RESEARCH ARTICLE

Optimization study of laccase production by *Flammulina velutipes* and the resistance research on laccase to tert-butyl hydroquinone toxicity

Haozheng Zhou[1], Lei Zhang[1], Zikai Lin[1], Ping Liu[1], Gang Li[1], Yonglin Han[2,*], Shaohong Wen[1,*]

[1]School of Life Sciences, Yantai University, Yantai, Shandong, China. [2]Yantai Science and Technology Innovation Promotion Center, Yantai, Shandong, China.

Received: January 15, 2025; **accepted:** May 26, 2025.

Laccase, a copper-dependent oxidase, can degrade toxic compounds, particularly phenolic substances. Its applications are primarily confined to environmental remediation and industrial processes with limited biomedical and biological exploration. Tert-butyl hydroquinone (TBHQ), a widely used lipid-soluble antioxidant, has been associated with adverse health effects and potential carcinogenicity upon chronic exposure. This study evaluated the protective effect of laccase against TBHQ-induced developmental abnormalities in zebrafish and aimed to optimize laccase production in *Flammulina velutipes*. A combined approach utilizing Plackett-Burman design and response surface methodology was applied to identify the optimal conditions for increasing laccase production by *Flammulina velutipes* LP03. Moreover, zebrafish embryos were exposed to 20 μM TBHQ for 120 hours, and three distinct laccase intervention groups were established to facilitate pathological assessment. The results showed that the optimal process conditions were 1.75% peptone, 0.46% glucose, 0.0798% $MgSO_4 \cdot 7H_2O$, pH of 6.11, and a liquid volume of 222.4 mL. The optimal combination for laccase production determined by the Response Surface Methodology (RSM) was 2,920.33 U/L. The results confirmed that laccase effectively alleviated TBHQ-induced zebrafish embryonic malformations, improved cardiac circuitry, and reduced pericardial edema. In addition, the study showed that TBHQ suppressed the expression of melanin-related genes including tyr, dct, oca2, and silv, while laccase intervention successfully counteracted the suppression of these genes. The optimized method was verified to significantly increase the production of laccase. In addition, laccase also alleviated the symptoms caused by TBHQ through the above molecular pathways.

Keywords: laccase; tert-butyl hydroquinone; melanin pigmentation; *Flammulina velutipes*; toxicity.

[*]**Corresponding authors:** Yonglin Han, Yantai Science and Technology Innovation Promotion Center, Yantai, Shandong, China. Shaohong Wen, School of Life Sciences, Yantai University, Yantai 264005, Shandong, China. E-mail: ytstipc@163.com (Han Y), wenshaohong1@163.com (Wen S).

Introduction

Laccase is a copper-containing oxidoreductase mainly produced by bacteria and fungi and widely used in wastewater treatment, wine clarification, and edible fungus cultivation in the food industry [1]. These enzymes catalyze the oxidation of a broad range of substrates including aromatic amines, phenolic compounds such as lignin-derived phenolics and chlorophenols, anthraquinone dyes, and, to a significant extent, certain polycyclic aromatic hydrocarbons (PAHs). Laccase transforms these toxic substances particularly phenolic compounds like phenol through oxidative coupling into water-insoluble products, therefore facilitating their removal [2]. Laccase exhibits a molecular weight ranging from 60 to 100 kilodaltons (kDa). In the past decade,

extensive research has focused on its biochemical characteristics, roles in bioremediation, and utility in various chemical transformations [3]. The scope of laccase research has evolved from elucidating its core catalytic mechanisms to embracing interdisciplinary applications, highlighting its significant promise in sustainable development and green chemistry. Future investigations should prioritize resolving challenges in large-scale production, improving catalytic efficiency, and expanding its application into emerging fields such as biomedical research and microplastic degradation to maximize its socio-economic impact. Growing interest in laccase is driven by its environmental benefits and potential applications in various fields, particularly due to its capacity to degrade harmful substances. *Flammulina velutipes* commonly known as Enoki mushrooms are a widely favored edible white-rot fungus [4], which is known for its ability to produce laccases. The optimizing the laccase production process in Enoki mushrooms through response surface methodology has been an area of significant research.

Tert-butyl hydroquinone (TBHQ) is a widely used lipid-soluble synthetic antioxidant primarily incorporated into food products such as biscuits and instant noodles [5]. It is also frequently used in cosmetics, particularly lipsticks and blushes, to improve product stability. However, it has been found that the average TBHQ intake in several countries surpasses the recommended maximum daily intake (MDI). Moreover, prolonged exposure to TBHQ has been associated with potential health risks including embryotoxicity and carcinogenicity [6]. Although TBHQ is considered safe within regulatory thresholds, emerging evidence suggests that excessive intake may lead to adverse outcomes. Animal studies have demonstrated that high doses of TBHQ can result in hepatomegaly, DNA damage [7], and cellular dysfunction. Given its widespread use, the potential health implications of chronic TBHQ exposure warrant careful consideration. The Plackett-Burman (P-B) design is recognized as an effective approach for identifying key factors influencing the response variables [8]. It enables the selection of significant variables from a large pool without evaluating interaction effects, thus substantially reducing the number of experimental trials required to assess main effects [9]. This design has been widely adopted in various studies. The response surface methodology often employs the central composite design (CCD) for further optimization. CCD can achieve rotatability when appropriately chosen axial distance (α). Rotatability is a geometric property related to spherical symmetry and is a valuable criterion when the experimental region is assumed to be spherical. For design that all the points of the factorial design and on the axis are placed on the spherical surface, the radius is √K and called spherical surface CCD [10]. Although strict rotatability is not critical for a well-performing design, α should be chosen to be √K from the perspective of a predetermined variance.

TBHQ has drawn increasing concern due to its reported adverse effects on human health. However, recent research primarily focuses on its antioxidant properties with limited attention given to its toxicity and detoxification mechanisms. This study investigated the *in vivo* effects of laccase intervention on TBHQ and explored new ideas for the use of laccase in the treatment of TBHQ-induced toxicity and its degradation in biological systems. The ability of laccase to degrade phenolic compounds and other toxic substances was explored. In addition, the potential protective effect of laccase on zebrafish embryos exposed to TBHQ was investigated. This research provided valuable knowledge on optimizing laccase production in Enoki mushrooms and highlighted its possible role in mitigating the harmful effects of TBHQ on human health.

Materials and methods

Plackett-Burman design (P-B)

The P-B design is a two-level factorial design based on the first-order model and was generated by using Design Expert (https://www.statease.com/software/design-expert/). The key factors influencing laccase production from eight variables including maize flour, peptone, glucose, pH, $MgSO_4 \cdot 7H_2O$, KH_2PO_4, Cu^{2+} concentration, and inoculation volume were identified through the experiments for subsequent optimization aimed at maximizing enzyme activity. Factors demonstrating a confidence level greater than 95% were considered the most influential in laccase production during the liquid-submerged fermentation of *Flammulina velutipes*.

The steepest ascent experiment

Using the multiple linear regression equations derived from the P-B test, the steepest ascent method was employed to design the ascent paths and step sizes for the primary factors, which was achieved by analyzing the signs (positive/negative) and magnitudes of the coefficients for the significant factors, thus pinpointing the region of maximum response. This approach provided an efficient and cost-effective means of approximating the optimal conditions in preparation for subsequent response surface methodology (RSM) experiments.

Central composite design (CCD)

A CCD that was generated using Design Expert 7.1.6 software and involved five factors at five levels was employed to assess the impact of factors on the response surface within the specified experimental range [11]. The factors that were selected for their significant influence on laccase production included peptone, glucose, pH, $MgSO_4$, and liquid medium volume. These factors were tested at two levels as high (+1) and low (−1) with center point set at a coded level of 0. A total of 50 experimental runs were conducted, and the results were calculated as follows.

$N = 2^k + 2k + 8$

where k was the number of factors. 2^k was the number of factorial points. 2k was the number of axial points. and 8 corresponded to the center point replications used to estimate pure error. Axial points were included with one factor set at an α value, while the others remained at 0. The fitting of the data to various models including linear, two factorial, quadratic, and cubic could be presented as follows.

$$Y = \beta_0 + + \sum \beta_i X_i + \sum \beta_{ii} X_i^{\,2} + \sum \beta_{ij} X_i X_j$$

Where Y was the response value. β_0 was the constant coefficient. β_i and β_{ij} were the second-order interaction coefficients where i and j were from 1 to 5. X_i was variables where i was from 1 to 5. The statistical test factor (F-test) and coefficient of determination (R2) were applied to assess the model equation's statistical significance and goodness of fit. Besides, the t-test was used to examine the importance of the regression coefficients [12].

Laccase concentrates

Key parameters affecting laccase activity were optimized using RSM to identify the optimal culture medium and conditions. *Flammulina velutipes* LP03 provided by Microbiology Laboratory at Yantai University (Yantai, Shandong, China) was cultured in a liquid medium based on the optimized conditions. MWCO Millipore 50 kDa ultrafiltration concentration tubes (Sigma-Aldrich, St. Louis, MO, USA) were employed for efficient concentration. Briefly, 15 mL aliquot of the liquid culture was centrifuged to obtain the supernatant that contained crude enzyme solution and then was transferred to the ultrafiltration tubes and centrifuged at 8,000 rpm at 4°C for 10 minutes. The filtrate underwent a second ultrafiltration step to minimize protein loss.

Laccase activity assay

Mycelium was harvested from liquid cultures at various time points. Culture broth samples were

centrifuged at 4,000 rpm for 2 minutes, and the supernatant was collected for enzyme activity measurement. Enzyme activity was determined spectrophotometrically using 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) as the substrate. Laccase activity was calculated using a molar extinction coefficient of 3.6×10^4/M·cm [13]. A commercially available laccase solution with an activity of 5,000 U/g (Anhui Cool Bio-engineering Co., Huaibei, Anhui, China) was used as the standard. A 100 mM citrate/phosphate buffer (pH 5.0) was prepared, and a 0.5 mM ABTS solution served as the standard solution [14, 15]. One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per minute.

Experimental animals

Zebrafish as vertebrates share a significant evolutionary relationship with humans [16]. Genomic analyses have shown that the zebrafish genome exhibits approximately 70% similarity to the human genome [17]. Moreover, 80% of zebrafish genes are arranged on identical chromosomes in the same sequence, and 84% are associated with human diseases. Wild-type AB line zebrafish were obtained from Hubei Chuangxin Biotechnology Co., Ltd. (Wuhan, Hubei, China) and fed twice daily with newly hatched brine shrimp in an aquaculture system with the temperature between 26 and 28.5°C and a light/dark cycle of 14/10 hours. The water pH was maintained between 6.8 and 7.5, conductivity was kept at 4, and salinity was adjusted to 50 –500 μs/cm. The pH was adjusted by adding $NaHCO_3$, while the conductivity was regulated with button salt. The fish feed was prepared by incubating shrimp eggs, sea salt, and deionized water in a ratio of 10g:40g:10L for 24 h. The zebrafish were evenly distributed on both sides of the breeding tank in a 1:1 ratio with a partition in the middle. The fish were allowed to acclimate for 12 hours before removal of the partition. The zebrafish were then given 2 to 3 h to mate. Following mating, the embryos were collected using filters, rinsed with embryo culture solution, and transferred to Petri dishes containing embryo culture medium for further development. Any dead embryos or contaminants were discarded.

Acute toxicity tests for laccase

The E3 embryo culture water was prepared by dissolving 0.127 g KCl, 2.867 g NaCl, 0.817 g $MgSO_4 \cdot 7H_2O$, and 0.365 g anhydrous $CaCl_2$ in 10 L ultrapure water. Laccase solutions with concentrations of 150 U/mL, 75 U/mL, and 37.5 U/mL were prepared in E3 water and applied to zebrafish embryos at the 6-hour stage with 20 embryos per group, while the group treated with E3 water alone served as the control. Both the mortality rate and the 72-hour hatching rate were monitored.

Treatment groups

The zebrafish embryos used in this study were healthy and selected at the 6 hours developmental stage. The experiment commenced at this stage with the culture medium replaced every 12 h over a total exposure period of 120 hours. A 20 μM TBHQ solution was prepared in E3 water for the exposure experiment. The experiment consisted of five groups with two replicates in each group and 20 embryos per replicate. The group treated with E3 water was the normal control, while the group treated with 20 μM TBHQ was designated the model group. The administration groups consisted of laccase solutions, where 20 μM TBHQ was used to dilute the laccase to concentrations of 80 U/mL, 40 U/mL, and 20 U/mL, respectively. A dose of 3 mL was administered per treatment.

Histopathological studies

A total of 8.0 mg of tricaine powder (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 50 mL of ultrapure water to prepare a 0.016% (w/v) tricaine solution. Zebrafish were cultured for 120 h and then anesthetized with benzocaine for 2 min before being fixed using sodium carboxymethyl cellulose and photographed for subsequent examination under an Leica DMi1 electron microscope (Leica Camera, Wetzlar, Germany). The yolk sacs, heart annulations, and pericardial areas were measured using ImageJ

Table 1. Primers' sequences for qPCR.

Gene	Forward primer	Reverse primer
β-Actin	TGCCCCTCGTGCTGTTTT	TCTGTCCCATGCCAACCAT
tyr	TTTACAGGATCCAGGTCAGCG	GCCACTGCGAAAACCGATG
dct	TGGACAGTAAACCCTGGGGA	CCGGCAAAGTTTCCAAAGCA
oca2	CTTATTTGGCAGCTGCGGTC	ACAGAGTCTGACTTTCGGCG
silv	CAGACATGGTGATGGTGGAG	GGACGATCTGCACACTCTCA

(https://imagej.net/ij/) software.

Quantitative Polymerase Chain Reaction (qPCR)

After 120 h of the experiment, 35 zebrafish from each group were homogenized in 700 µL of tissue lysis buffer, and the total RNAs were extracted using the Tissue and Cell RNA Rapid Extraction Kit (Shandong Sikejie Biotechnology Co., Ltd., Jinan, Shandong, China) following the manufacturer's instructions. After the determination of the RNA concentration and purity using a spectrophotometer, 1 µL aliquot of the sample was used for cDNA synthesis by using SPARKscript II RT Plus Kit (Shandong Sikejie Biotechnology Co., Ltd., Jinan, Shandong, China) following manufacturer's instructions. The resulting cDNA was then used for qPCR using the SYBR Green qPCR Mix Kit (Shandong Sikejie Biotechnology Co., Ltd., Jinan, Shandong, China) following the manufacturer's instructions. 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) was employed for qPCR reaction with the primers listed in Table 1 where β-actin served as the reference control gene.

Statistical analysis

Data were expressed as mean ± SEM and were analyzed using GraphPad Prism (https://www.graphpad-prism.cn/). A *P* value less than 0.05 was considered statistically significant.

Results

Selection of key factors using P-B design

The results showed that the fitted first-order model equation for laccase activity could be expressed as below.

$$Y = +1279.96 + 50.24X_1 - 320.47X_2 - 153.18X_3 - 245.31X_4 + 143.45X_5 + 31.10X_6 - 85.73X_7 + 237.60X_8$$

The effects of various factors on the response and their significance levels demonstrated that, based on the model equation, corn flour (X_1), $MgSO_4 \cdot 7H_2O$ (X_5), KH_2PO_4 (X_6), and liquid volume (X_8) had the positive influences on laccase production in *Flammulina velutipes* LP03. However, peptone (X_2), glucose (X_3), pH (X_4), and Cu^{2+} (X_7) showed a negative impact. This evaluation facilitated the identification of key variables for subsequent optimization.

Path of steepest ascent experiment

Based on the P-B design model equation analysis, the direction and magnitude of the optimization steps were determined. Accordingly, it was predicted that increasing the levels of corn flour, $MgSO_4 \cdot 7H_2O$, KH_2PO_4, and liquid volume while reducing the concentrations of peptone, glucose, and Cu^{2+} would increase laccase yield. The results showed that the third experimental group exhibited the highest laccase activity among all groups, suggesting that the conditions in run 3 were likely close to the optimal parameters for laccase production (Table 2). Therefore, this combination was selected as the central point for the subsequent response surface composite design.

ANOVA analysis and fitting of the quadratic model of CCD

Table 2. Path of steepest ascent design.

Run	Peptone (%)	Glucose (%)	pH	$MgSO_4$ (%)	Liquid volume (mL)	Laccase activity (U/L)
1	1.45	0.95	7.0	0.055	170	1,576.26 ± 122
2	1.35	0.85	6.5	0.065	190	1,954.34 ± 40
3	1.25	0.75	6.0	0.075	210	2,533.50 ± 76
4	1.15	0.65	5.5	0.085	230	1,856.11 ± 43
5	1.05	0.55	5.0	0.095	250	1,827.39 ± 70
6	0.95	0.45	4.5	0.105	270	1,738.43 ± 68
7	0.85	0.35	4.0	0.115	290	1,578.11 ± 79

Table 3. Coded and actual levels of variables considered for CCD.

Factors	Level				
	Lowest	Low	Center	High	Highest
	-2.236	-1	0	1	2.236
Peptone (%)	0.132	0.75	1.25	1.75	2.368
Glucose (%)	0.0792	0.45	0.75	1.05	1.421
pH	3.76	5.00	6.00	7.00	8.24
$MgSO_4$ (%)	0.0079	0.025	0.075	0.125	0.142
Liquid volume (mL)	120.6	170	210	250	299.4

A central composite design comprising five factors at five levels with a total of 50 experimental runs was utilized. The coded and actual values of the variables selected for the CCD were shown in Table 3, and the mathematical expression of the relationship between the enzyme activity and the variables in terms of coded factors were shown.

$$Y = 2808.67 + 34.98X_1 - 120.55X_2 + 100.19X_3 + 61.29X_4 + 12.12X_5 + 24.10X_1X_2 - 31.06X_1X_3 - 57.22X_1X_4 + 51.52X_1X_5 - 23.98X_2X_3 + 6.92X_2X_4 + 43.22X_2X_5 + 129.10X_3X_4 - 66.56X_3X_5 + 72.80X_4X_5 + 4.61X_1^2 - 291.71X_2^2 - 326.25X_3^2 - 125.22X_4^2 - 98.92X_5^2$$

Under the condition of $R^2 = 0.9859$, the model statistics (F = 26.24, P = 0.0107) included the variables X_1 (F = 1.93, P = 0.2587), X_2 (F = 78.64, P = 0.003), X_3 (F = 17.96, P = 0.024), X_4 (F = 46.08, P = 0.00065), X_5 (F = 15.76, P = 0.0286), X_6 (F = 0.74, P = 0.4528), X_7 (F = 5.63, P = 0.0983), X_8 (F = 43.22, P = 0.0072). The significance of each coefficient was determined by Student's t-test. The ANOVA results indicated that X_2, X_3, X_3X_4, X_2^2, X_3^2, X_4X_5, and X_5^2 had significant effects on laccase production, while the remaining factors and interactions showed no significant influence (Table 4). To evaluate the adequacy of the response surface model, three statistical tests were performed including the overall significance of the regression model, the importance of individual model coefficients, and the lack of fit test [18]. The results showed that the model was highly significant ($P < 0.0001$), suggesting it effectively captured the relationship between the experimental variables and laccase production across a broad operational range. The lack-of-fit test yielded a non-significant result (P = 0.3134), indicating that the model fit the data well within the tested variable range. The coefficient of determination (R^2) was 0.8817, which was acceptably close to 1, further supporting the model's reliability. An adequate precision value above 4 was desirable, and the obtained value of 13.404 confirmed that the model had a satisfactory signal-to-noise ratio (Table 5). Furthermore, the residual plot results demonstrated that residuals were approximately normally distributed, aligning along a straight line and validating the normality assumption in the error terms (Figure 1) [19].

Table 4. ANOVA for response surface quadratic model.

Variable	Coefficient	Standard Error	F Value	p-value
Intercept	2808.67	79.63	10.80	< 0.0001
X_1	34.98	34.75	1.01	0.3225
X_2	-120.55	34.75	12.03	0.0017
X_3	100.19	34.75	8.31	0.0073
X_4	61.29	34.75	3.11	0.0884
X_5	12.12	34.75	0.12	0.7298
$X_1 X_2$	24.10	39.81	0.37	0.5497
$X_1 X_3$	-31.06	39.81	0.61	0.4417
$X_1 X_4$	-57.22	39.81	2.07	0.1614
$X_1 X_5$	51.52	39.81	1.67	0.2059
$X_2 X_3$	-23.98	39.81	0.36	0.5516
$X_2 X_4$	6.92	39.81	0.030	0.8631
$X_2 X_5$	43.22	39.81	1.18	0.2866
$X_3 X_4$	129.10	39.81	10.51	0.0030
$X_3 X_5$	-66.56	39.81	2.80	0.1053
$X_4 X_5$	72.80	39.81	3.34	0.0778
X_1^2	4.61	33.37	0.019	0.8911
X_2^2	-291.71	33.37	76.42	< 0.0001
X_3^2	-326.25	33.37	95.59	< 0.0001
X_4^2	-125.22	33.37	14.08	0.0008
X_5^2	-98.92	33.37	8.79	0.006

Table 5. ANOVA result for quadratic equation of the response.

Source	Sum of Squares	df	Mean Square	F value	P value
Residual	1.471E + 006	29	50,725.68		
Lack of fit	1.209E + 006	22	54,941.20	1.47	0.3134
Pure error	2.623E + 005	7	37,476.89		
Cor total	1.243E + 007	49			
R-Squared 0.8817	Adj R-Squared 0.8001	Adeq Precision	13.404		

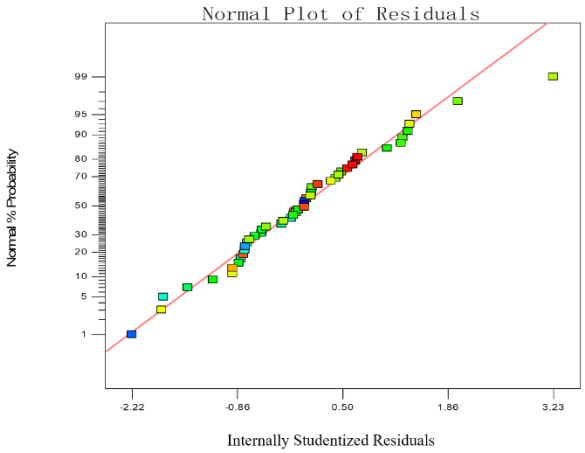

Figure 1. Normal plot of residuals.

The response surface plots generated by the regression model demonstrated the interactive effects of two independent variables on laccase production while keeping the remaining variables constant at their central (zero) levels. The results showed that, in the interaction between glucose concentration and pH, the laccase activity was minimal when the lower pH values and reduced glucose concentrations were presented (Figure 2a). The maximum enzyme activity was observed within the pH 6.0 - 6.5 and glucose concentrations of 0.7% - 0.75%. Although increased glucose generally promoted mycelial growth, it did not directly correlate with increased laccase production. On the other hand,

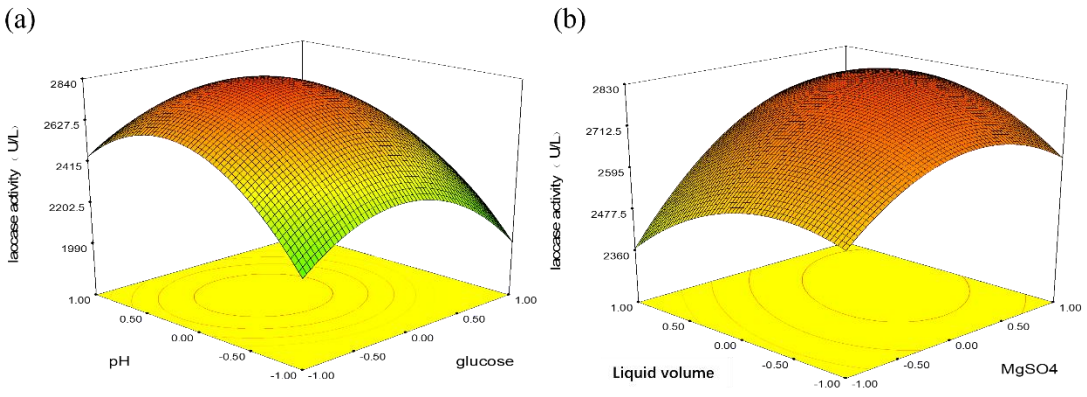

Figure 2. 3D response surface curve. (**a**) The full quadratic model predicted the 3D response surface curve of glucose and pH. (**b**) The full quadratic model predicted the 3D response surface curve of $MgSO_4$ and liquid volume.

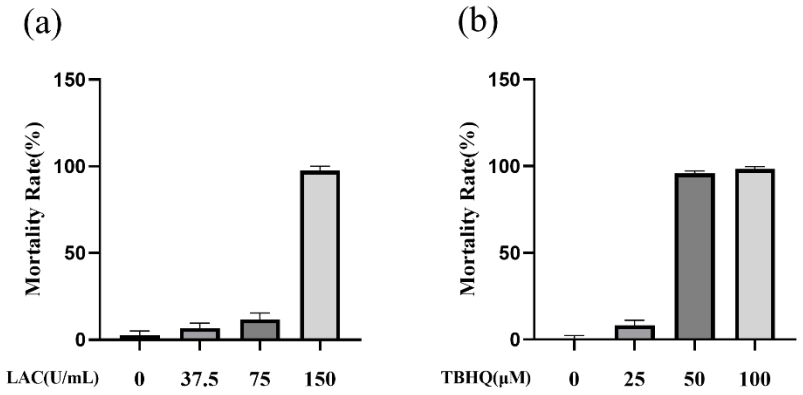

Figure 3. Acute toxicity test results of laccase. (**a**) Lethality rates at different laccase concentrations. (**b**) Lethality rates at different TBHQ concentrations.

the laccase activity increased with rising concentrations of $MgSO_4$ and liquid medium volume with the peak near the central point of the design space (Figure 2b). The optimum of the variables was calculated from the data obtained [20]. The optimal actual values of the key factors for laccase production were 1.75% peptone, 0.46% glucose, 0.0798% $MgSO_4$, pH 6.11, and 222.4 mL of liquid medium, which were validated through a series experiments to validate the model's predictive capability. The results demonstrated that the actual laccase activity reached 2,920.33 U/L, which closely matched the model's predicted peak value of 2,869.32 U/L. The results represented a 4.85-fold increase compared to the initial activity of 499.58 U/L before optimization. These findings confirmed the robustness and accuracy of the response surface model in forecasting laccase production, demonstrating that the optimized culture conditions significantly improved laccase secretion in *Flammulina velutipes* LP03.

Acute toxicity test of laccase

The mortality rate of zebrafish embryos was evaluated after 120 hours of development. The results showed that embryo mortality reached 100% at a concentration of 150 U/mL within the 3 mL system by the 120-hour mark. However, the minimal mortalities were observed at 75 U/mL and 37.5 U/mL concentrations, which remained within acceptable limits (Figure 3a). The highest enzyme activity concentration of 80 U/mL deemed safe and was selected as the reference

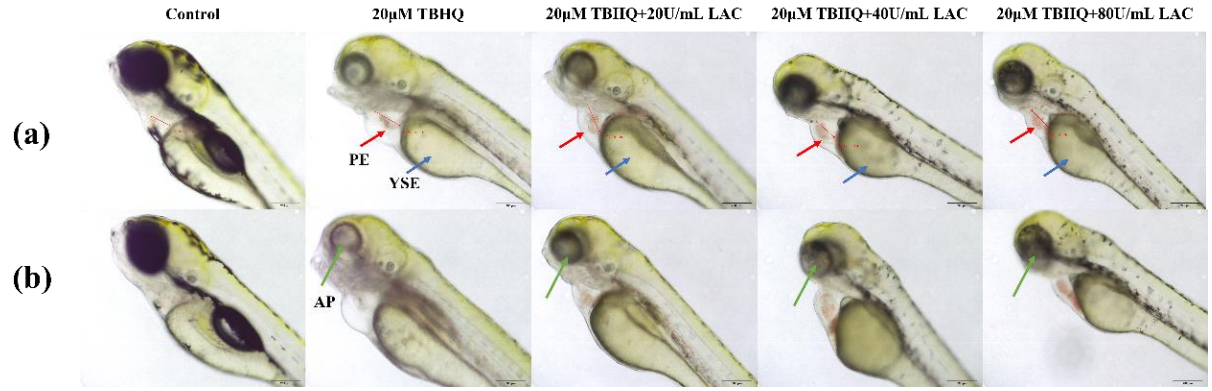

Figure 4. Histopathological studies of zebrafish embryo (4×, 200μm). (**a**) Effects of laccase on TBHQ on zebrafish malformations. →PE: primitive heart tube elongation and looping. →YSE: yolk sac expansion. (**b**) Effects of laccase on TBHQ on zebrafish pigmentation. →AP: abnormal pigmentation.

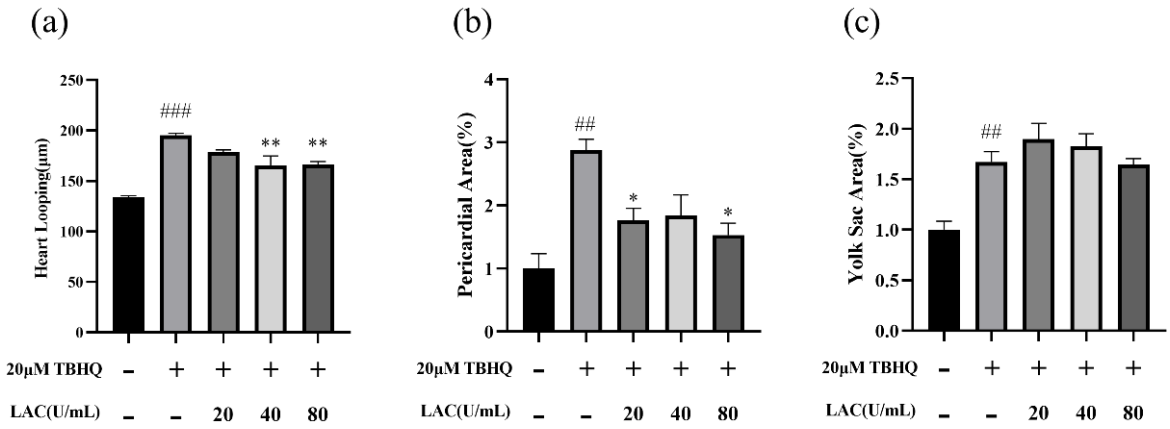

Figure 5. Effects of laccase on TBHQ induced malformations in zebrafish embryo. (**a**) Heart looping. (**b**) Pericardial area. (**c**) Yolk sac area. ###: $P < 0.001$ *vs.* control. ##: $P < 0.01$ *vs*. control. **: $P < 0.01$ *vs.* 20 μM TBHQ. *: $P < 0.05$ *vs.* 20 μM TBHQ.

point for serial dilution. From this baseline, two additional concentrations of 40 U/mL and 20 U/mL were derived and used as the upper and lower exposure thresholds, respectively. The zebrafish embryos were exposed to 100 μM TBHQ and exhibited mortality beginning on the second day with the vast majority succumbing by day three. Similarly, embryos treated with 50 μM TBHQ demonstrated significant mortality after 120 hours of exposure. However, only limited lethality was observed at 25 μM TBHQ (Figure 3b). Based on these results, a concentration of 20 μM TBHQ was selected as the modeling concentration to minimize TBHQ-induced mortality while maintaining experimental integrity.

Effects of laccase on TBHQ induced zebrafish embryo malformations

The heart is the first organ to form and begin functioning during zebrafish development. Cardiac looping and the size of the pericardial area serve as important indicators for assessing cardiac development and identifying potential malformations. The results showed that zebrafish exposed to 20 μM TBHQ exhibited pronounced cardiac looping and pericardial edema, indicative of cardiac injury or developmental defects. However, zebrafish co-treated with laccase showed a marked improvement in these parameters, suggesting a protective or ameliorative effect of laccase on cardiac morphology (Figure 4). Furthermore, yolk

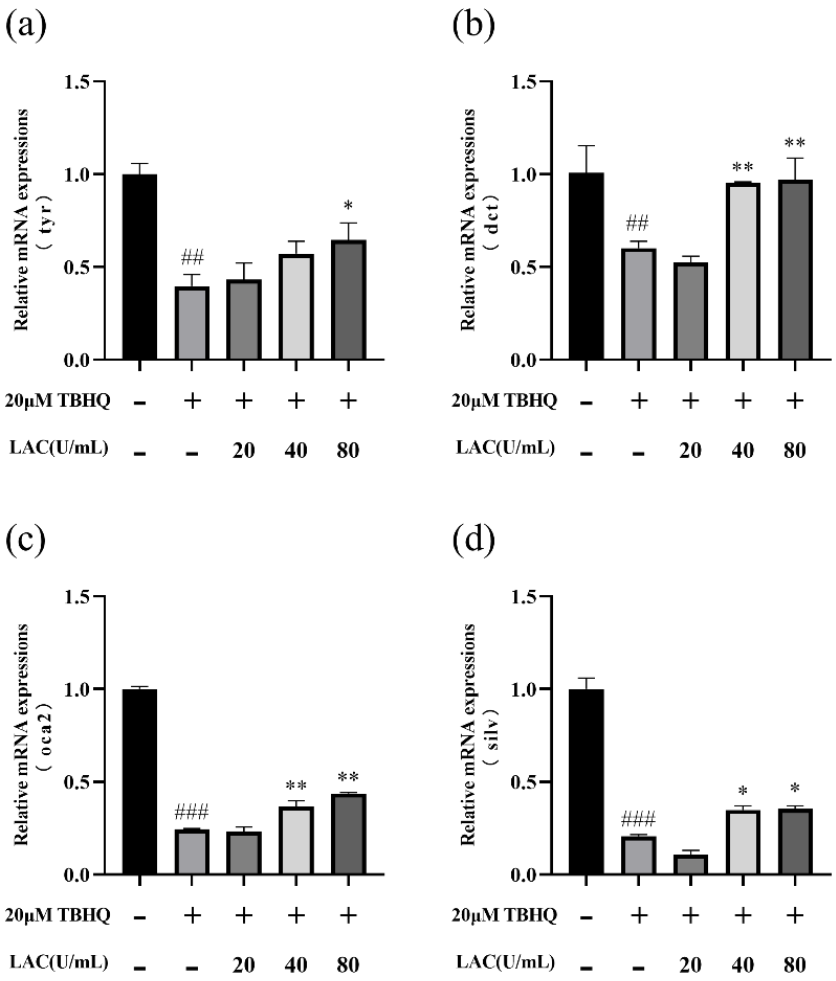

Figure 6. Laccase upregulated the mRNA levels of *tyr*, *dct*, *oca2*, *silv* in zebrafish embryo affected by TBHQ. **(a)** *tyr*. **(b)** *dct*. **(c)** *oca2*. **(d)** *silv*. ###: $P < 0.001$ *vs*. control. ##: $P < 0.01$ *vs*. control. **: $P < 0.01$ *vs*. 20 μM TBHQ. *: $P < 0.05$ *vs*. 20 μM TBHQ.

sac enlargement is another indicator of developmental abnormality. A significant expansion of the yolk sac was observed in embryos exposed to 20 μM TBHQ. However, treatment with laccase did not substantially alleviate this malformation, indicating that its protective effects might be organ-specific and less effective in correcting yolk sac-related anomalies (Figure 5).

Effects of laccase on TBHQ induced zebrafish pigmentation

It was hypothesized that TBHQ exposure would lead to hypopigmentation in zebrafish, resulting in albino or translucent phenotypes during early development. The results demonstrated that there were clear differences in pigmentation, particularly in the eyes and body markings, which were evident between the treatment and control groups (Figure 4) [21]. Key genes involved in melanin biosynthesis including *tyr*, *dct*, *oca2*, and *silv* were selected for expression analysis based on their established roles in pigmentation pathways [22]. The results showed that zebrafish embryos exposed to TBHQ demonstrated significant downregulation of these pigmentation-related genes, which were consistent with the observed reduction in melanin. However, co-treatment with laccase at varying concentrations effectively mitigated this suppression. The extent of gene expression recovery was positively correlated with the

laccase concentration, indicating a dose-dependent protective effect (Figure 6).

Discussion

Laccase, a polyphenol oxidase enzyme containing copper, is primarily derived from bacteria and fungi. It is an environmentally friendly and versatile oxidizing agent with widespread applications in food processing and environmental management industries. Given its ability to degrade harmful substances, laccase has attracted considerable interest for its potential in environmental applications. While much of the current research focuses on laccase's use in various chemical reactions, this research explored its role in mitigating the bio-pathological damage caused by TBHQ. Moreover, this study investigated and optimized the conditions for laccase production, providing valuable insights for improving this enzyme's production and practical applications. TBHQ, a commonly encountered antioxidant, exhibits relatively potent anti-lipid peroxidation capabilities and is extensively used in various food products such as potato chips and instant noodles [23]. However, research has confirmed that prolonged exposure to TBHQ is linked to carcinogenic potential, and the possibility of its accumulation in the human body and subsequent harmful effects cannot be ruled out [24]. Furthermore, the specific pathogenic mechanisms underlying the effects of TBHQ remain poorly understood and warrant further investigation. As a polyphenolic compound, TBHQ belongs to the class of phenolic substances. Phenols and their derivatives serve as substrates for laccases that can degrade them into harmless compounds and water. Therefore, exploring the potential mitigating effect of laccases on the pathological damage induced by TBHQ presents a promising avenue for its application. RSM has been widely used in chemical and biochemical processes for various purposes, which has several advantages compared to the classical experimental and optimization methods using the one-variable-at-a-time technique [25]. Using RSM in the research allows for investigating the interaction effects between independent parameters on the response, which helps reduce the number of experimental runs required to obtain statistically valid results. RSM not only offers alternative experimental strategies but also provides criteria for evaluating them [26].

The Plackett-Burman design, CCD, and response surface methodology were jointly employed in this research to determine the optimal levels of key factors [27, 28]. After determining the concentration of TBHQ based on the previous studies, the enzyme activity concentrations of laccase were screened for acute toxicity. TBHQ was found to induce malformations in zebrafish embryos with cardiac looping abnormalities, pericardial edema, and enlarged yolk sacs being the primary features of these malformations [6]. After 120 h of incubation, zebrafish in the TBHQ group exhibited more pronounced malformations. Data analysis revealed significant deformities in the TBHQ-exposed zebrafish, confirming that the model was successfully established. Zebrafish treated with laccase showed some alleviation of cardiac annulation and pericardial edema as laccase enzyme activity concentration increased. However, no significant effect was observed on the enlargement of the yolk sac. The results suggested that laccase mitigated cardiac annulation and pericardial edema malformations in zebrafish. TBHQ induced a reduction in pigmentation. Microscopic images of zebrafish obtained after 120 h of incubation revealed that juveniles in the TBHQ-treated group exhibited a remarkably decrease in melanin pigmentation, approaching a translucent state. However, those in the laccase-treated group showed improved recovery. The key genes involved in melanin synthesis including *tyr*, *dct*, *oca2*, and *silv* were investigated [29], and the results showed that all four genes were downregulated in the zebrafish larvae exposed to TBHQ. However, the extent of downregulation was less severe in the larvae treated with laccase with an evident trend of improvement as the laccase enzyme activity concentration increased,

which suggested that laccase had a mitigating effect on the pigmentation reduction caused by TBHQ.

This research aimed to ascertain an optimized approach to produce laccases and explore the mechanism underlying the mitigating effect of laccases concerning the pathological damage induced by TBHQ. According to the RSM, the maximum laccase activity was achieved under the 1.75% peptone, 0.46% glucose, 0.0798% $MgSO_4$, pH 6.11, and 222.4 mL of liquid medium. The observed enzyme activity of 2,920.33 U/L closely aligned with the predicted peak activity of 2,869.32 U/L as determined by the response surface model, which represented a 4.85-fold increase compared to the initial enzyme activity of 499.58 U/L before optimization. Laccase was found to alleviate deformities in zebrafish induced by TBHQ and modulate the reduction in pigmentation by influencing melanin-related genes including *tyr*, *dct*, *oca2*, and *silv*. The optimal production conditions for laccase had been established through this study, and the potential strategies for its application in mitigating pathological effects had also been proposed.

Acknowledgements

This research was funded by Shandong Provincial Natural Science Foundation, China (Grant No. ZR2024MC209) and the Graduate Innovation Foundation of Yantai University (Grant No. GCIFYTU2428).

References

1. Janusz G, Pawlik A, Świderska-Burek U, Polak J, Sulej J, Jarosz-Wilkołazka A, *et al*. 2020. Laccase properties, physiological functions, and evolution. Int J Mol Sci. 21(3):966.
2. Mate DM, Alcalde M. 2017. Laccase: A multi-purpose biocatalyst at the forefront of biotechnology. Microb Biotechnol. 10(6):1457-1467.
3. Mate DM, Alcalde M. 2015. Laccase engineering: From rational design to directed evolution. Biotechnol Adv. 33(1):25-40.
4. Hao R, Zhou X, Zhao X, Lv X, Zhu X, Gao N, *et al*. 2023. *Flammulina velutipes* polysaccharide counteracts cadmium-induced gut injury in mice via modulating gut inflammation, gut microbiota and intestinal barrier. Sci Total Environ. 877:162910.
5. Gutiérrez-Del-Río I, López-Ibáñez S, Magadán-Corpas P, Fernández-Calleja L, Pérez-Valero Á, Tuñón-Granda M, *et al*. 2021. Terpenoids and polyphenols as natural antioxidant agents in food preservation. Antioxidants (Basel). 10(8):1264.
6. Khezerlou A, Akhlaghi AP, Alizadeh AM, Dehghan P, Maleki P. 2022. Alarming impact of the excessive use of tert-butylhydroquinone in food products: A narrative review. Toxicol Rep. 9:1066-1075.
7. Leonard ER, Marques ES, Roy MA, Conlin SM, Ranjan R, Timme-Laragy AR. 2023. Dietary exposure to the food preservative tert-butylhydroquinone (tBHQ) impairs zebrafish (*Danio rerio*) survival, growth, organ development, and gene expression in Nrf2a-dependent and independent ways. Food Chem Toxicol. 176:113788.
8. Analytical Methods Committee Amctb No. 2013. Experimental design and optimization (4): Plackett-Burman designs. Anal Methods. 5(8):1901-1903.
9. Freitas M, Souza P, Cardoso S, Cruvinel K, Abrunhosa LS, Ferreira Filho EX, *et al*. 2021. Filamentous fungi producing l-asparaginase with low glutaminase activity isolated from Brazilian Savanna soil. Pharmaceutics. 13(8):1268.
10. Marrubini G, Dugheri S, Cappelli G, Arcangeli G, Mucci N, Appelblad P, *et al*. 2020. Experimental designs for solid-phase microextraction method development in bioanalysis: A review. Anal Chim Acta. 1119:77-100.
11. Eliseo Cristiani-Urbina, Alma Rosa Netzahuatl-Muñoz, Francisco J Manriquez-Rojas, Cleotilde Juárez-Ramírez, Nora Ruiz-Ordaz, Juvencio Galíndez-Mayer, *et al*. 2000. Batch and fed-batch cultures for the treatment of whey with mixed yeast cultures. Proc Biochem. 35(7):649-57.
12. Kim HC, Kwon YR, Kim JS, Kwon M, Kim JH, Kim DH. 2022. Computational approach to the surface-crosslinking process of superabsorbent polymer *via* central composite design. Polymers (Basel). 14(18):3842.
13. Zheng F, An Q, Meng G, Wu XJ, Dai YC, Si J, *et al*. 2017. A novel laccase from white rot fungus *Trametes orientalis*: Purification, characterization, and application. Int J Biol Macromol. 102:758-770.
14. Eichlerová I, Šnajdr J, Baldrian P. 2012. Laccase activity in soils: Considerations for the measurement of enzyme activity. Chemosphere. 88(10):1154-1160.
15. Xu KZ, Wang HR, Wang YJ, Xia J, Ma H, Cai YJ, *et al*. 2020. Enhancement in catalytic activity of CotA-laccase from *Bacillus pumilus* W3 *via* site-directed mutagenesis. J Biosci Bioeng. 129(4):405-411.
16. Bambino K, Chu J. 2017. Zebrafish in toxicology and environmental health. Curr Top Dev Biol. 124:331-367.
17. Rosa JGS, Lima C, Lopes-Ferreira M. 2022. Zebrafish larvae behavior models as a tool for drug screenings and pre-clinical trials: A review. Int J Mol Sci. 23(12):6647.
18. Weremfo A, Abassah-Oppong S, Adulley F, Dabie K, Seidu-Larry S. 2023. Response surface methodology as a tool to optimize

the extraction of bioactive compounds from plant sources. J Sci Food Agric. 103(1):26-36.
19. Vyavahare GD, Gurav RG, Jadhav PP, Patil RR, Aware CB, Jadhav JP. 2018. Response surface methodology optimization for sorption of malachite green dye on sugarcane bagasse biochar and evaluating the residual dye for phyto and cytogenotoxicity. Chemosphere. 194:306-315.
20. Wang ZW, Liu XL. 2008. Medium optimization for antifungal active substances production from a newly isolated *Paenibacillus* sp. using response surface methodology. Bioresour Technol. 99(17):8245-8251.
21. Wang H, Kang G, Ma C, Lian H, Zhao K, Zhao B, *et al*. 2024. Inhibitory effect of acetaminophen on ocular pigmentation and its relationship with thyroxine in zebrafish embryos. Bull Environ Contam Toxicol. 112(2):39.
22. Yang X, Sun Z, Wang W, Zhou Q, Shi G, Wei F, *et al*. 2018. Developmental toxicity of synthetic phenolic antioxidants to the early life stage of zebrafish. Sci Total Environ. 643:559-568.
23. Srinivasan P, Rajaji U, Sethuraman MG, Karuppiah C, Yusuf K, Liu TY. 2024. Rapid detection of tertiary butylated hydroquinone in food samples using Mn-MOF@f-CNF composite-modified screen-printed electrode. Mikrochim Acta. 191(10):576.
24. Eskandani M, Hamishehkar H, Ezzati Nazhad Dolatabadi J. 2014. Cytotoxicity and DNA damage properties of tert-butylhydroquinone (TBHQ) food additive. Food Chem. 153:315-320.
25. Rajewski J, Dobrzyńska-Inger A. 2021. Application of response surface methodology (RSM) for the optimization of chromium (III) synergistic extraction by supported liquid membrane. Membranes (Basel). 11(11):854.
26. Abbasi S, Li Z, Dastan D, Tao L. 2023. The effect of individual factors, their binary and ternary interactions on photodegradation rate of organic contaminants using photocatalysts based on multi-walled carbon nanotubes (MWCNTs): Statistical analysis based on ANOVA and RSM. Environ Monit Assess. 195(10):1191.
27. Hu X, Shen X, Zhu S, Zeng H, Shuai Y. 2023. Optimization of glutathione production in Saccharomyces cerevisiae HBSD-W08 using Plackett-Burman and central composite rotatable designs. BMC Microbiol. 23(1):11.
28. Abbas M, Ejaz U, Shafique M, Naz SA, Sohail M. 2023. Biological pretreatment of sugarcane bagasse for the production of fungal laccase and bacterial cellulase. J Basic Microbiol. 63(7):722-733.
29. Zhou S, Zeng H, Huang J, Lei L, Tong X, Li S, *et al*. 2021. Epigenetic regulation of melanogenesis. Ageing Res Rev. 69:101349.