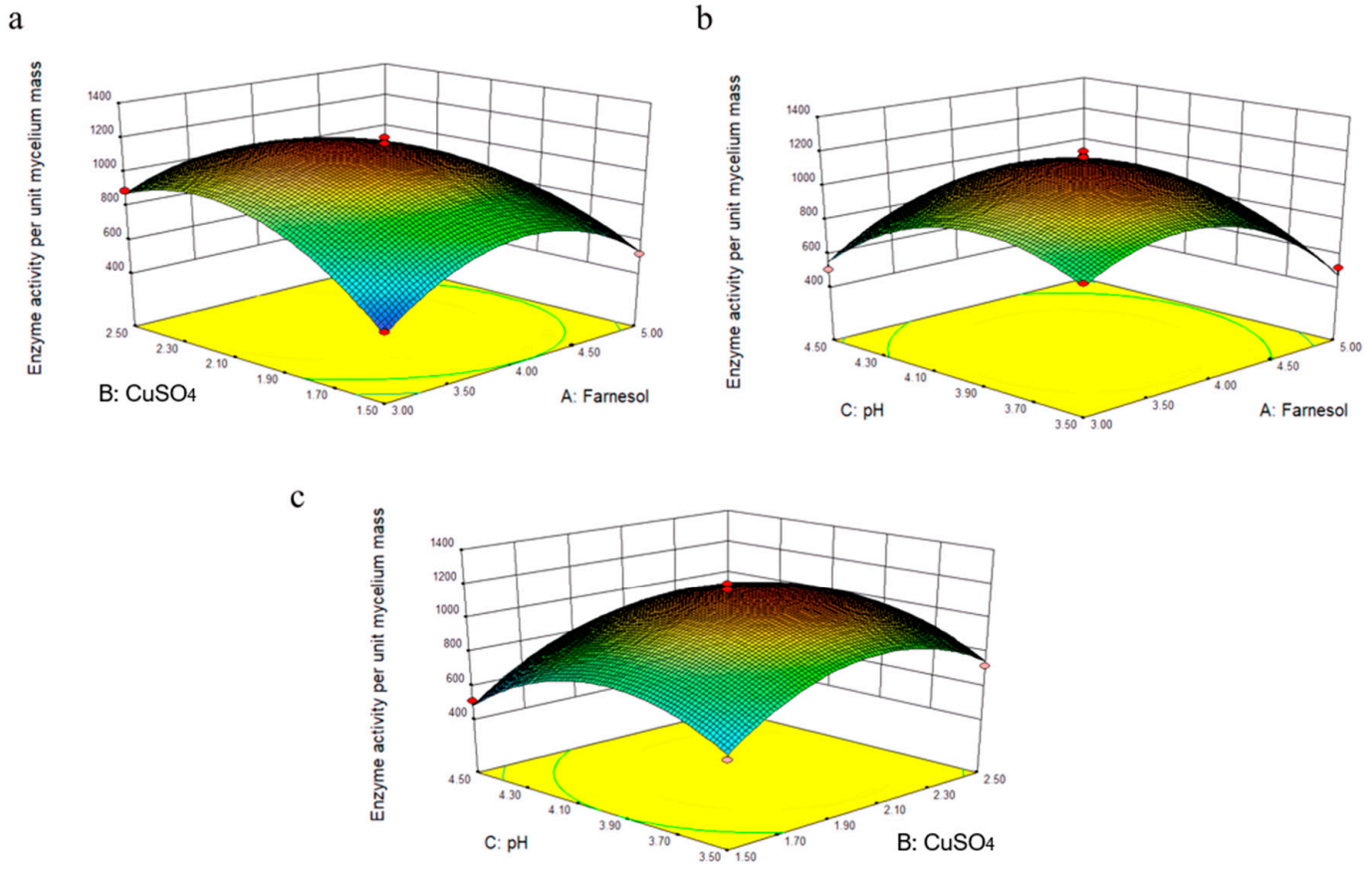

Figure 8. Response surface curves and contour plots of the mutant strain TA-04 under various conditions: (**a**) response surface curves and contour plots of farnesol addition and copper sulfate addition; (**b**) response surface curves and contour plots of farnesol addition and pH; and (**c**) response surface curves and contour plots of copper sulfate addition and pH. The types of plots and colors (circular or elliptical) indicated whether the mutual interactions between the variables were significant.

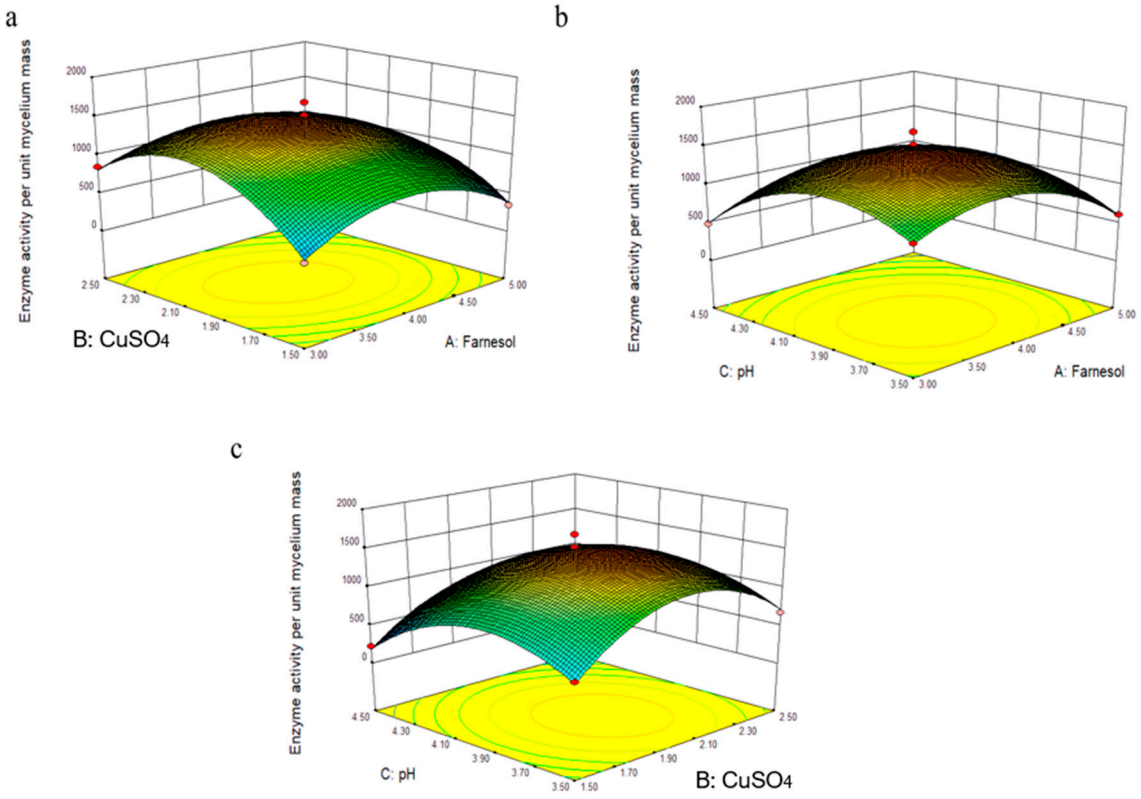

Figure 9. Response surface curves and contour plots of the mutant strain TA-15 under various conditions: (**a**) response surface curves and contour plots of farnesol addition and copper sulfate addition; (**b**) response surface curves and contour plots of farnesol addition and pH; and (**c**) response surface curves and contour plots of copper sulfate addition and pH. The types of plots and colors (circular or elliptical) indicated whether the mutual interactions between the variables were significant.

3.5. Analysis of Sequencing and Homology Modeling for Mutant Laccases

Mutation sites for the two laccase isoenzymes, Lcc1 and Lcc2, were analyzed based on the obtained sequencing results (Figure 10). In mutant strain TA-04, Lcc1 exhibits mutations including R43G, G236R, and E270A, while Lcc2 displays mutations N54Y and N249I. Similarly, in mutant strain TA-15, Lcc1 shows mutations H174L and N357I, whereas Lcc2 presents mutations R22L, N217K, Q252L, and T335P. In the mutant strain TA-04, R43G in Lcc1, a positively charged alkaline amino acid, underwent conversion to an uncharged residue; meanwhile, G236R transitioned from being uncharged to becoming positively charged. Simultaneously, E270A, a negatively charged acidic amino acid, was transformed into an uncharged residue. Conversely, in the mutant strain TA-15, H174L in Lcc1 and R22L in Lcc2 shifted from being positively charged alkaline residues to uncharged ones, whereas N217K in Lcc2 transitioned from being uncharged to becoming positively charged. We hypothesize that alterations in the charges of several amino acids within the active site may impact laccase's hydrogen bonding and hydrophobic interactions, thereby promoting the variation in bonds between coordinating amino acids and metals to enhance the enzymatic activity of laccase through the modulation of its internal redox potential. Concurrently, point mutations N249I, as well as Q252L, and T335P within the substrate binding region of Lcc2 in TA-04 and TA-15, respectively, lead to the substitution of hydrophobic amino acids. Consequently, a hydrophobic microenvironment is created within the enzyme's active site, thereby facilitating substrate binding reactions. The N54Y mutation site of Lcc2 in TA-04, N357I in Lcc1, and R22L in Lcc2 in TA-15 are coincidentally situated at the binding site within the secondary structural region. These mutations induce alterations in the enzyme structure's rigidity, promoting the binding of enzymes and substrates.

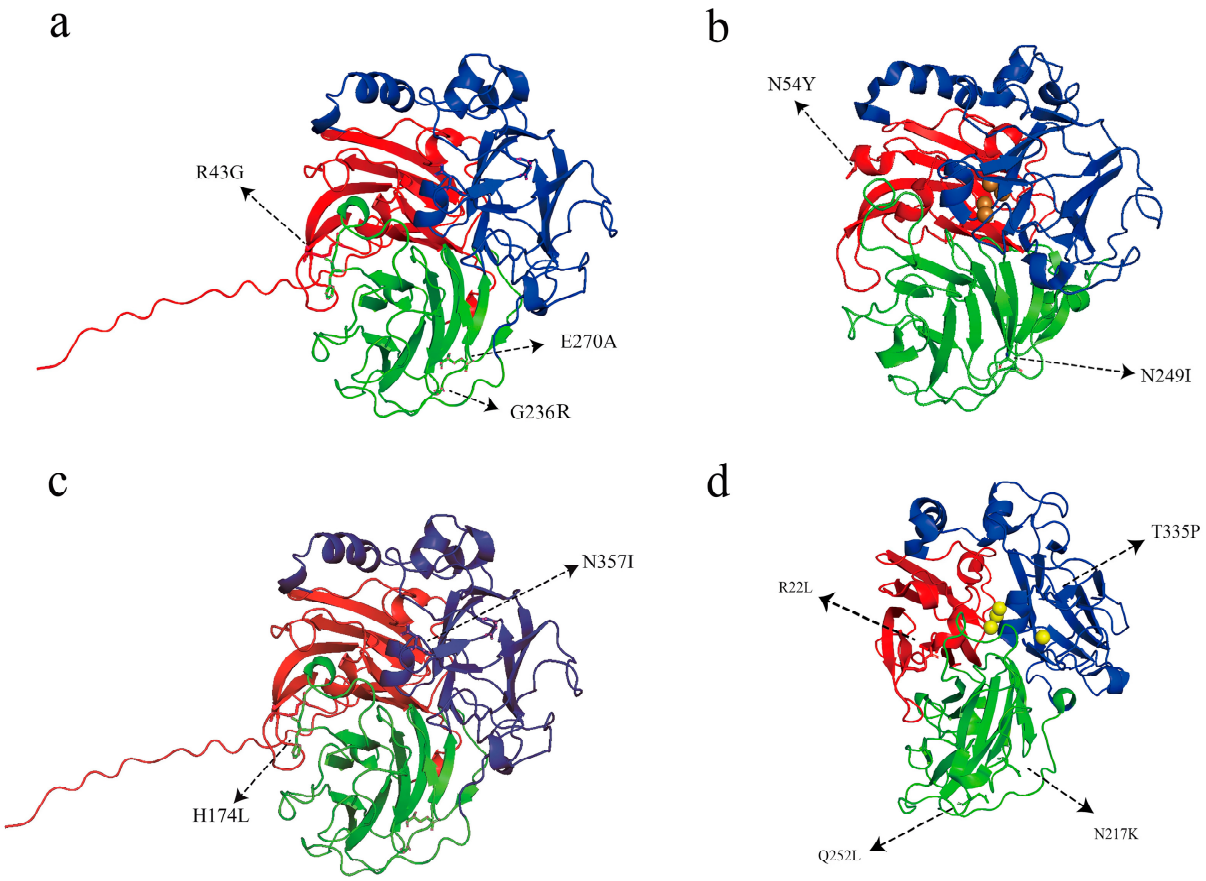

Figure 10. Three-dimensional structures are predicted by Lcc1 (**a**) and Lcc2 (**b**) from mutant strain TA-04 and Lcc1 (**c**) and Lcc2 (**d**) from mutant strain TA-15. The model was built with the PyMOL molecular graphics system according to its sequence. The three structural domains within laccase are labeled in red, blue, and green, respectively. Copper ions are drawn as yellow spheres.

4. Conclusions

The degradation of polyphenols and aromatic compounds is efficiently catalyzed by laccase, a multicopper oxidase, which is widely applied in environmental processes such as wastewater treatment and dye decolorization. *Trametes versicolor* laccases are well known for their broad compound transformation abilities. However, the application of these enzymes is curtailed due to their low enzymatic activity, as well as their limited tolerance to heat and pH fluctuations. Significant enhancements via genetic modification have been hindered by the structural stability of laccase. Firstly, the two mutant strains demonstrated a significantly augmented laccase production capacity, exhibiting 1.021-fold and 1.354-fold higher levels compared to the wild strains, respectively. Additionally, a comprehensive analysis was conducted to evaluate the catalytic activity of both mutant strains under varying pH, temperature, and salinity conditions. The results revealed a significant enhancement in the laccase catalytic characteristics. The consequences presented herein demonstrate the efficacy of ARTP as a mutational strategy for enhancing the catalytic properties of laccase in *Trametes versicolor*. To elucidate the factors contributing to the enhanced catalytic properties of laccase, this study employed sequencing and homology modeling methods to preliminarily analyze the impact of amino acid residue alterations on both the structure and function of laccase. This analysis provides a theoretical basis for further augmenting laccase activity using ARTP mutagenesis or other rational genetic approaches. The production conditions of laccase were optimized through one-way and Box–Behnken experiments, thereby concurrently enhancing its yield. In conclusion, the ARTP mutation method proves to be a highly effective approach for enhancing the catalytic properties of laccase. In future,

we will integrate the findings of this study with rational genetic engineering approaches to further enhance the catalytic properties of laccase. Additionally, a yeast heterologous expression system would be employed to optimize the yield of laccase, thereby establishing a solid theoretical foundation for its subsequent practical applications.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9120995/s1, Figure S1: Response surface curves and contour plots of *Trametes versicolor* ATCC20869 under various conditions; Table S1: *Trametes versicolor* ATCC20869's Response Surface Quadratic Model ANOVA results.

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