RESEARCH ARTICLE

Process optimization and flavor enhancement of tobacco stem fermentation using *Ganoderma lucidum* enzyme solution

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Tobacco stems are a major by-product of the tobacco industry, which are resource waste and environmental pollution. To enhance the utilization rate of tobacco stem and improve its quality, this research investigated *Ganoderma lucidum* enzyme solution for fermentation. The absorption and the fermentation process were optimized through single factor experiments and response-surface methodology (RSM). The quality-improving effects were further evaluated by scanning electron microscopy (SEM), gas chromatography-mass spectrometry (GC-MS) analysis of mainstream smoke compounds, and sensory evaluation. The enzyme types and functions in the enzyme solution were also identified through enzyme purification and molecular docking. The results showed that the optimized absorption process conditions were 53℃, 32% water content, and 5.2 min of drum working time, which was further optimized by RSM. Along with the fermentation process, the structure of tobacco stem was loosened, and the lignin could be degraded. The sensory evaluation quality was improved, and the roasting sweet, fresh, and floral aroma compounds such as 5-hydroxymethylfurfural, maltol, methyl cyclopentenolone, vanillin, and isovanillin in the mainstream smoke of tobacco stem significantly increased. Laccase, pectinase, amylase, cellulase, and xylanase activities were found in the enzyme solution, among which laccase activity was the highest of 35,466.67 U/L. Three laccase isoenzymes were identified with Lac3 having the highest activity and being purified. Lac3 possessed a molecular weight of 58.4 kDa and contained 500 amino acids. The three-dimensional structure model of Lac3 was predicted by using AlphaFold 3, and it was revealed as a typical trinuclear copper center enzyme. The results suggested that *G. lucidum* enzyme solution contained multiple enzyme activity and mainly laccase, which could improve the quality of tobacco stem through lignin degradation effects.

Keywords: *Ganoderma lucidum*; enzyme solution; tobacco stem; laccase; lignin degradation.

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Introduction

Tobacco stems are a major agricultural by-product of tobacco industry, which brings a huge challenge for both economic and environment. Every year, about 299,800 tons of tobacco stems are produced in China, and around 60% of which become tobacco wastes [1, 2]. The waste tobacco stems are mainly stored or burned, which not only causes waste of resources, but also brings certain environmental pollution [3, 4]. Therefore, improving internal quality and enhancing the

utilization rate of tobacco stems has become one of the hotspots for tobacco industry [5]. To solve this problem, microbial fermentation and enzymatic hydrolysis technology have emerged as promising strategies for improving the quality of tobacco stems [6, 7]. In the process of cigarette processing, the use of appropriate exogenous enzymes and microorganisms to treat tobacco stems can accelerate the degradation of unfavorable chemical components of tobacco stems and improve the sensory quality of tobacco stems [8]. For example, the use of *Bacillus pumilus*, white rot fungus, and lactic acid bacteria can reduce the content of cellulose, pectin, lignin, and protein in tobacco stem, thus improving the smoking quality of tobacco stem [9-11].

Ganoderma lucidum enzyme solution is used as the enzyme source to carry out biological fermentation treatment of tobacco stem. The fermented tobacco stem is used as the raw material to make cut stems, which provide important technical support for the comprehensive utilization of tobacco stem resources by biological fermentation technology [12]. However, the absorption and fermentation process of *G. lucidum* enzyme solution on tobacco stem remain unclear, and its enzymatic structure and mechanism of action have not been reported, which has brought limitations for the application of this enzyme solution.

This research investigated the effects of preset temperature, water content of tobacco stem, drum working time, fermentation time, and other factors on the absorption and fermentation process of *G. lucidum* enzyme solution. In combination with microscopic image analysis, mainstream smoke compounds determination, and sensory evaluation, the quality-improving effects of *G. lucidum* enzyme solution were studied. Further, the enzymatic purification and molecular docking techniques were used to explain the enzymatic types and activity in the enzyme solution. This study not only revealed the absorption and fermentation process of *G. lucidum* enzyme solution on tobacco stems but also clarified the enzymatic mechanism of quality improvement of enzyme solution, which provided some support for application of *G. lucidum* enzyme solution to tobacco industry.

Materials and methods

Determination of laccase activity

Ganoderma lucidum enzyme solution and selected long tobacco stems were provided by China Tobacco Sichuan Industrial Co., Ltd. (Chengdu, Sichuan, China). A reaction mixture consisting of 2 mL of citrate–phosphate buffer (pH 3.0), 0.5 mL of diluted *G. lucidum* enzyme solution, and 0.5 mL of 1 mM 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) solution was prepared and quickly placed in a 45℃ constant temperature water bath for 5 min. UV absorption at 420 nm was measured using 1800 UV-visible spectrophotometer (Metada Instruments Co., Ltd., Shanghai, China). Laccase activity was quantified as follows.

$$\text{Laccase (U/L)} = \frac{10^6 \times V_{total} \times A_{420}}{\varepsilon \times V_{laccase} \times \Delta_t} \quad (1)$$

where ε was 3.6×10^4 (L/(mol·cm)). V_{total} was the total volume of the reaction system (mL). $V_{laccase}$ was the volume of added enzyme solution (mL). Δt was the reaction time (min).

Determination of enzyme absorption rate

Ganoderma lucidum enzyme solution was diluted to laccase activity of 8,000 U/L, and 1.5 mL of diluted solution was sprayed thoroughly on the tobacco stems. Under a certain temperature, the stems were stirred for different tested time, and 148.5 mL of sterile water was added to extract the residual enzyme solution for 30 s. The laccase activity of the residual enzyme solution on the surface was tested by using the ABTS method.

Determination of tobacco stems water content

Totally 10 g of tobacco stems were soaked in 300 mL sterile water at 50℃ for 1, 2, 3, 5, 10, 15, and 20 min. The water on surface was wiped off, and

the samples were dried at 100°C for 3 h to constant mass. The weight of tobacco stems was measured, and the water contents of tobacco stems were calculated corresponding to different washing times.

Single factor experiments

By using enzyme absorption rate of tobacco stem as the response value, single factor experiments were performed. The conditions for optimization were selected as water content of tobacco stem that was corresponding to tobacco stem washing time of 1, 2, 3, 5, 10, 15, and 20 min, the preset temperature of tobacco stem at 40, 45, 50, 55, and 60 ℃, and the drum working time at 1.0, 2.5, 5.0, 7.5, and 10.0 min.

Response surface methodology (RSM) optimization

Building on one-factor screening, Box-Behnken design (Synad Technologies Limited, Armonk, NY, USA) was applied. Water content (A), temperature (B), and action time of drum (C) were used to carry out the central composite design optimization experiment. Design Expert 10 software (Synad Technologies Limited, Armonk, NY, USA) was used to optimize experimental conditions. Each factor was examined at three coded levels of -1, 0, +1, which corresponded to water contents of 26%, 33%, 40%; temperature at 45℃, 50℃, 55℃; and drum time of 2.5 min, 5.0 min, 7.5 min, respectively. By using absorption rate as response value, seventeen response-surface tests of a three-factor and three-level Box–Behnken design were carried out. Each test was performed in triplicate, and the data were presented as mean ± standard deviation (mean ± SD).

Quality improvement of tobacco stem using enzyme solution at different fermentation times

The water content of tobacco stem was maintained at 33%, and 1.5 mL of enzyme solution with laccase activity of 8,000 U/L was sprayed. The drum working time was set as 5 min. Solid-state fermentation was performed at 50℃, and samples were taken out at 2, 4, 6, and 8 h for subsequent scanning electron microscopy (SEM) analysis and sensory evaluation determination.

SEM analysis

The tobacco stem samples at different fermentation times were dried, sliced, and dispersed on the conductive adhesive. After gold spraying, the microstructure of tobacco stem slices was observed using Regulus 8100 scanning electron microscope (Hitachi, Tokyo, Japan). SEM imaging was conducted at 3 kV with magnifications ranging from 500× to 2,500×. The effects of *G. lucidum* enzyme solution were evaluated by the morphological changes of tobacco stem.

Sensory evaluation method

Tobacco stems at different fermentation times of 2, 4, 6, 8 h were cut and made into cigarettes as the experimental groups. Control group was treated with sterile water for 8 h under the same conditions. Sensory evaluation team was composed of 10 evaluators and performed the sensory evaluation according to the China national standard GB/T 5606.4-2005 cigarettes-part 4: sensory technical requirements [13]. The sensory evaluation items were mainly chosen as woody, flammability, irritation, sweetness, aroma intensity, and offensive odor. The intense of each item was given a score of 0 to 5, while higher score meant better quality.

Gas chromatography-mass spectrometry (GC-MS) analysis of mainstream smoke volatile compounds

Tobacco stems at different fermentation times of 4 and 8 h were cut and made into testing cigarette using original tobacco stems as control group. 20 test cigarettes were selected from each group. An rM20H rotary smoking machine (Bowat Casey, Hamburg, Germany) was used to collect mainstream smoke compounds according to the standard requirements of the Canadian deep smoking mode [20]. The test was performed at the suction capacity of 55 mL, duration of 3 s, suction interval of 30 s, and 11 puffs. Cambridge filter papers with a diameter of 92 mm were used to collect mainstream smoke

compounds of cigarettes. After suction, the Cambridge filter paper and the filter rod were transferred into 150 mL Erlenmeyer flasks containing 100 mL dichloromethane. The ultrasonic oscillation extraction was performed for 30 min and then the flasks were transferred into a 4-h incubation in a thermostatic shaker. The extract was combined and concentrated, and 50 µL of 0.882 mg/mL of phenyl acetate solution was added as an internal standard. The solutions were filtered through a 0.22 µm membrane before GC–MS analysis. GC-MS was performed on an Agilent 6890-5973 gas chromatograph-mass spectrometer (Agilent, Santa Clara, California, USA) equipped with an DB-5 MS column (30 m × 0.25 mm i.d. × 0.25 µm d.f.) and an injection volume of 1.0 µL. High-purity helium was used as the carrier gas with the flow rate of 1.0 mL/min and a split ratio of 10:1. The initial temperature was 45℃ for 5 min, then increased to 200°C at a rate of 10°C/min followed by increasing to 240°C at a rate of 5°C/min, and maintained for 10 min with solvent delay of 8 min. The temperature of transmission line was 270°C, and ion source temperature was 230°C. The ionization mode was EI (electron energy, 70 eV), and the mass scanning range was set to 35–550 m/z. For qualitative analysis, GC-MS peaks with matching scores > 85 were selected. The retention index (RI) values were calculated and compared with previous reports. Several key compounds were verified by their retention times with the in-house standard compounds. For quantitative analysis, the internal standard method was used with phenethyl acetate as the internal standard.

Determination of pectinase activity

A mixture of 0.9 mL 1% (w/v) pectin and 0.1 mL of enzyme solution was incubated at 40°C for 30 min before adding 2.0 mL of 3,5-dinitrosalicylic acid (DNS). The mixture was boiled for 2 min and diluted to 15 mL. The standard curve of galacturonic acid was obtained by using standard solutions of 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL. UV absorption at 540 nm was measured. Galacturonic acid was determined based on its calibration curve [14]. Enzyme activity (U) was defined as the quantity of catalyzed enzyme that released 1 µmol of galacturonic acid per minute under the standard condition (40°C, pH 4.6).

Determination of amylase activity

Amylase activity was determined by incubating the reaction mixture at 50°C and pH 4.6 with the substrate of 1% soluble starch solution. The amount of glucose yielded from soluble starch by amylase was calculated [15], and the enzyme activity was determined in units (U).

Determination of cellulase activity

The cellulase activity was determined according to the national standard GB/T 23881-2009. One unit (U) was defined as the enzyme quantity that released 1 µmol of glucose per minute from filter paper under standardized conditions (37°C, pH 5.5).

Determination of hemicellulase activity

Hemicellulase activity was determined by incubating the reaction mixture at 40°C and pH 4.6 with the substrate of 1% xylan solution [16].

Separation, purification, structure analysis, and active site prediction of laccase from *Ganoderma lucidum* enzyme solution

G. lucidum enzyme solution was centrifuged at 10,000 rpm at 4°C for 10 min and then membrane filtered. Ammonium sulfate powder was gradually added to make a 40% saturation solution. The solution was kept at 4℃ for 12 h before centrifugation to obtain supernatant. The purification was repeated using 80% of ammonium sulfate saturation solution. The pellet was collected and redissolved using disodium hydrogen phosphate-citric acid (0.2 - 0.1 mol/L, pH 6.0) to 20 mL, which was transferred into a dialysis bag for dialysis overnight. The crude laccase was redissolved to 5 mL, and gel filtration chromatography was performed on the protein purification system (AKTATM Pure 25L) using Sephadex G-200 column with the elution flow rate of 2.0 mL/min. Eluent was collected for each of 5 mL in a tube, and enzyme activity was determined. The eluent with enzyme activity was collected. The eluent of the same peak was

combined for SDS-polyacrylamide gel electrophoresis, and the gel imager was used to scan the lane strips to detect the purification effect. Laccase was further purified by non-denaturing electrophoresis gel extraction [17]. After electrophoresis, the gel was stained with 1 mmol/L of ABTS, and the corresponding parts of the gel coloration position were cut off. The gel pieces were immersed in disodium hydrogen phosphate-citric acid buffer for recovery. The non-denaturing electrophoresis was applied to verify the purification after gel cutting recovery by mixing 5 µL of the three isozyme solutions after gel cutting recovery with loading buffer. After electrophoresis, the gel was stained with 1 mmol/L guaiacol for band observation and determination of purification effect. The laccase samples with better purification effect were selected, and the molecular weight of the target protein was estimated. The purified laccase was analyzed by SCIEX TripleTOF 6600 liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Applied Biosystems, Boston, MA, USA) for full-length sequencing and the analysis by Shanghai Shenggong Bioengineering Co., Ltd (Shanghai, China). The tertiary structure of laccase was predicted using AlphaFold 3 (Google DeepMind, San Francisco, CA, US). The prediction model was uploaded to SAVES (https://saves.mbi.ucla.edu/) for model evaluation. The model of laccase and copper ligand was established by Alpha Fold 3, and the active center position was predicted. The constructed laccase-copper active center model was visualized and refined in PyMOL (https://www.pymol.org/).

Statistical analysis

All measurements were performed using three parallel experiments, and the results were expressed as mean ± standard deviation (SD). Statistical analysis was performed using SPSS 25.0 software (IBM, Armonk, New York, USA). One-way analysis of variance (ANOVA) was used for significant comparisons among multiple groups. When ANOVA indicated significant differences, post-hoc pairwise comparisons were conducted using the LSD test. *P* value less than 0.05 was defined as statistically significant difference.

Results and discussion

Optimization of tobacco stem absorption process

(1) Single factor results

The results showed that, with the increase of stem washing time, the water content of tobacco stem increased, and finally reached equilibrium (Figure 1a). The water content of tobacco stem reached 33% after washing for 5 min, and the final water content could be 46% to 47%. When the water content was between 27% and 35%, the overall enzyme solution absorption rate was higher with the highest value at water content of 33% (Figure 1b). The effect of the preset temperature of tobacco stem on the absorption rate of enzyme solution was initially increased and then decreased with a higher adsorption rate observed at 50°C (Figure 1c). For drum working time, two platform periods of 2.5 - 5.0 min and 7.5 - 10.0 min were observed. Considering the actual production condition, the drum working time was selected as 5.0 min (Figure 1d).

(2) RSM results

Based on the RSM analysis results, a coded mathematical model for the absorption rate was established as below.

$$Y = 61.13 - 1.8A + 0.97B + 4.12C + 0.34AB + 1.59AC + 2.69BC - 5.25A^2 - 0.23B^2 - 2.21C^2 \quad (2)$$

where Y was the absorption rate. A was the water content. B was the temperature. C was the drum working time. ANOVA analysis showed that the regression model was statistically significant (P = 0.0002). The correlation coefficient of the model R^2 was 0.9691, while the correction coefficient of determination R^2_{adj} was 0.9295, which indicated the high accuracy for the model prediction. Analysis of the significance of the three factors suggested that the drum working time (C) > water content (A) > B (temperature). The interaction term AC, BC and the quadratic term A^2, C^2 were

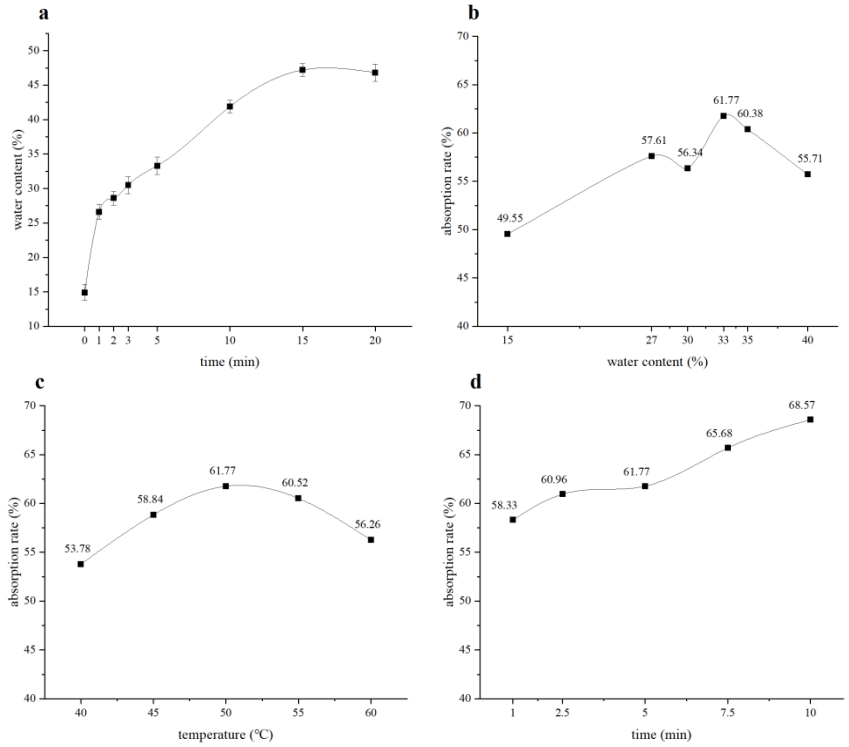

Figure 1. The effect of stem washing time on the water content of tobacco stems (**a**) and the absorption rate of *Ganoderma lucidum* enzyme solution on tobacco stems under different water content (**b**), preheating temperature (**c**), and drum working time (**d**).

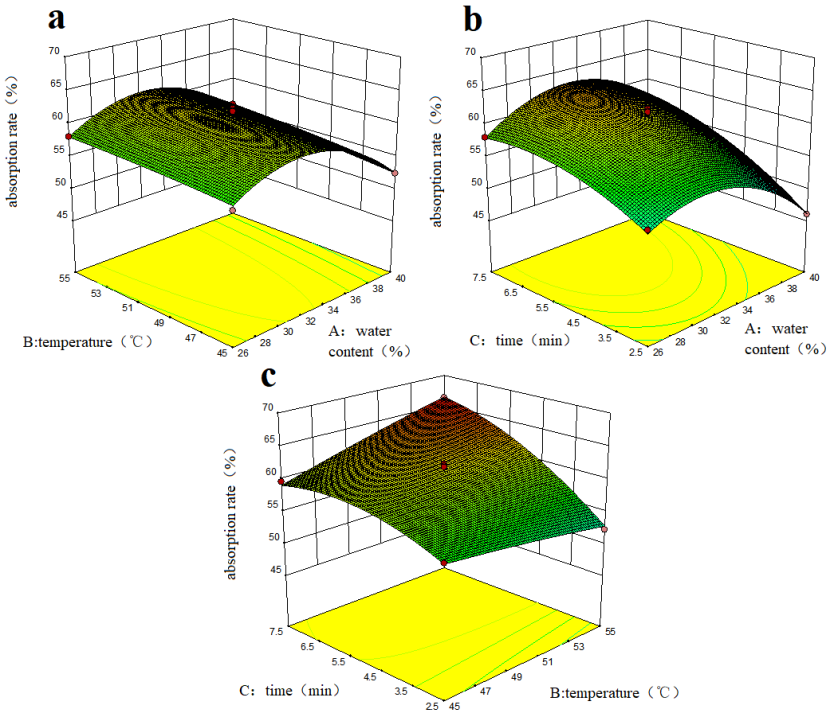

Figure 2. Response surface three-dimensional models of the interaction among three factors. **a.** water content with temperature on absorption rate. **b.** water content with time on absorption rate. **c.** temperature with time on absorption rate.

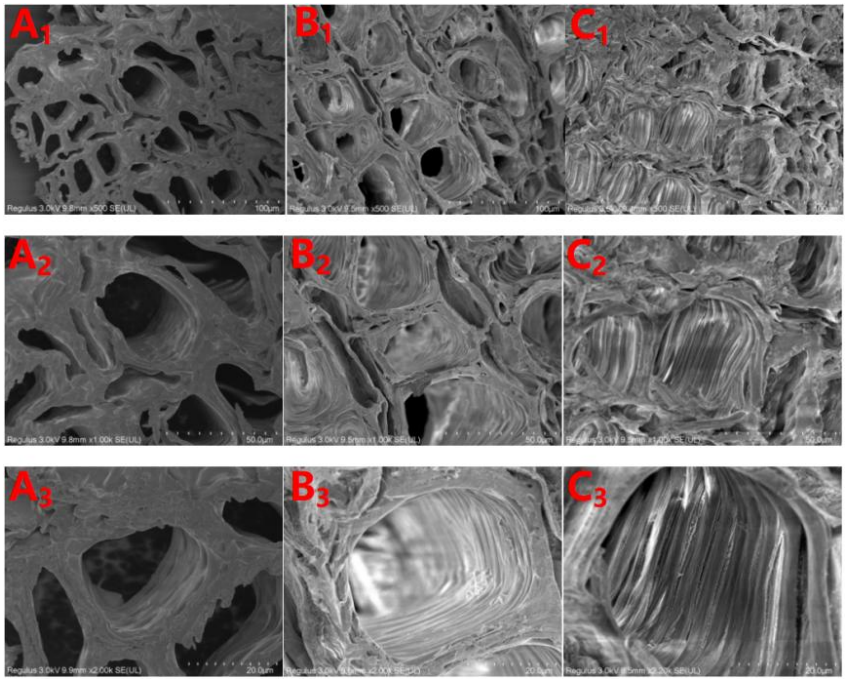

Figure 3. SEM observation of tobacco stem slices. A1, A2, A3 were untreated tobacco stem (500×, 1,000×, 2,000×). B1, B2, B3 were treated with *Ganoderma lucidum* enzyme solution for 4 h (500×, 1,000×, 2,000×). C1, C2, C3 were treated with *Ganoderma lucidum* enzyme solution for 8 h (500×, 1,000×, 2,000×).

all significant ($P < 0.05$). These results confirmed the rationality of the experimental design and the reliability of the data. The contour plots and response surfaces generated by the quadratic regression model showed that there was interaction between water content (A) and temperature (B) on the adsorption rate of the enzyme solution but without significant. The interaction between water content (A) and drum working time (C) was significant, and the interaction between B and C was also significant (Figure 2). Through the optimization of response surface model, the predicted suitable parameters were the preset temperature of 52.986℃, the water content of 32.011%, and the drum working time of 5.191 min. The parameters were slightly adjusted to 53℃, the water content of 32%, and the drum working time of 5.2 min. The average absorption rate of the enzyme was determined as 61.232%, which was highly in accordance with the predicted value of 62.150%. These results indicated that the regression model was accurate and that the optimized enzymatic hydrolysis conditions obtained by RSM were reliable.

SEM observation

The results of SEM observation showed that the original dense structure of tobacco stems was destroyed, and many holes and pits appeared on the surface, along with increase of fermentation time [19] (Figure 3). The results suggested that the *G. lucidum* enzyme solution had the ability to degrade some lignin in tobacco stems.

Sensory evaluation and GC-MS volatile compounds analysis of fermented tobacco stems

The sensory evaluation results showed that the sensory evaluation quality and scores were enhanced along with the fermentation time, and the highest quality was found for fermentation time of 8 h. Overall, the sweetness taste was enhanced, and the wood odor was highly improved. The aroma intense increased and the

Table 1. Sensory evaluation for tobacco stems at different fermentation times.

	Woody	Flammability	Irritation	Sweetness	Aroma intensity	Offensive odor
Original Sample	1.00 ± 0.08$^{e\#}$	3.47 ± 0.05^{a}	1.10 ± 0.08^{d}	2.00 ± 0.08^{d}	1.03 ± 0.05^{e}	1.47 ± 0.05^{d}
Control Sample	1.20 ± 0.08^{d}	3.50 ± 0.08^{a}	2.03 ± 0.05^{c}	2.33 ± 0.05^{c}	1.27 ± 0.05^{d}	2.03 ± 0.05^{c}
2 h	1.50 ± 0.08^{c}	3.50 ± 0.08^{a}	2.20 ± 0.08^{bc}	2.53 ± 0.12^{b}	1.80 ± 0.08^{c}	2.23 ± 0.05^{b}
4 h	1.60 ± 0.08^{c}	3.53 ± 0.05^{a}	2.27 ± 0.09^{b}	2.57 ± 0.05^{b}	1.93 ± 0.05^{b}	2.33 ± 0.05^{b}
6 h	2.00 ± 0.08^{b}	3.47 ± 0.05^{a}	2.20 ± 0.08^{bc}	2.83 ± 0.05^{a}	2.03 ± 0.05^{b}	2.33 ± 0.05^{b}
8 h	3.10 ± 0.08^{a}	3.53 ± 0.05^{a}	2.63 ± 0.05^{a}	3.00 ± 0.08^{a}	2.53 ± 0.05^{a}	2.73 ± 0.05^{a}

Notes: the small case letters (a-e) and $^{\#}$ within the same row denoted statistically significant differences ($P < 0.05$).

offensive odor was significantly improved [20] (Table 1). GC-MS analysis results of mainstream smoke compounds of tobacco stems at different fermentation times demonstrated that, along with increase of fermentation time, the total volatile compounds increased, especially for the aldehyde, ketone, and phenol components. With the extension of fermentation time, the contents of roasting sweet flavor compounds such as 5-methylfuran aldehyde, 5-hydroxymethylfurfural, maltol, methyl cyclopentenolone, ethylcyclopentenolone, 2,4-dihydroxy-2,5-dimethyl-3(2*H*)-furan-3-one, and 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) increased, thus the sweetness taste was enhanced. The results indicated that cellulose might be degraded into glucose during fermentation and converted into roasting sweet flavor components after combustion. The contents of vanillin, isovanillin, neophytadiene, 4-hydroxystyrene, and acetyl eugenol increased, which could further enhance the characteristics of freshness sweet and floral aroma. Phenols such as 4-vinyl-2-methoxyphenol, phenol, 2-methoxy-6-(1-propenyl) phenol, 4-vinyl-2,6-dimethoxy-phenol, and 4-allyl-2,6-dimethoxyphenol increased first and then decreased. In comparison with the original sample, the content of phenols increased after fermentation for 4 h, indicating that the lignin component in the tobacco stem was degraded. Moreover, as the fermentation time increased to 8 h, these phenolic compounds were further converted into phenolic aroma components such as 4-hydroxystyrene, vanillin, isovanillin, acetyl eugenol, and 2,4,5-trimethoxybenzaldehyde, and the flavor of tobacco stem was further improved.

Table 2. Extracellular enzyme activity in the enzyme solution.

Enzyme type	Enzyme activity (U/L)
Pectinase	281.90
Amylase	1,642.98
Cellulase	21.02
Xylanase	336.50
Laccase	35,466.67

Enzymatic types and activity determination of *Ganoderma lucidum* enzyme solution

To explore the degradation potential of *Ganoderma lucidum* enzyme solution on cell wall macromolecules in tobacco stems, several extracellular enzyme activities were determined [21]. A serial standard curves for the determination of pectinase, amylase, and hemicellulase activity were established as $y = 0.9665x - 0.0533$ ($R^2 = 0.9984$) for pectinase, $y = 1.145x - 0.0256$ ($R^2 = 0.9986$) for amylase, and $y = 1.145x - 0.0256$ ($R^2 = 0.9971$) for hemicellulase. The results of extracellular enzyme activity determination showed that the laccase activity in the *G. lucidum* enzyme solution reached 35,466.67 U/L (Table 2). Laccase could effectively degrade lignin in tobacco stems. It has been reported that laccase treatment can significantly reduce the offensive odor and irritation of cigarettes and effectively improve the aroma quality [22]. The high laccase activity of the enzyme solution indicated its strong lignin oxidation ability, making it suitable for degrading and modifying lignin in tobacco stems. In addition to laccase activity, the activities of pectinase, amylase, cellulase, and xylanase were also detected, indicating that the enzyme solution of *G. lucidum* had the ability of multi-enzyme

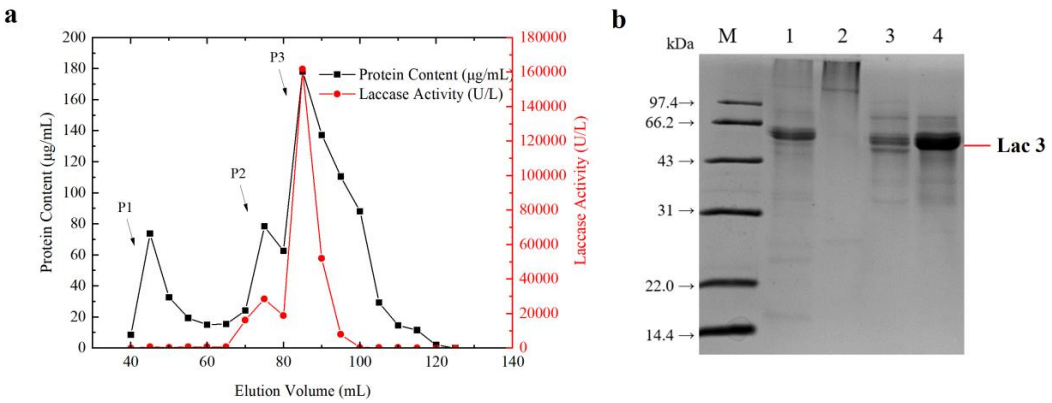

Figure 4. Electrophoretic analysis of *Ganoderma lucidum* enzyme solution and Lac3. **a.** Gel filtration chromatography results. **b.** SDS-PAGE analysis. M: marker. Lane 4: Lac3.

synergy to degrade pectin, starch, cellulose, hemicellulose, and other macromolecules of tobacco stems [23]. In general, most of the existing research focused on the post-treatment of tobacco stem using single enzyme. *G. lucidum* enzyme solution in this study possessed a multiple degradation enzyme system.

Separation, purification, structure analysis, and active site prediction of laccase from *Ganoderma lucidum* enzyme solution

Electrophoretic analysis was performed on both the *G. lucidum* enzyme solution and the purified laccase (Lac3). The results showed that, after non-denaturing polyacrylamide gel electrophoresis (Native-PAGE) and staining, three distinct bands appeared, indicating the presence of three isoenzymes. Following molecular sieve purification, the most active Lac3 variant exhibited a single band on both Native-PAGE and SDS-PAGE (Figure 4). The molecular weight of Lac3 was determined as 58.4 kDa [24]. The full-length sequence of Lac3 protein demonstrated that there were 500 amino acids in the enzyme. Prior research on protein crystallography has shown that laccase copper sites are classified into three types including type I copper, a blue copper center (T1), type II copper, a normal copper center (T2), and type III copper, a coupled binuclear copper center (T3). These copper sites reside within the central cavity of the laccase protein could form a trinuclear copper cluster active center with a distinct three-dimensional arrangement [25]. The three-dimensional structure of Lac3 and the location of copper sites were then predicted (Figure 5). The trinuclear copper cluster was situated at the center of the Lac3 protein, whereas the type I copper resided in an external cavity. Typically, substrates were captured and oxidized in the vicinity of the type I copper. [25]. The binding sites of Cu^{2+} were predicted, and the protein-Cu ligand structure model was established to predict the amino acid sites with strong binding ability to copper ions. The specific binding sites were speculated as that type I copper ion was coordinated with two histidines and one cysteine (HIS-396, HIS-459, CYS-454), while type II copper ion was coordinated with two histidines (HIS-65, HIS-399), two type III copper ions and three histidines (HIS-67, HIS-110, HIS-455; HIS-112, HIS-401, HIS-453). Two type III copper ions and one type II copper ion formed a trinuclear copper center.

Conclusion

The results of single factor and response surface optimization showed that the optimized conditions for the absorption and fermentation process of tobacco stem by *G. lucidum* enzyme solution were preset temperature of 53℃, water content of 32%, and drum working time of 5.2

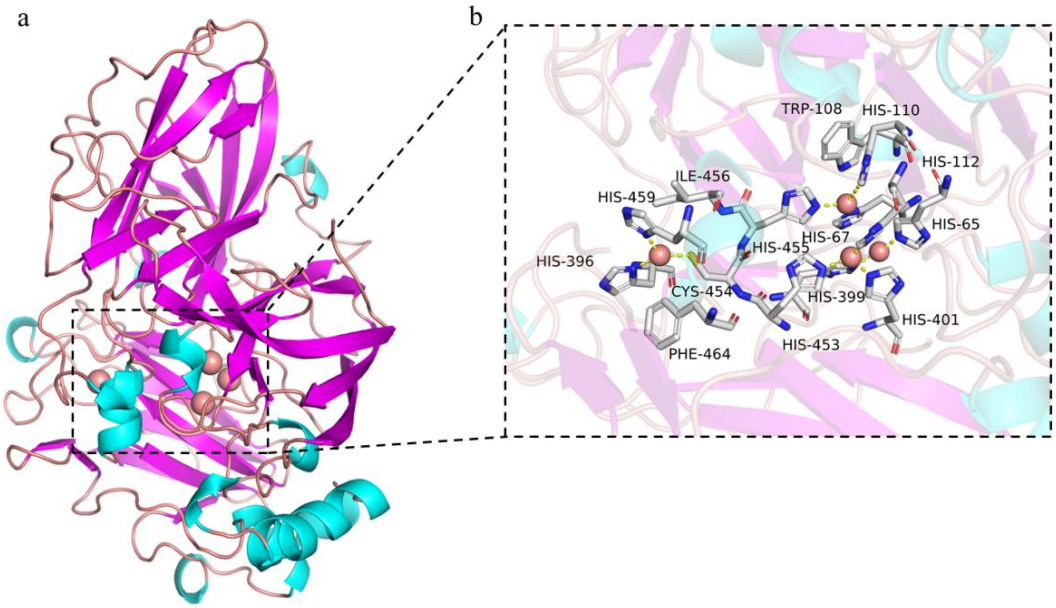

Figure 5. Three-dimensional structural model (**a**) and active sites of Lac3 (**b**).

min. SEM observation suggested that the original dense structure of tobacco stem was destroyed and the lignins were degraded. GC-MS analysis in combination with sensory evaluation results suggested that, along with fermentation time, the total volatile compounds of tobacco stem mainstream smoke compounds gradually increased. The sensory evaluation quality was improved, and the roasting sweet, fresh, and floral aroma compounds increased. The enzyme solution of *G. lucidum* contained laccase, pectinase, amylase, cellulase, and xylanase activities, among which laccase activity was the highest and possessed three kinds of laccase isozymes. Three-dimensional structure model of Lac3 was predicted to be a typical trinuclear copper central enzyme. This study provided theoretical guidance and technical support for the establishment of tobacco stem fermentation process control method and would help the improvement of stem quality and the comprehensive utilization of tobacco stem resources.

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