Optimizing Laccase Production in Auricularia Cornea by Submerged Fermentation With Wheat Bran Extract: Applications in Decolorization of Malachite Green Dye

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Original article

Keywords: Wood ear mushroom, Laccase, Decolorization, Biodegradation

Posted Date: April 29th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-464777/v1

Optimizing laccase production in *Auricularia cornea* by submerged fermentation with wheat bran extract: Applications in decolorization of malachite green dye

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Abstract:

Wheat bran extract may enhance laccase activity of *Auricularia cornea* in submerged fermentation. The laccase activity of *A. cornea* was maximal (768.78 U/mL) at 26.9 ℃, pH 5, a time point of 6 d, 22 h, and an inducer concentration of 10%. Laccase from *A. cornea* AC5 decolorized 83.27% of 75 mg/L malachite green within 12 h. High performance liquid chromatography (HPLC) analysis of the extracted metabolites suggested that the decolorization occurred through biodegradation. Malachite green induced cytotoxic damage including formation of a micronucleus and chromosome bridge at anaphase. Degradation products of malachite green proved to be less toxic and had negligible effects on chromosomal aberrations.

Key words:

Wood ear mushroom, Laccase, Decolorization, Biodegradation

Introduction

Robust growth of the worldwide textile industry in recent years has driven a commensurate increase in the use of synthetic dyes and consequent environmental pollution due to the discharge of contaminated wastewater (Saratale et al. 2011). Triphenylmethane dyes, including malachite green, are among the most widely used group of synthetic colorants for dyeing cotton, wool, silk, nylon, etc. (Ayed et al. 2009). However, malachite green is highly toxic to mammalian cells. It promotes hepatic tumor formation in rodents and causes reproductive abnormalities in rabbits and fish (Fernandes et al. 1991; Rao 1995). Moreover, removal of dye chemicals from dye-bearing wastewater is a complex problem because of difficulty in wastewater treatment using conventional methods (Kumar et al. 2006), such as, ozonation, photooxidation, electrocoagulation, adsorption, reverse osmosis, membrane filtration, etc. (Daneshvar et al. 2007; Jain et al. 2003). Therefore, developing a new, practical method for their removal from wastewater is of utmost importance (Ayed et al. 2011).

Auricularia cornea is a type of white-rot fungus which has been intensively studied for its dye-decolorizing properties. Non-specific lignin-modifying enzymes in white-rot fungi enable them to transform a wide range of organic compounds (Wesenberg et al. 2003). There are a considerable number of recent studies on decolorization and degradation of individual synthetic dyes including triphenylmethane (Vršanská et al. 2018), azo (Tauber et al. 2005), anthraquinone (Tychanowicz et al. 2004), etc. by white-rot fungi (Asgher et al. 2006). The biodegradation ability of white-rot fungi is assumed to be associated with the production of lignolytic enzymes such as lignin peroxidase and laccase (Couto and Herrera 2006; Ghodake et al. 2008). In addition, most studies have found that fungi with the highest laccase activity,

47 for instance, *Trametes versicolor* (Li et al. 2014), *Fomes fomentarius* (Neifar et al. 2011),
48 *Ganoderma* sp. (Zhuo et al. 2011), *Agaricus bisporus* (Othman et al. 2018), *Thelephora* sp.
49 (Selvam K et al. 2003), and *Pleurotus* sp. (Tychanowicz et al. 2004; Meng and Li 2017) can
50 decolorize dyes the most efficiently (Erkurt et al. 2007). This suggests that laccase plays a key
51 role in this process.

52 Laccase (EC 1.10.3.2, benzenediol/oxygen oxidoreductase) is a multicopper oxidase,
53 widely distributed among plants, fungi, and bacteria (Claus 2003). When different sources of
54 laccase catalyze the same substrate, the enzymatic characteristics are different. When the
55 same laccase catalyzes different substrates, the enzymatic characteristics are also different. In
56 order to better apply the use of laccase for remediation, optimization of cultivation conditions
57 is an important strategy to maximize laccase enzyme productivity (Bhattacharya et al. 2011).
58 Some researchers have reported increases in laccase activity following the addition of
59 different inducers to media, such as veratryl alcohol, Tween-80, copper sulphate or
60 2,5-xylidine (Churapa et al. 2007; Sondhi and Saini 2019; Galhaup and Haltrich 2001). In
61 addition, many studies have shown that the nitrogen (N) source, as one of the important
62 nutritional factors in fungal metabolism, can regulate the production of ligninolytic enzymes
63 in wood-rotting basidiomycetes (Galhaup et al. 2002; Sun et al. 2004). Laccase production by
64 *Trametes modesta* can be significantly influenced by wheat bran, yeast extract and incubation
65 temperature (Nyanhongo et al. 2002). The laccase activity of *Ganoderma lucidum* 447 was
66 the highest in submerged fermentation processes using wheat or soybean bran (Songulashvili
67 et al. 2007), with evidence that enzyme activity was influenced the N source.

68 This study aims to investigate the potential of *A. cornea* for decolorizing a solution

containing a cationic textile dye, malachite green. Correlation of laccase activity with various parameters (time, temperature, inducer concentration and pH) were characterized. The cytogenotoxicity of the products formed after decolorization were studied.

Materials and methods

Organisms and preparation of crude enzyme solution

An *A. cornea* AC5 strain was provided by the Collection Center of Mushrooms in Jilin Agricultural University. The strain was maintained on potato dextrose agar (PDA) medium at 18 ℃. Five 0.7-cm potato dextrose agar discs of mycelia from a Petri dish were inoculated in a flask with 100 mL complete yeast medium (CYM) (1% maltose, 2% glucose, 0.2% yeast extract, 0.2% tryptone, 0.05% $MgSO_4 \cdot 7H_2O$, 0.46% KH_2PO_4), followed by shaking the culture at 26°C and 160r/min. After seven days, the cultures were centrifuged at 6000r/min for 10min, and the cell-free supernatant was used as a crude enzyme solution.

Effects of different physicochemical parameters on laccase activity

To obtain a representative picture of laccase activity, preliminary experiments were conducted at different temperatures (22-34 °C) at pH 5, at various other pH ranges (3-9) at 26 °C, as well as at pH 5, at different inducer concentrations (0-20%) at 26 °C and pH 5, and with different cultivation periods (1-9 d) at 26 °C and pH 5. The factors affecting the laccase production of A. *cornea* AC5 were screened on the basis of these preliminary experiments. The effects of these parameters on laccase activity were examined by varying only one factor at a time, where time, temperature, inducer concentration and pH were considered as key variables. All of these experiments were performed under aseptic conditions under isothermal incubation at a constant agitation of 160 rpm in a 250 mL Erlenmeyer flask with a 5% inducer

concentration (except experiments measuring the effects of different inducer concentrations).

Laccase activity assay

Laccase activity was determined by monitoring the A_{420} change related to the rate of oxidation of 1 μmol of 2,2-azino-bis-[3-ethylthiazoline-6-sulfonate] (ABTS) in 20 mM of a sodium acetate buffer (pH 4.5). Assays were performed in a 1 mL spectrophotometric cuvette at 30 ℃ with an adequately diluted culture liquid. One unit of laccase activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of ABTS per minute.

Selection of significant parameters by Plackett-Burman (PB)

A set of 27 experiments was designed using the Plackett-Burman design of Design Expert (version 8.0.6.1) software for four variables (Table 1, 2) that were analyzed as possible factors affecting production. The parameters evaluated were as follows: A: time, B: temperature, C: inducer concentration, D: pH. Ideal concentration levels were calculated based on single factor analysis. All the trials were carried out in triplicate and the mean laccase yield was calculated.

Optimization of laccase production using response surface methodology (RSM)

Central composite design (CCD) of RSM in Design Expert (version 8.0.6.1) was employed at an α value of ±1 to further optimize the levels of significant variables (Table 1). Laccase activity was recorded as the response. Response data were analyzed by the software to generate 3D plots indicating the optimum conditions and interactions among these factors. A regression analysis was performed on the data obtained. A second-order polynomial equation was used to fit the data by a multiple regression procedure. A quadratic model was obtained as per the following equation:

$$Y = \delta_0 + \Sigma \delta_i V_i + \Sigma \delta_{ii} V_{i2} + \Sigma \delta_{ij} V_i V_j$$

Where, Y is the predicted response [laccase activity (U/mL)], δ_0 is the constant term, δ_i the linear coefficients, δ_{ii} the squared coefficients and δ_{ij} the interaction coefficients. The quality of fit by the polynomial model equation was expressed using the coefficient of determination, R^2.

Analytical methods in dye decolorization

Dye colors were determined spectrophotometrically (Abadulla et al. 2000). Color removal was determined based on absorbance in the UV-visible spectrum using a previously described equation (Jadhav et al. 2008). The decolorization rate (%) was calculated as $(A_i - A_t) / A_i \times 100$, where A_i and A_t are the initial and final absorbance units, respectively.

The decolorization of malachite green dye was determined by High Performance Liquid Chromatography (HPLC). Chromatographic separation of samples was performed using an Eclipse Plus 95Å C18 column (4.5 × 250mm, 5 µm). The column temperature was maintained at 30 °C. Mobile phase A was 0.125 mol/mL ammonium acetate solution and mobile phase B was acetonitrile. The elution profile was set as following: 0.01 min, 80% B; 20min, 80% B. The flow rate was 1.00 mL/min, the injection volume was 20 µL and absorbance was measured at 614 nm.

Cytogenotoxicity test

Allium sativum was used to study the cytotoxicity of malachite green. The meristematic cells of root tips were exposed to malachite green dye for 24 h. These root tips were later fixed in Carnoy's Fluid for 3h. Fixation was followed with treatment with 1 M HCl at 60 °C for 8 min, followed by a distilled water rinse. Root tips were squashed on a glass slide in

Schiff reagent for 10 min. The prepared slide samples were sealed with a coverslip and observed at 400× and 1000× magnification.

Results

Determination of optimum time, temperature, inducer concentration and pH for laccase activity

Adaptation of the fungus to a broader range in pH and temperature may improve its suitability for laccase production. Figure 1C shows that laccase activity was maximal (625.14 U/mL) at 7.5% inducer concentration. The laccase activity was 386.61 U/mL, 321 U/mL and 346.94 U/mL at 9 d, 26 ℃ and pH 5, respectively (Figure 1A, B, D). The laccase activity of *A. cornea* increased proportionally with culture time. However, the liquid medium became considerably more viscous by the 9th day. Therefore, we focused on laccase activity at 3-7 d for further study.

Optimization of laccase activity of *A. cornea* AC5 using CCD by RSM approach

The experiments were conducted as per the combination listed in Table 2. Multiple regression analysis was carried out and fitted to a second degree polynomial equation, which relates dependent and independent variables, as indicated:

$$R1=576.47983+33.275152779167A+156.64087497083B-69.960944458333C-115.68750001667D+101.702166625AB-11.748708375AC+35.5289166625AD-21.723541675BC-118.0526667125BD-175.587916675CD-171.30556249792A^2-143.77193753542B^2-16.914166679167C^2-113.57749997917D^2$$

The fitness of the quadratic polynomial equation generated was statistically verified by an F-test (ANOVA). Table 3 shows the analysis of variance (ANOVA) for the generated

157 model for the process, with a significance level of $p< 0.05$. An R^2 value of 99.93% measured
158 for laccase production indicated a good fit for the generated model. The "Lack of Fit" *p*-value
159 of 0.0706 (non-significant) supported this inference. The variations in responses as a function
160 of the assessed parameters are represented in a 3D response surface plot as shown in Figure 2.
161 A response surface contour map directly reflects the influence of various factors on the value
162 of a specific response, so as to determine the best process parameters and their interactions.
163 The higher the slope of the surface, the steeper the slope, indicating that a significant
164 interaction beyond a certain degree of slope. In addition, laccase activity decreased gradually
165 when the color of the surface changed from red to green. The center of the smallest ellipse of
166 the contour on the surface was the highest point of the response surface, corresponding to
167 maximal enzyme activity. The model forecasted that peak laccase production of *A. cornea*
168 *was* 787.87 U/mL at 26.9 ℃ and pH 5, with an incubation time of 6d, 22 h, and an inducer
169 concentration of 10%. The optimal extraction conditions were verified experimentally, and
170 the results indicated that the laccase yield was 768.78 U/mL, which was consistent with the
171 model.

172 **Decolorization of malachite green dye**

173 Laccase from *A. cornea* AC5 decolorized 83.27% of malachite green at an initial
174 concentration of 75 mg/L within 12 h. High performance liquid chromatography (HPLC)
175 analysis results revealed that the peak elution time of malachite green was at 2.373 min, the
176 peak height was 696.445 mAU and the peak area was 34.595 mAU*min (Figure 3 A).
177 Degraded malachite green dye was also detected by HPLC, with a peak height of 16.479
178 mAu at an elution time of 1.997 min; the peak area was 0.653 mAu *min (Figure 3 B). HPLC

179 analysis of the extracted metabolites suggested that the decolorization occurred through
180 biodegradation.

181 **Cytogenotoxicity**

182 Malachite green dye is known to generate carcinogenic compounds after breakdown in
183 some instances. *Allium sativum* is a well-accepted and reliable model for testing
184 cytogenotoxic effects. As demonstrated in Figure 4, malachite green treatment was associated
185 with an increase in chromosomal aberrations. Malachite green induced cytotoxic damage
186 including micronucleus (Figure 4A) and chromosome bridge at anaphase (Figure 4B). The
187 degradation products of malachite green after treatment with *A. cornea* AC5 were
188 considerably less cytogenotoxic and had a negligible effect on induced chromosomal
189 aberrations.

190 **Discussion**

191 In this study, we found that wheat bran extract significantly enhanced the laccase activity
192 of *A. cornea* in submerged fermentation. This result was consistent with that wheat bran could
193 improve the bacterial laccase activity (Muthukumarasamy et al. 2015). The concentration of
194 wheat bran extract was directly correlated to the production of laccase up to a concentration
195 of 7.5%. Above this concentration, laccase production was inversely correlated to the
196 concentration of wheat bran extract. This suggests that the presence of wheat bran extract
197 increased the amount of N in the liquid media (Bagewadi et al. 2017). Above a certain point,
198 an excess N:C ratio increases the viscosity of the fermentation broth, which is not conducive
199 to the growth of mycelia.

200 Laccases can efficiently degrade different types of dyes (Legerská et al. 2016), for

instance, triphenylmethane, anthraquinone, thiazine, azo, etc. In this study, we found that laccase from *A. cornea* AC5 degraded the triphenylmethane dye, malachite green. The decolorization rate of malachite green (75 mg/L) was 83.27% within 12 h. To understand the bioremediation mechanisms involved, we predict the propensity of redox enzymes to degrade dyes, based on bioinformatics research and molecular modeling docking tools (Lohning et al. 2017). We obtained a laccase protein sequence in *A. cornea* by Protein BLAST. The PDB Format laccase and Mol2 Format small dye molecules were simulated by AutoDock software (Figure 5), and a minimum binding energy of -28.37 kJ/mol was obtained, which was plotted by PyMoL and LigPlus for analysis. It is generally accepted that an energy well occurs when a stable conformation of the ligand and its receptor binding is present (Lin et al. 2020). With a value of binding energy lower than -5 kJ/mol, the better the binding effect, and the greater the possibility of action. The laccase-dye complex was tested in vitro to verify the effectiveness of dye bioremediation. The results indicated that the combination of laccase structure prediction via modeling and in vitro analysis is an effective preliminary screening method, which can be further applied to the real-time treatment of toxic wastewater in the textile industry.

Declarations

Ethical approval Not applicable Not applicable

Consent to participate All the authors agree to participate.

Consent to publish We consent to publish this manuscript in the Environmental Science and Pollution Research. This manuscript has not been published in whole or in part nor is it being considered for publication elsewhere.

Availability of data and materials All data generated or analyzed during this study are included in this manuscript.

Competing interests The authors declare no competing interests.

Funding This work was financially supported by National Key Research and Development Program of China [grant number 2018YFD1001001].

Authors' contribution Conceived and designed the experiments: LM, LW, WW. Performed the experiments: XRB and SYZ. Analyzed the data: QJW and XRB. Wrote the paper: LM and XRB. Editing and supervision: ZL and XBL. All authors read and approved the final manuscript.

Acknowledgements The authors would like to thank to all who contributed to this study.

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Figure Captions

Figure 1 Effects of time (A), temperature (B), inducer concentration (C) and pH (D) on the laccase activity of *Auricularia cornea*.

Figure 2 The influence of the interactions of two combinations of factors on laccase production in *Auricularia cornea*. (A) Interaction between inducer concentration and pH. (B) Interaction between inducer concentration and time. (C) Interaction between inducer concentration and temperature. (D) Interaction between temperature and time.

Figure 3 Decolorization analysis of malachite green dye by HPLC. (A) Original sample of malachite green dye. (B) Malachite green after degradation by *A. cornea* AC5 for 12 h.

Figure 4 Cytotoxic damage induced in root meristematic cells of *Allium sativum* on treatment with malachite green. The black arrow in the picture refers to the chromosome variant cells. (A) micronucleus, (B) chromosome bridge.

Figure 5 Structural model of molecular docking complex. (A) 3D model of molecular docking complex of laccase from *A. cornea* AC5 and Malachite green. (B) 2D ligand-receptor interaction diagram. Ligand interaction diagram indicating in detail how these residues interact with the ligand. Note the interactions between amino groups and acidic residues, specifically at Asn82, Ser135, Glu175, Ala537, Asp539, Phe536, and Asp178.

359 Table 1 Factors and coded levels of the central composite design

Factors	Code	Level		
		-1	0	1
Time (d)	A	3	5	7
Temperature (℃)	B	22	26	30
Inducer concentration (%)	C	5	7.5	10
pH	D	5	6	7

360 Table 2 Experimental design and results of preliminary experiments on laccase activity in
361 *Auricularia cornea*

Run No.	Factors				Laccase activity (U/mL)
	A: Time (h)	B: Temperature (℃)	C: Inducer concentration (%)	D: pH	
1	0	-1	0	1	61.91
2	-1	0	0	1	282.59
3	-1	0	1	0	205.56
4	1	-1	0	0	302.23
5	0	1	0	1	218.62
6	0	-1	1	0	310.66
7	0	0	-1	-1	466.35
8	-1	0	-1	0	220.78
9	-1	-1	0	0	118.98
10	0	-1	0	-1	404.03
11	0	0	1	-1	654.33
12	-1	1	0	0	0.62
13	0	0	0	0	593.35
14	-1	0	0	-1	224.28
15	1	1	0	0	590.67
16	0	1	0	-1	418.63
17	0	1	-1	0	537.51
18	0	0	0	0	580.91
19	0	-1	-1	0	485.06
20	0	0	-1	1	572.27
21	1	0	1	0	535.76
22	1	0	0	1	226.03
23	1	0	-1	0	637.88
24	0	0	0	0	555.19
25	1	0	0	-1	639.93
26	0	0	1	1	57.90
27	0	1	1	0	316.11

362 Table 3 Regression variance results of the experimental model

Sources	Sum of squares	Df	Sum of squares	F value	P value	Significant
Model	995899.3315	14	71135.66653	16.37894561	< 0.0001	significant
A-A	13286.82951	1	13286.82951	3.059284722	0.1058	
B-B	294436.3645	1	294436.3645	67.79380069	< 0.0001	
C-C	58734.40499	1	58734.40499	13.52356239	0.0032	
D-D	160603.1719	1	160603.1719	36.97878638	< 0.0001	
AB	41373.32278	1	41373.32278	9.526183368	0.0094	
AC	552.1285939	1	552.1285939	0.127127286	0.7276	
AD	5049.215677	1	5049.215677	1.162578956	0.3021	
BC	1887.649052	1	1887.649052	0.43463009	0.5222	
BD	55745.72847	1	55745.72847	12.83542137	0.0038	
CD	123324.4659	1	123324.4659	28.39538614	0.0002	
A^2	156509.844	1	156509.844	36.03630002	< 0.0001	
B^2	110241.9735	1	110241.9735	25.3831499	0.0003	
C^2	1525.808184	1	1525.808184	0.351316442	0.5644	
D^2	68799.19201	1	68799.19201	15.84097371	0.0018	
Residual	52117.39626	12	4343.116355			
Lack of fit	51359.82189	10	5135.982189	13.55901791	0.0706	not significant
Pure error	757.5743644	2	378.7871822			
Cor total	1048016.728	26				

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Figures

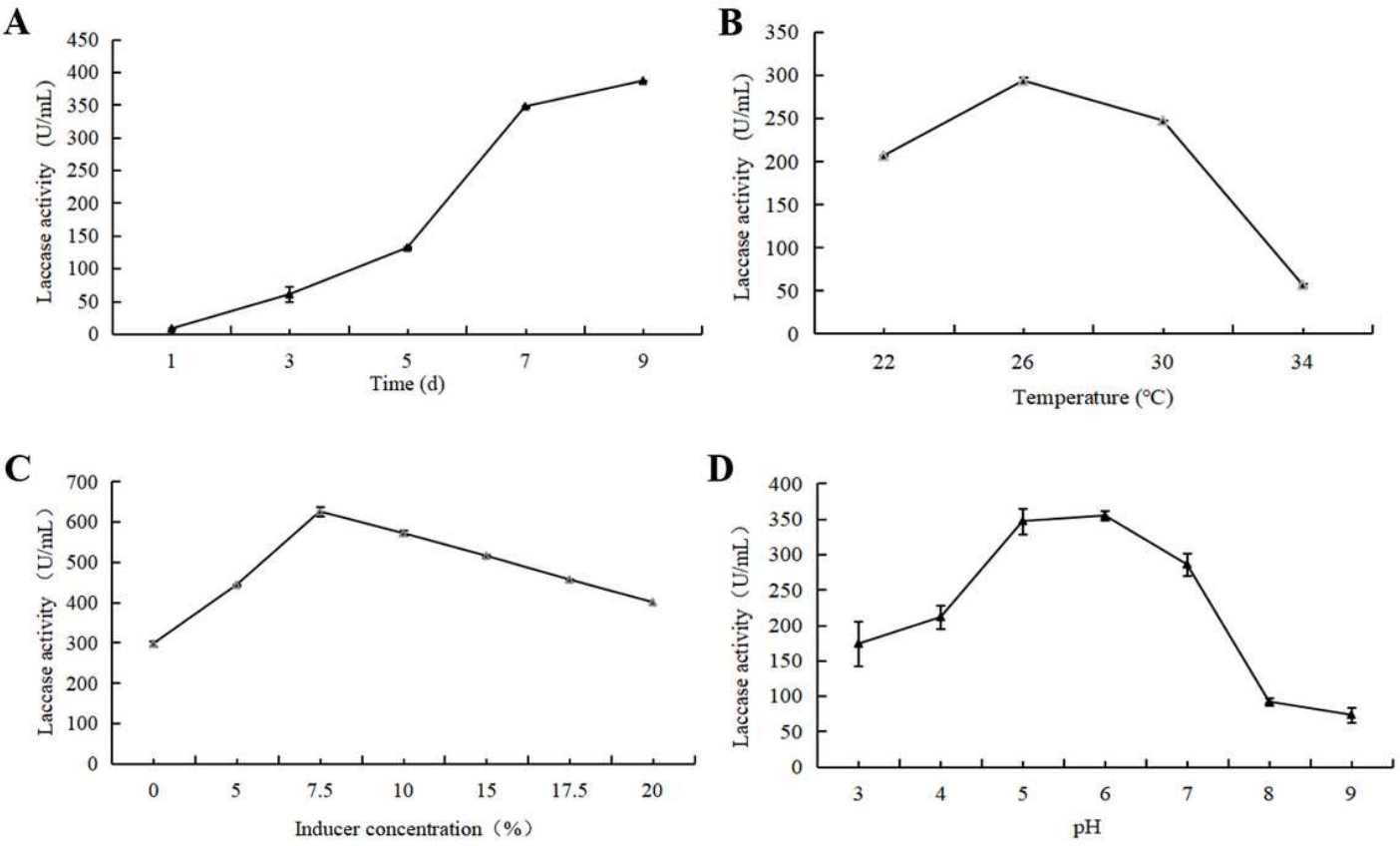

Figure 1

Effects of time (A), temperature (B), inducer concentration (C) and pH (D) on the laccase activity of Auricularia cornea.

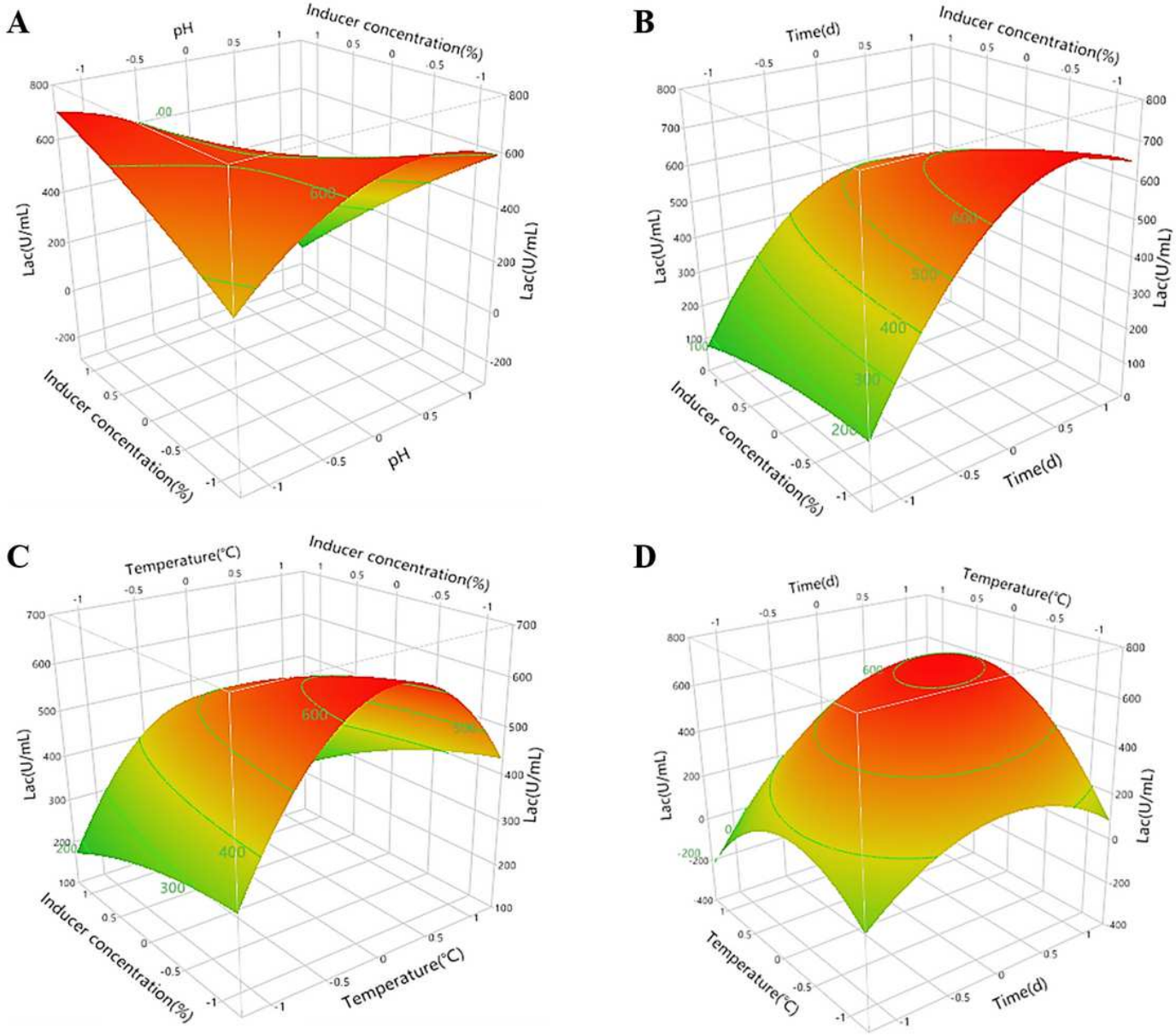

Figure 2

The influence of the interactions of two combinations of factors on laccase production in Auricularia cornea. (A) Interaction between inducer concentration and pH. (B) Interaction between inducer concentration and time. (C) Interaction between inducer concentration and temperature. (D) Interaction between temperature and time.

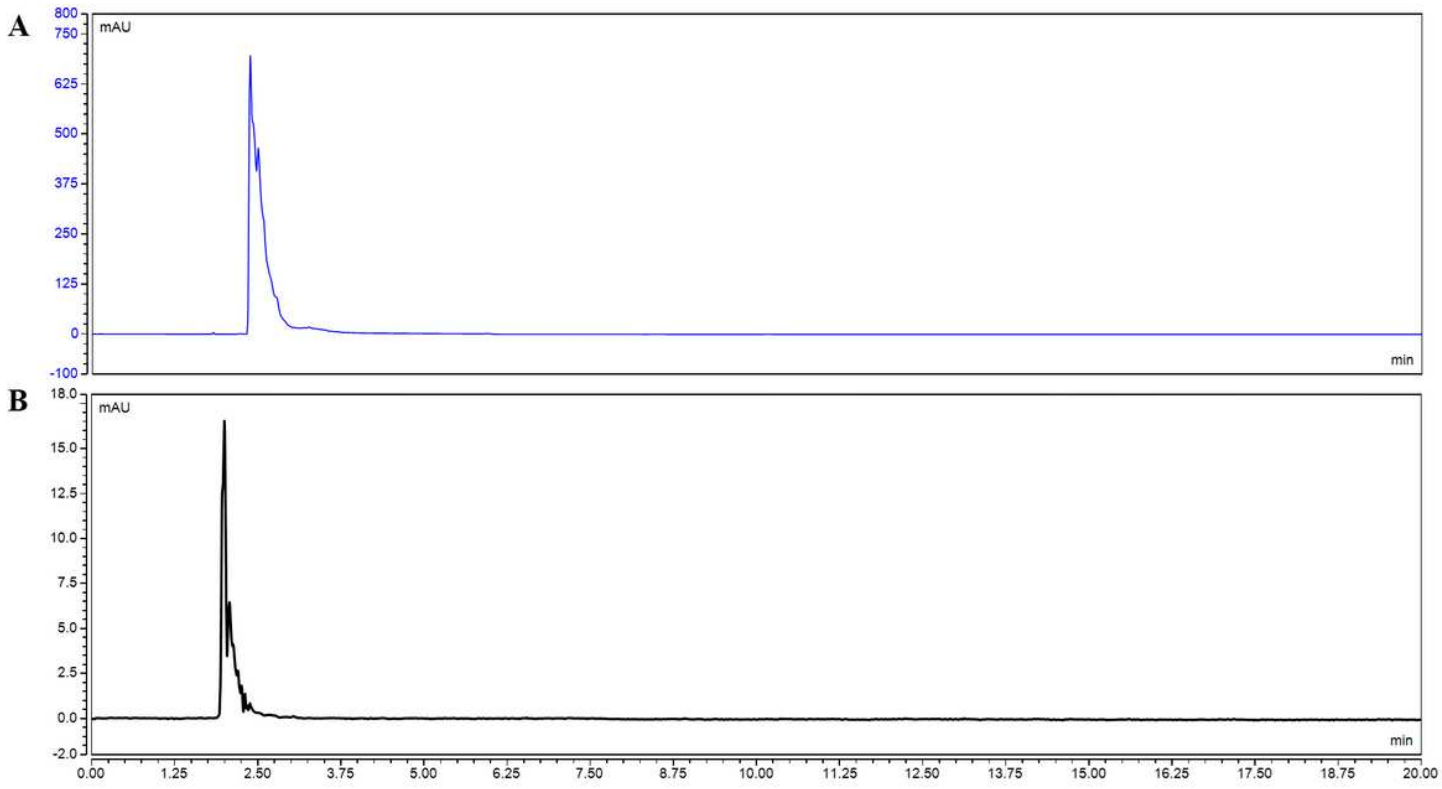

Figure 3

Decolorization analysis of malachite green dye by HPLC. (A) Original sample of malachite green dye. (B) Malachite green after degradation by A. cornea AC5 for 12 h.

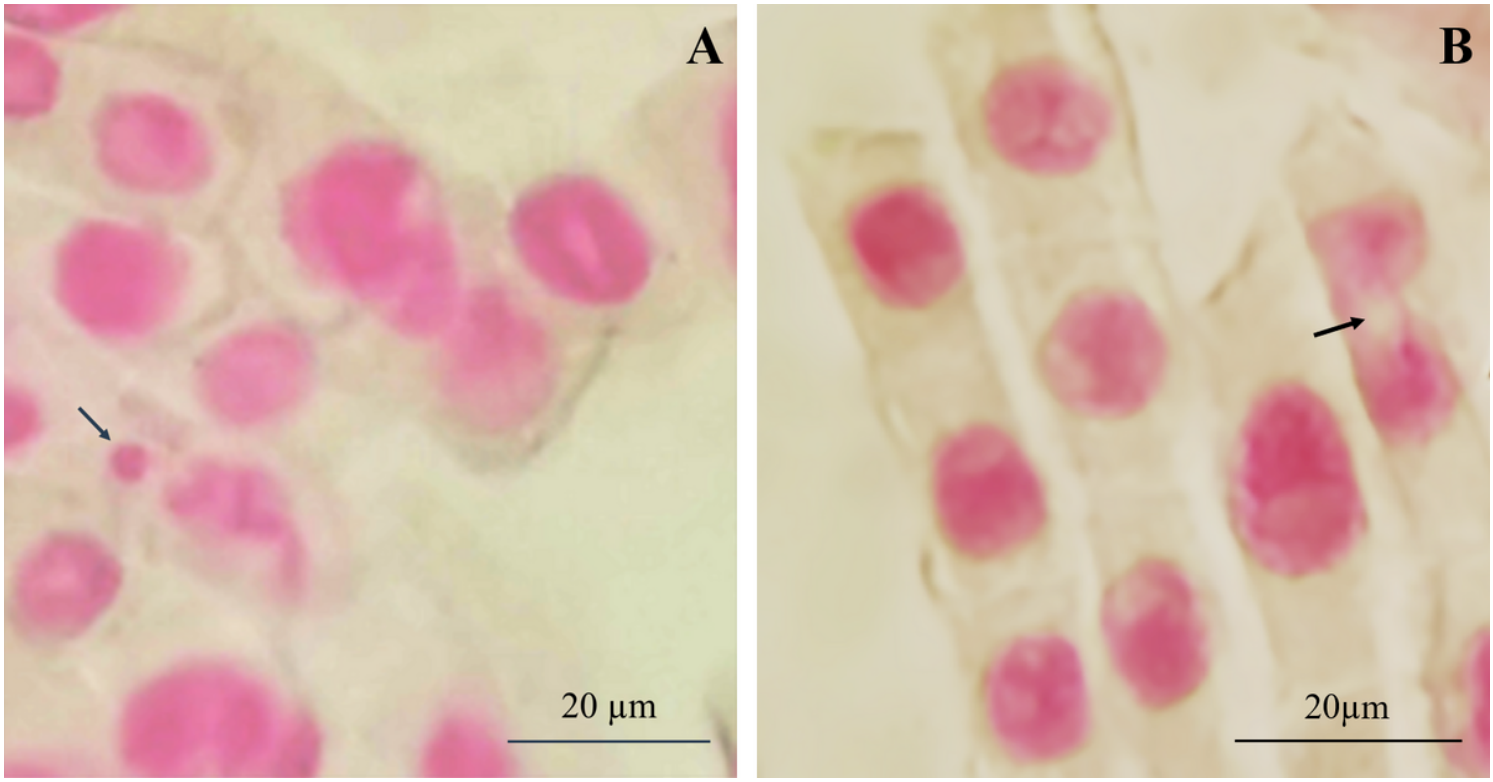

Figure 4

Cytotoxic damage induced in root meristematic cells of Allium sativum on treatment with malachite green. The black arrow in the picture refers to the chromosome variant cells. (A) micronucleus, (B) chromosome bridge.

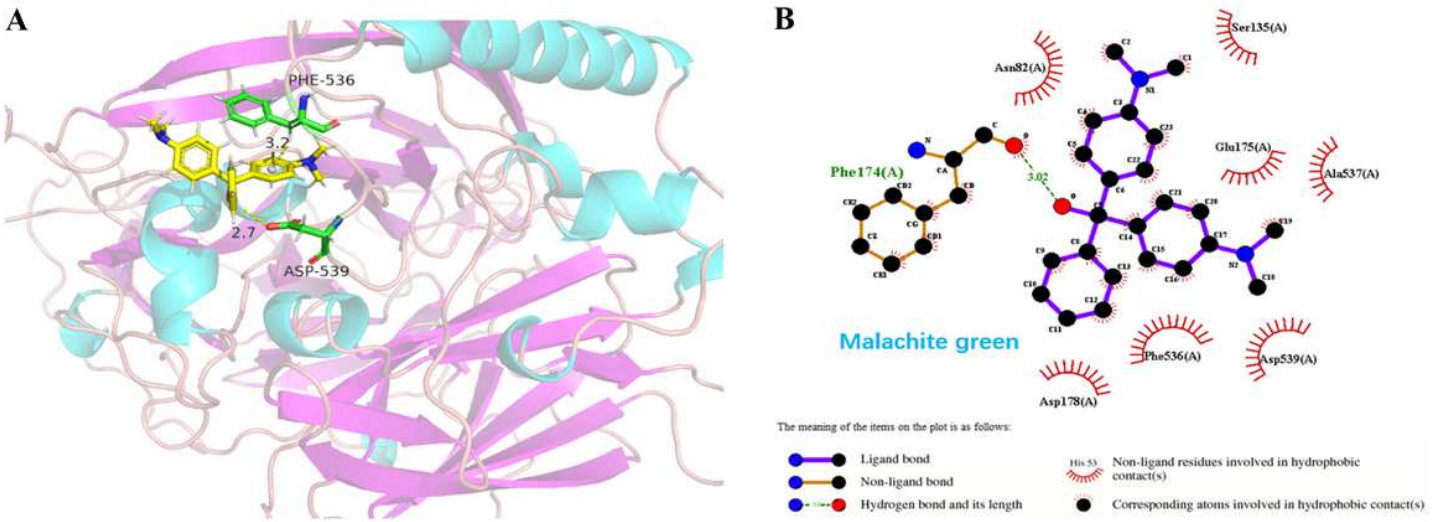

Figure 5

Structural model of molecular docking complex. (A) 3D model of molecular docking complex of laccase from A. cornea AC5 and Malachite green. (B) 2D ligand-receptor interaction diagram. Ligand interaction diagram indicating in detail how these residues interact with the ligand. Note the interactions between amino groups and acidic residues, specifically at Asn82, Ser135, Glu175, Ala537, Asp539, Phe536, and Asp178.