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RESEARCH

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Insight into the role of antioxidant in microbial lignin degradation: Ascorbic acid as a fortifier of lignin-degrading enzymes

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Abstract

Background Microbial-driven lignin depolymerization has emerged as a promising approach for lignin degradation. However, this process is hindered by the limited activity of lignin-degrading enzymes. Antioxidants are crucial for maintaining redox homeostasis in living cells, which can impact the efficiency of enzymes. Ascorbic acid (AA) is well-known for its antioxidant properties, while *Trametes versicolor* is a commonly used lignin-degrading fungus capable of secreting laccase (Lac) and manganese peroxidase (MnP). Thus, AA was selected as model antioxidant and added into the culture medium of *T. versicolor* to examine the effect of antioxidants on the activity of lignin-degrading enzymes in the fungus.

Results The presence of AA resulted in a 4.9-fold increase in the Lac activity and a 3.9-fold increase in the MnP activity, reaching 10736 U/L and 8659 U/L, respectively. This increase in enzyme activity contributed to a higher lignin degradation rate from 17.5% to 35.2%, consistent with observed morphological changes in the lignin structure. Furthermore, the addition of AA led to a reduction in the molecular weights of lignin and an increase in the content of degradation products with lower molecular weight, indicating more thorough degradation of lignin. Proteomics analysis suggested that the enhancement in enzyme activity was more likely to attributed to the reinforcement of AA on oxidative protein folding and transportation, rather than changes in enzyme expression.

Conclusions The addition of AA enhanced the performance of enzymes responsible for lignin degradation in terms of enzyme activity, degradation rate, lignin structural change, and product mapping. This study offers a feasible strategy for enhancing the activity of lignin-degrading enzymes in the fungus and provides insights into the role of antioxidant in microbial lignin degradation.

Keywords Lignin degradation, *Trametes versicolor*, Ascorbic acid, Laccase, Manganese peroxidase

Introduction

Lignin is a significant constituent of lignocellulose, constituting 15–30% of its total mass [1]. Despite its abundance, lignin has traditionally been considered a waste product in the paper and pulp industry, with its utilization lagging behind that of cellulose and hemicellulose. As the commercialization of lignocellulosic biofuels has led to a surge in waste lignin production, the need for valorization of lignin has become increasingly urgent [2]. Lignin, as the largest aromatic heteropolymer in nature,

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holds potential as a renewable source of aromatic chemicals, which are essential for the production of value-added materials, fine chemicals, and pharmaceuticals [3]. Lignin depolymerization is an important starting point for its valorization, but is challenged by the inherent heterogeneity and recalcitrance of lignin [4]. Efficient approaches to degrading lignin polymer are critical to maximizing the potential of this valuable resource. Biological depolymerization of lignin using enzymes from microorganisms has emerged as a promising and environmentally friendly alternative, offering advantages such as mild reaction conditions, low energy consumption, and reduced organic chemical requirements [5].

Despite the natural recalcitrance of lignin, a variety of microorganisms, such as fungi and bacteria, have been found to possess the ability to degrade it. However, their efficiency in breaking down lignin is hindered by inadequate activity of the enzymes responsible for lignin degradation. Various strategies have been utilized to improve the activity of lignin-degrading enzymes. For instance, the consortium composed of *Trametes versicolor* and other microorganisms has been employed to boost the activity of lignin-degrading enzymes through microbial synergism [6, 7]. Additionally, the Chinese herbal medicine *Polygonum cuspidatum* was used to enhance laccase production in *T. versicolor* [8]. Although these studies have provided several feasible strategies, it is still necessary to develop new approaches to improve the activity of lignin-degrading enzymes in microorganisms. The importance of developing new methods lies not only in achieving efficient lignin degradation platforms, but also in gaining a better understanding of the production mechanism of microbial lignin-degrading enzymes.

Microorganisms degrade lignin through the secretion of extracellular lignin-degrading enzymes. Oxidative protein folding in endoplasmic reticulum (ER) is essential for the formation of active conformation and secretion of these enzymes [9]. However, this process also leads to the generation of reactive oxygen species as a byproduct, contributing to ER stress, inefficient protein folding, and activation of the unfolded protein response [10]. Furthermore, the depolymerization of lignin by lignin-degrading enzymes results in the generation of various free radicals [11, 12]. Therefore, the maintenance of the redox homeostasis is crucial for the production of lignin-degrading enzymes and lignin degradation. The addition of an appropriate antioxidant has the potential to regulate microbial redox status and further influence the activity of lignin-degrading enzymes.

Ascorbic acid (AA) is widely recognized for its antioxidant properties in free radical-mediated oxidation processes [13, 14]. It is believed to play an essential role in maintaining the intraluminal oxidative environment in the ER [15, 16]. Besides, AA has been shown to act as a pro-oxidant, enhancing the pro-oxidant activity of copper and iron, which are critical components of important lignin-degrading enzymes such as laccase (Lac) and manganese peroxidase (MnP) [17]. These suggest that AA has the potential to amplify the activity of microbial lignin-degrading enzymes. The fungus *T. versicolor*, known for its ability to secrete Lac and MnP, is an efficient microorganism for lignin depolymerization [7, 18]. However, the degradation efficiency of *T. versicolor* is limited by the low activity of its lignin-degrading enzymes. In this study, AA was added into the culture medium of *T. versicolor* to investigate the impact of antioxidant on enzyme activity. The effects of AA on Lac and MnP activity were examined and analyzed, as well as the rates of lignin degradation, changes in lignin structure, and detailed product mapping. Furthermore, label-free differential proteomics analysis was performed to obtain an in-depth understanding of the molecular mechanisms underlying the AA-enhanced activity of lignin-degrading enzymes. The study demonstrates that the addition of AA is an effective way to enhance the activity of lignin-degrading enzymes, which was more likely to be achieved through reinforcing protein folding and transportation.

Materials and methods

Chemicals and microorganism

The *T. versicolor* was purchased from China General Microbiological Culture Collection Center. Ascorbic acid was purchased from Beijing Solarbio Science & Technology Co., Ltd. Lignin, which was extracted from corn stalks, was supplied by Shandong Longlive Bio-Technology Co., Ltd. The extraction procedure for the lignin was as follows: the 70 g of raw corn stalks underwent treatment with 1 M NaOH at a temperature of 50 °C for 3 h, utilizing a solid-to-liquid ratio of 1:20 (g/ml). Following the reaction, the resulting liquid fraction was filtered and its pH was adjusted to a range of 5.0–6.0 using 6 M HCl. Subsequently, the solution was concentrated and precipitated in ethanol to eliminate hemicellulosic components. The aqueous ethanol filtrate, which contained lignin, was then concentrated under vacuum and reprecipitated through acidification with 6 M HCl to pH 2.0. Ultimately, the purified lignin was obtained, yielding 3.2 g after being dried in a vacuum oven at 50 °C. All other common chemical reagents were analytically pure and purchased from China National Pharmaceutical Group Corporation.

Culture medium and conditions

The *T. versicolor* was maintained on potato dextrose agar (PDA) plates. The cell culture of *T. versicolor* was performed at 30℃ and 200 rpm in lab-modified medium

containing (per liter) glucose 20 g, peptone 5 g, yeast extract 2 g, KH_2PO_4 2 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, $MnSO_4$ 75 mg, $CaCl_2{\cdot}2H_2O$ 0.1 g, succinic acid 1.18 g, ammonium tartrate 1.84 g, thiamine 1 mg, tween-80 0.5 mL, and trace element 70 mL (pH 5.4). The trace element components composed of the following components (in grams per liter): $MgSO_4{\cdot}7H_2O$ 3.0, $MnSO_4{\cdot}H_2O$ 0.5, NaCl 1.0, $FeSO_4{\cdot}7H_2O$ 0.1, CoCl 0.1, $ZnSO_4{\cdot}7H_2O$ 0.1, $CuSO_4{\cdot}5H_2O$ 0.1, $KAl(SO_4)_2{\cdot}12H_2O$ 0.01, H_3BO_3 0.01, $NaMo_4{\cdot}2H_2O$ 0.01. A loop of colonies of *T. versicolor* from PDA plates was transferred in 250-mL flasks for seed cultures. A 10% (vol/vol) inoculum was used for all experiments. Varying quantities of AA solution (200 g/L) were added into the culture medium to achieve needed final concentrations of AA in the culture system before the seed solutions transferred. The effect of AA on activity of lignin-degrading enzymes was determined with AA concentrations ranging from 0 to 2.0 g/L. The enzyme activity in the presence of lignin and enzyme activity in a 1.0-L scale-up cultivation and lignin degradation were studied with 1.5 g/L AA, and the cultivation without added AA was used as the control group. The sterilized glass beads with a diameter of 0.5 cm were applied in all liquid cultures for *T. versicolor* growth.

Enzyme assays

The supernatant was collected from 1.0 mL culture solution by centrifugation at 8000 rpm for 10 min. The activity of lignin-degrading enzymes was assayed using ultraviolet-visual (UV-visual) spectrophotometry. The catalytic activity of Lac was determined by monitoring the change in absorbance at 420 nm [19]. The assay mixture (1.0 mL) for Lac activity contained 0.2 mL ABTS (0.5 mM), 0.1 mL collected supernatant and 0.7 mL malonic acid–malonate buffer (50 mM, pH 4.5). The MnP activity was measured by monitoring the oxidation of 2,6-dimethoxyphenol (2,6-DMP) as the substrate at 470 nm [20]. The assay mixture (1.0 mL) for MnP activity contained 50 μL 2,6-DMP solution (10 mM), 50 μL $MnSO_4$ solution (10 mM), 50 μL H_2O_2 (10 mM), 0.1 mL collected supernatant and 0.7 mL malonic acid–malonate buffer (50 mM, pH 4.5). The enzyme activity was calculated with the UV absorption data according to reported methods [21].

Determination of degradation rates

The degradation rate of lignin was determined by the Laboratory Analysis Protocol from the National Renewable Energy Laboratory [22]. According to the protocol, the contents of acid-soluble and acid-insoluble lignin need to be determined separately. Then, the lignin degradation rate was calculated based on the mass change by combing the acid insoluble and soluble portions of the sample. The equation used is as follows:

$$\text{Lignin degradation rate} = \frac{W - W_{\text{acid-insoluable}} - W_{\text{acid-soluable}}}{W} 100\% \quad (1)$$

where W is the amount of lignin added in the cultivation system; $W_{\text{acid-insoluable}}$ the amount of acid-insoluble lignin at the end of the cultivation; and $W_{\text{acid-soluable}}$ the amount of acid-soluble lignin at the end of the cultivation.

Characterization of lignin in cultivation with or without ascorbic acid

The changes of lignin morphology and structure were characterized using a scanning electron microscope (SEM, TESCAN MALA3 LMH, Czech Republic). The samples were prepared by centrifuging the culture solution at the end of cultivation at 8000 rpm for 10 min and the precipitate was collected and lyophilized for 48 h to achieve a constant weight. Samples were coated with gold powder by a spray meter beforehand. The weight-averaged (Mw) and number-averaged (Mn) molecular weights were determined at 40 ℃ in tetrahydrofuran solution by a Malvern OmniSEC system (Malvern Panalytical Ltd., Malvern, UK). The FT-IR spectrum of water soluble fraction was obtained on a Nicolet iS50 FT-IR spectrometer (Thermo Fisher Scientific Co., Waltham, MA, USA).

Analysis of lignin degradation products

Samples (50 mL) from the cultivation culture were centrifuged at 8000 rpm for 10 min to remove the cells. Then, the pH of the centrifuged supernatant was adjusted to approximately 2.0 by addition of 6.0 M HCl. Subsequently, the mixture was derivatized according to reported procedures [23]. The derivatized sample (1.0 μL) was injected into the Gas chromatography-mass spectrometry (GC–MS, Thermo Fisher Scientific Trace ISQ, America) equipped with capillary column HP-5 using Helium as the carrier gas. The injection temperature, ion source temperature, and transfer line temperature were 280, 250, and 200 ℃, respectively. The initial column temperature of 50 ℃ was held for 5 min, then raised to 300 ℃ at a rate of 10 ℃/min, and then maintained for 5 min. The solvent delay time was 180 s. The ionization mass spectrum in the range of 30–550 m/z was recorded in Full Scan mode and 70 eV electron energy. The results were compared with standard mass spectrometry databases to determine the products after lignin degradation [24].

Label-free differential proteomics analysis

The separation of peptides was performed by SCIEX's eksigent ultra 2D model nanoliter liquid phase chromatograph with a tandem self-packed C18 column (75 μm internal diameter, 3 μm column size, 20 cm column length). The liquid phase chromatography separated peptides were passed to an ESI tandem mass spectrometer: TripleTOF 5600 (SCIEX, Framingham, MA, USA), the ion source was Nanospray III source (SCIEX, Framingham, MA, USA), and the emitter was a needle (New Objectives, Woburn, MA, USA) drawn from quartz material. For data acquisition, the mass spectrometer parameters were set as follows: ion source spray voltage 2300 V, nitrogen pressure 35 psi, spray gas 15 and spray interface temperature 150 ℃. Scanning in high sensitivity mode, the MS1 scan cumulative time was 250 ms, and the scan quality range was 350–1500 Da. Based on the MS1 scanning information, according to the ionic strength in the MS1 spectrum from high to low, the first 30 ions exceeding 150 cp were selected for fragmentation and the MS2 information was scanned. The raw datum was identified using the integrated Andromeda engine of MaxQuant [25], and then MaxQuant performed quantitative analysis based on the peak intensity, peak area, and LC retention time of the peptides related to the first-order mass spectrometry, and performed a series of statistical analysis and quality control. At the spectrum level, filtering was performed with PSM-level FDR < = 1%, and at the protein level, further filtering was performed with Protein-level FDR < = 1% to obtain significant identification result. Then based on the identification results, this process will automatically complete analysis of functional annotations such as GO, KOG, Pathway. Based on the quantitative results, the search for differential proteins between different comparison groups is completed, and the functional analysis of the corresponding differentially enriched proteins is finally performed. Welch's t-test was used for quantitative analysis of proteomics data.

Results and discussion

Effect of AA on activity of lignin-degrading enzymes

AA is very popular for its antioxidant properties. The effects of AA on physiological processes of various microorganisms have been widely identified [26–28]. In view of this, AA is a good model compound for testing the effect of antioxidant on the activity of lignin-degrading enzymes. Therefore, AA in different concentrations was added into the culture medium of *T. versicolor* and the activity of lignin-degrading enzymes was analyzed after 10 days of cultivation. The enzymatic activity of Lac and MnP was enhanced in the presence of AA as expected (Fig. 1a and b), while the cell growth of *T. versicolor* was not obviously affected (Table S1). Particularly, the highest activity of Lac (10736 U/L) and MnP (8659 U/L) was achieved when the concentration of AA was 1.5 g/L, which was 4.9- and 3.9-fold greater than those of the control sample, respectively. Moreover, it was noted that the activity of lignin-degrading enzymes decreased with further increase of AA concentration up to 2.0 g/L. It might be attributed to the high concentrations of AA lead to the excessive elimination of free radicals [29–31], which commonly exert a series of advantageous physiological effects such as sensing of oxygen tension, enhancement of signal transduction, and oxidative stress response [32, 33]. These functions are necessary for maintaining cellular metabolism and inevitably affect the activity of lignin-degrading enzymes. Thus, the delicate balance between the advantageous and detrimental effects is clearly an important aspect of the increased activity of lignin-degrading enzymes driven by antioxidant.

The activity of lignin-degrading enzymes was determined in the presence of lignin to further confirm the reinforcement effect of the AA. As shown in Fig. 1c and d, the addition of AA also produced an enhancement on the activity of Lac and MnP during the whole cultivation at the additive amount for 1.5 g/L. Both Lac and MnP activities were also highest on the 7th day in the cultivation with or without lignin. In the presence of lignin, the highest activity of Lac and MnP was 12694 U/L and 8790 U/L, respectively. The enhancement of Lac and MnP activity was 4.9- and 3.3-fold than those of the control. These outcomes demonstrated that the addition of AA can still enhance the activities of lignin-degrading enzymes in the presence of lignin. Therefore, this strategy could be directly applied to the degradation of lignin. More encouragingly, the activity of lignin-degrading enzymes in the presence of AA was also much higher than those of the control in a 1.0-L scale-up cultivation (Fig. 1e and f). Bioprocess scale-up is a vital step in process development. Generally, the decrease of indicators such as biological activity, biomass, and product yield was observed during the scale-up process [34]. The activity of both Lac and MnP was higher in the scale-up cultivation than which in 250-mL shake flask. It is of great significance to construct a scalable system for the activity enhancement of lignin-degrading enzymes.

Lignin degradation and products mapping

It is expected that the increase in Lac and MnP activities could indeed enhance the lignin degradation ability of *T. versicolor*. Thus, the degradation rate and morphological change of lignin in cultivation systems with or without AA were compared. As shown in Fig. 2a, the lignin degradation rate of the cultivation with AA was determined to be.

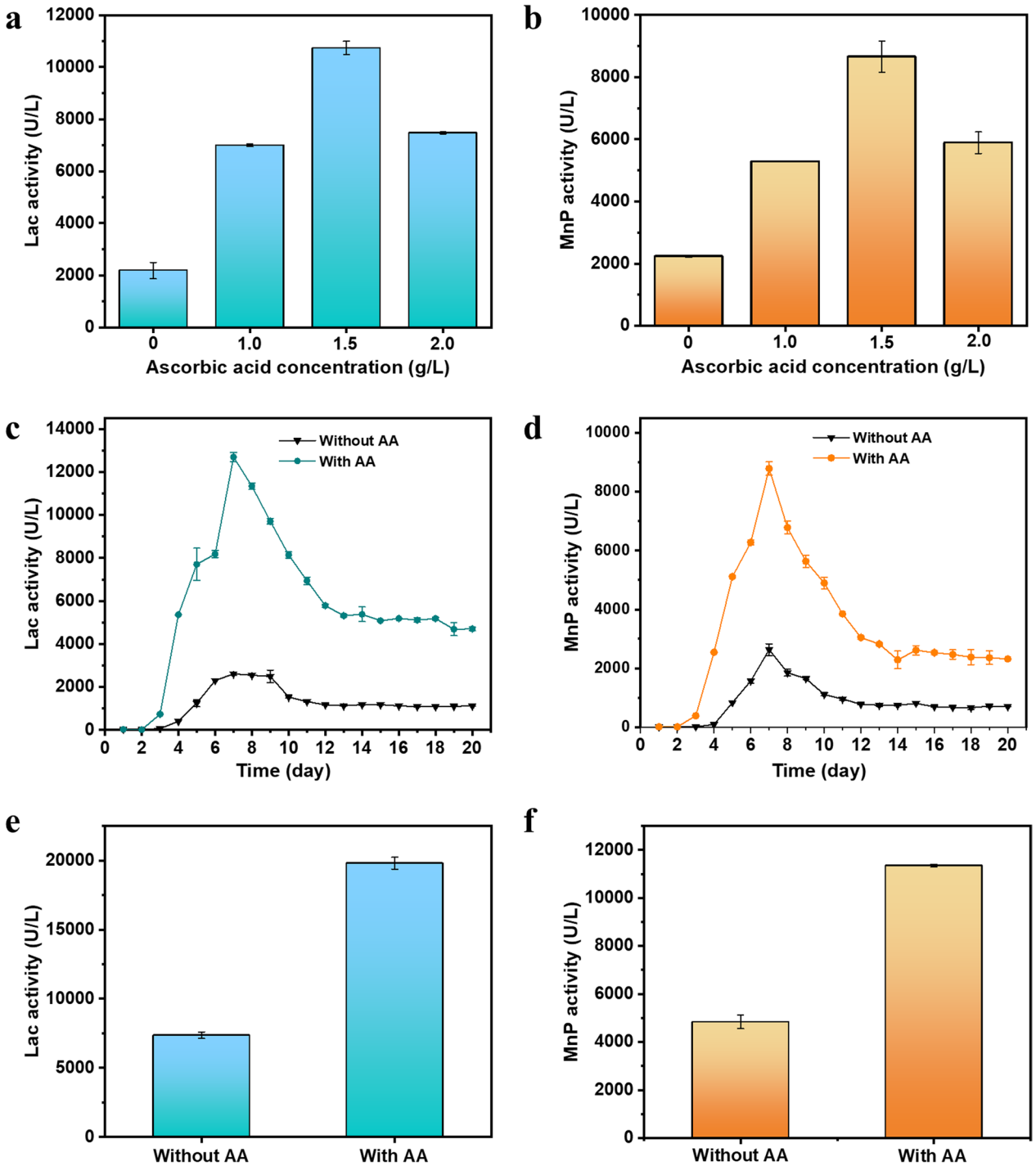


Fig. 1 Effects of ascorbic acid addition on lignin-degrading enzyme activity. **a**-**b** Effect of ascorbic acid concentration; **c**-**d** Enzyme activity in the presence of lignin; **e**–**f** Enzyme activity in a 1.0-L scale-up cultivation. Error bars indicate standard deviation

35.2%, which was much higher than that of the control group (17.5%). The results of lignin morphological changes obtained through SEM were in good agreement with the experimentally determined degradation rate (Fig. 2b-f). Changes in the shape and size of lignin suggested that the cultures with AA indeed possessed stronger lignin degradation capability. The particle size of lignin in the cultivation with AA was smaller than those in the control group after 7 days. A more obvious difference was observed after 20 days. The morphology of

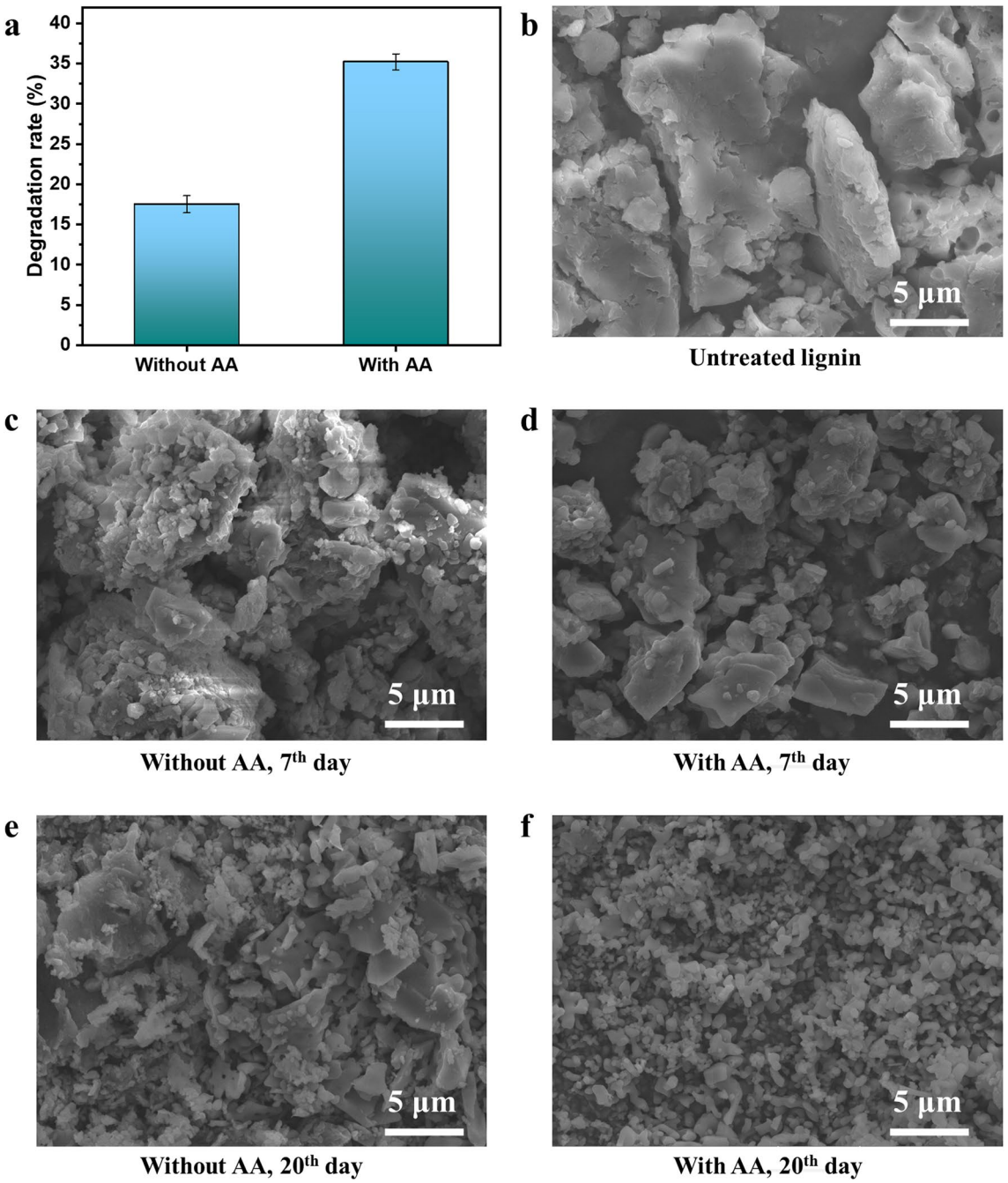


Fig. 2 Lignin degradation in cultivation systems with or without ascorbic acid. **a** Lignin degradation rate. **b**-**f** SEM images. Error bars indicate standard deviation

lignin in the cultivation with AA was more irregular and the particles were more densely packed (Fig. 2f).

To gain a better understanding of the reinforcement of AA on the activity of lignin-degrading enzymes, the molecular weights, chemical composition, and degradation products were identified and characterized by GPC, FT-IR, and GC–MS. As shown in Fig. 3a and b, the lignin in the sample was continuously degraded and the molecular weights gradually decreased. The original lignin has number-averaged (Mn) and weight-averaged (Mw) molecular weights of 50400 g/mol and 68700 g/mol, respectively. After 7 days of treatment, the Mn and Mw of lignin decreased in both cultivation with or without AA, and further decrease of lignin molecular weights was

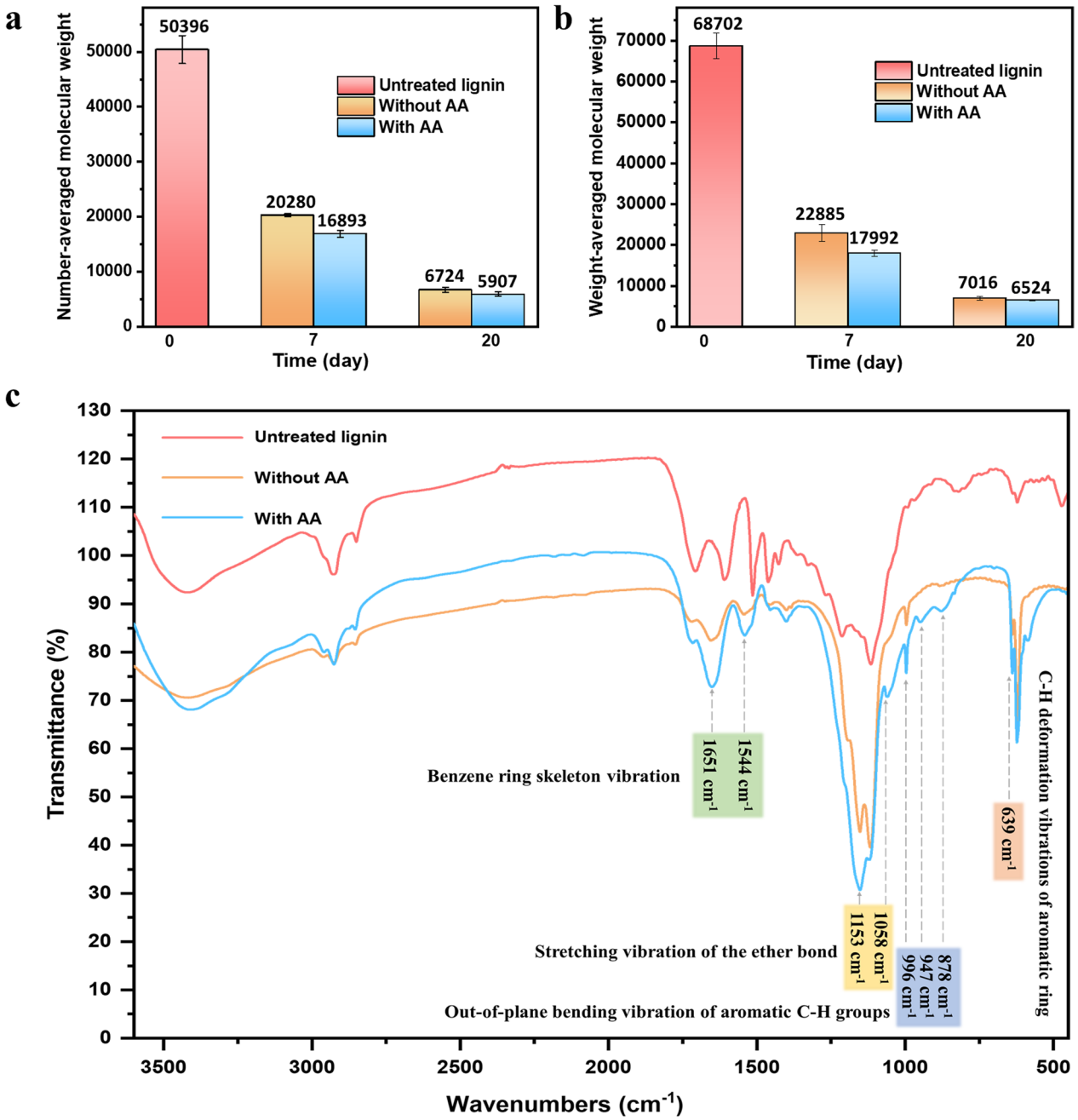
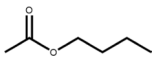
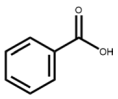
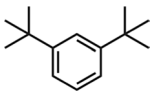
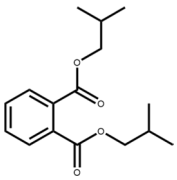
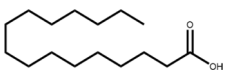
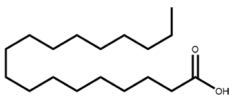
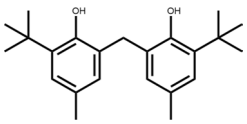
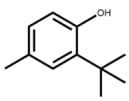
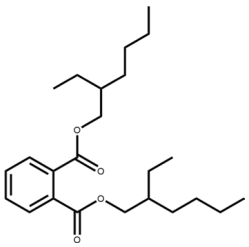


Fig. 3 Characterization of lignin in cultivation systems with or without ascorbic acid. **a** Number-averaged molecular weights; **b** Weight-averaged molecular weights; **c** FT-IR spectrometry. Error bars indicate standard deviation

observed after 20 days of treatment. The difference is that the Mn and Mw of lignin in the cultivation with AA were lower than those in control group. This result implied that the depolymerization of lignin occurred to a greater extent in cultivation with AA. It was further confirmed by the structure changes of lignin. As shown in Fig. 3c, more new or more changed peaks appeared in the FT-IR spectra of lignin degradation with AA, indicating the lignin in cultivation with AA exhibited more structure changes than the lignin in cultivation without AA. For example, the signals at 1058 cm^{-1}, 947 cm^{-1}, and 878 cm^{-1} were observed only in the lignin cultivated with AA, while the band at 1651 cm^{-1}, 1544 cm^{-1}, 1153 cm^{-1}, 996 cm^{-1}, and 639 cm^{-1} showed relatively higher intensity. These bands mainly originated from out-of-plane bending vibration of aromatic C-H groups (996 cm^{-1}, 947 cm^{-1}, and

878 cm^{-1}), C-H deformation vibrations of aromatic ring (639 cm^{-1}), benzene ring skeleton vibration (1651 cm^{-1} and 1544 cm^{-1}), and stretching vibration of the ether bond (1058 cm^{-1} and 1153 cm^{-1}). These results indicated that the lignin in cultivation with AA experienced more substitutions at aromatic ring and more alterations in the connection of the constituent units. Moreover, the increased activity of lignin-degrading enzymes also contributes to the degradation of lignin into smaller products. As shown in Table 1 and Fig. S1, the high molecular weight products such as $C_{16}H_{22}O_4$, $C_{23}H_{32}O_2$, and $C_{24}H_{38}O_4$ have a higher content in the cultivation without AA, while a higher content of the low molecular weight compounds including $C_6H_{12}O_2$, $C_7H_6O_2$, $C_{14}H_{22}$, and $C_{11}H_{16}O$ was detected in the cultivation with AA. These results demonstrated that AA-mediated increase in lignin-degrading enzyme activity not only enhances lignin degradation rate, but also encourages further formation of smaller molecules.

Table 1 Differential products of lignin degradation identified by GC–MS

Retention time (min)	Formula	Product name	Product structure	Relative amount[a]	
				Without AA	With AA
5.260	$C_6H_{12}O_2$	Butyl acetate		+	+ +
14.380	$C_7H_6O_2$	Benzoic acid		+	+ +
14.470	$C_{14}H_{22}$	1,3-Di-tert-butylbenzene		+	+ +
22.535	$C_{16}H_{22}O_4$	Diisobutyl phthalate		+ +	+
24.405	$C_{16}H_{32}O_2$	Palmitic acid		+	+ +
26.315	$C_{18}H_{36}O_2$	Stearic acid		+	+ +
27.940	$C_{23}H_{32}O_2$	6,6′-Methylenebis(2-(tert-butyl)-4-methylphenol)		+ +	+
28.155	$C_{11}H_{16}O$	2-(Tert-butyl)-4-methyl phenol		+	+ +
28.925	$C_{24}H_{38}O_4$	Bis(2-ethylhexyl) phthalate		+ +	+

[a] +, detected; + +, detected with a higher amount

As a selected model antioxidant, AA indeed enhanced the activity of lignin-degrading enzymes and the lignin degradation ability of *T. versicolor*. In addition to AA, it has been confirmed that a variety of enzymatic antioxidants, non-enzymatic antioxidants, and synthetic antioxidants play a critical role in the cellular metabolism [35, 36]. These antioxidants possess the ability to delay oxidation reaction, obstruct the development of free radicals, or break the generation of the autoxidation chain reaction [14, 37]. They also act as reducing agents and metal chelators, transforming hydroperoxides and metal prooxidants into stable forms [37]. The mentioned roles of antioxidants are closely linked to the production of enzymes, the provision of cofactors, the proper folding of proteins, and the secretion of enzymes. Naturally, these antioxidants might produce effects that are comparable to or even superior to those of AA. It is necessary to conduct more extensive screening for effective antioxidants.

Proposed mechanism for the enhanced activity of lignin-degrading enzymes

The addition of AA was a straightforward approach for enhancing the activity of lignin-degrading enzymes in *T. versicolor*; however, it simultaneously raises considerations regarding the economic feasibility and scalability in practical applications. The construction of microorganisms that can autonomously synthesize appropriate amount of AA, or the engineering of microorganisms based on the mechanism of AA enhancing the lignin-degrading enzyme activity, presents a viable alternative to the addition of exogenous AA.

To obtain an in-depth understanding of the molecular mechanisms underlying the AA-enhanced activity of lignin-degrading enzymes, label-free differential proteomics analysis was performed. Respectively, 1758 and 2201 proteins were identified in the cultures with AA and the cultures without AA, including 1676 that were common to both groups (Fig. 4a). Compared with the cultures without AA, a total of 261 differential proteins were identified, of which 179 proteins were significantly up-regulated (fold change > 1.5 and p-value < 0.05) and another 82 proteins were significantly down-regulated (fold change < 0.6 and p-value < 0.05), respectively (Fig. 4b). KEGG pathway enrichment analysis showed that the differential proteins mapped to metabolism were mainly enriched in the carbohydrate metabolism and amino acid metabolism (Fig. 4c). Meanwhile, several ribosomal proteins, protein translation factors, and amino acid-tRNA ligases were up-regulated (Fig. 5a and Fig. S2). Hence, it was originally speculated that the increase in Lac and MnP activities was mainly caused by the enhancement of their expression, benefiting from the improvement in carbohydrate metabolism and amino acid metabolism driven by AA addition. However, the expression level of both Lac and MnP did not show significant changes (Table S2). Obviously, the AA-enhanced activity of Lac and MnP was not achieved by simply or directly changing their intracellular expression levels.

Further analysis revealed that the impact of AA on cellular processes is mainly reflected in transport and catabolism, while the genetic information processing associated with the differential proteins mainly involved in the translation, folding, sorting and degradation (Fig. 4c). In particular, the differential proteins involved in the assistance of the newly synthesized polypeptide folding and prevention of the misfolded protein aggregation were found to be up-regulated in the presence of AA (Fig. 5a and Fig. S2). The up-regulation of these proteins was able to help more lignin-degrading enzymes to be able to form the correct structure faster. Particularly, the predicted structure of Lac and MnP from *T. versicolor* contains multiple disulfide bonds. The up-regulation of protein disulfide-isomerase (PDI) and peptidyl-prolyl *cis–trans* isomerase (PPI) could promote the correct folding of Lac and MnP because PDI plays pivotal roles in disulfide bond formation of proteins, while the efficiency of PDI is markedly improved by PPI [38, 39]. As shown in Fig. 5b, the contribution of AA to this positive effect was mediated by the oxidized AA form dehydroascorbic acid (DHA), which could act as an activator of PDI [40]. Furthermore, the up-regulation of hydroxyacylglutathione hydrolase and thioredoxin reductase, both of which are involved in the synthesis of glutathione (GSH), was also observed. One of the basic roles of GSH is to restore AA via AA-GSH cycle (Fig. 5b). It is noteworthy that DHA is also reduced back to AA in the process of PDI-mediated disulfide bond formation. AA regeneration through the above two pathways is also beneficial for ER to exert its protein synthesis and folding functions properly, because the antioxidant effect of AA could efficiently scavenges reactive oxygen species formed in oxidative protein folding maintaining the intralumenal redox homeostasis [17]. It is important for promoting the formation of active Lac and MnP.

The up-regulation of proteins associated with the protein transportation and the regulation of cell membrane was also identified (Fig. 5a and Fig. S2). It is generally recognized that the extracellular secretion capacity represents a major hurdle in microbial-driven lignin conversion. The up-regulation of proteins associated with protein transportation and cell membrane is bound to enhance the secretion of Lac and MnP, consequently resulting the promotion of the lignin-degrading ability of *T. versicolor*. In addition, the expression of coproporphyrinogen oxidase, which is engaged in the

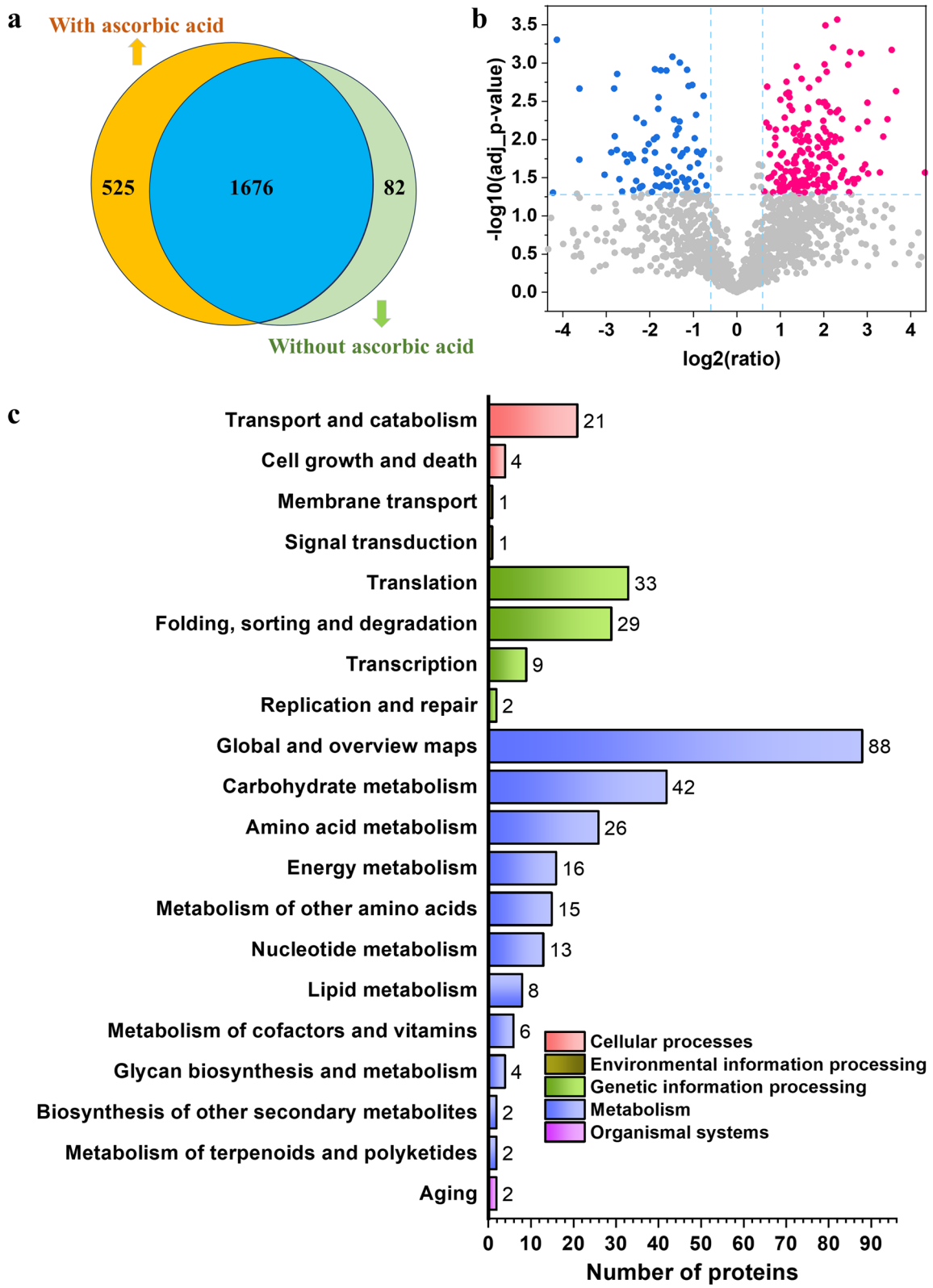


Fig. 4 Label-free differential proteomics analysis. **a** Overlap of the identified proteins between sample with AA and without AA; **b** Volcano plot of differential proteins; **c** Differential protein pathway classification

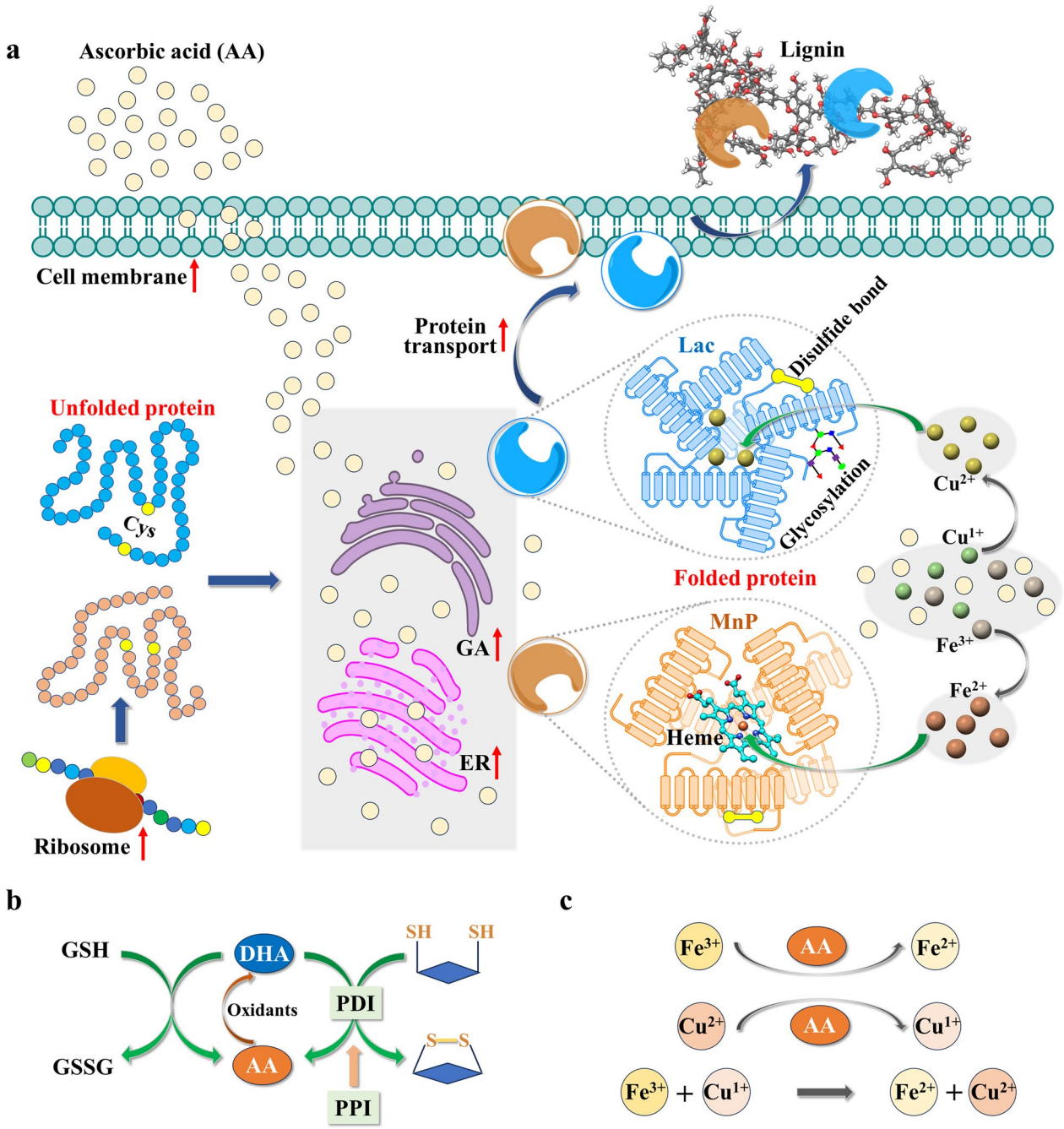


Fig. 5 Proposed mechanism for the reinforcement of AA on the activity of lignin-degrading enzymes. **a** Distribution of differential proteins; **b** PDI activation by DHA and disulfide bond formation; **c** AA-mediated alteration of the redox state of copper and iron. *ER* Endoplasmic reticulum, *GA* Golgi apparatus, *GSH* Glutathione, *GSSG* Oxidized glutathione, *DHA* Dehydroascorbic acid, *PDI* Protein disulfide-isomerase tigA, *PPI* Peptidyl-prolyl *cis–trans* isomerase. Red arrow, site with up-regulated protein

heme synthesis, was found to be up-regulated (Fig. 5a and Fig. S2). It is beneficial for the active structure formation of MnP as heme is an essential cofactor of MnP. Besides, as a reducing agent, AA is able to reduce catalytic metals such as Fe^{3+} and Cu^{2+} to Fe^{2+} and Cu^{1+} (Fig. 5c). Meanwhile, the reduced copper could transfer an electron to iron [41]. The alteration of the redox state of copper and iron by AA addition was also the positive factor for the active conformation formation of Lac and MnP. The reason is that Cu^{2+} is essential component of

active Lac, while Fe^{2+} is an important raw material for the synthesis of MnP cofactor heme. Moreover, glycosylation is one of the most important post-translational modifications and has a significant effect on the structure and functions of proteins [42]. It was found that the differential proteins associated with the protein glycosylation were up-regulated (Fig. 5a and Fig. S2). Several potential glycosylation sites were predicted in the Lacs of *T. versicolor*. Thus, it was speculated that the enhancement of Lac activity was partially benefits from the strengthening of glycosylation. The results of proteomics analysis provided information for us to find the mechanisms underlying the AA-enhanced activity of lignin-degrading enzymes. Taken together, the activity enhancement of lignin-degrading enzymes was more likely to benefit from the reinforcement of AA on the protein folding and transportation rather than change enzyme expression levels. The proper folding and effective secretion of lignin-degrading enzymes are critical determinants of the lignin degradation capacity of microorganisms [43–45]. Enhancing these processes is generally advantageous for improving microbial lignin degradation capability. Therefore, the enhancement of AA on the lignin degradation ability of *T. versicolor* did not appear to be specific, suggesting that this approach holds potential for application in other lignin-degrading microorganisms.

Conclusion

As the most abundant aromatic polymer found in nature, lignin represents a significant resource for the development of sustainable bioproducts. The valorization of lignin not only mitigates reliance on non-renewable fossil fuels but also enhances the economic viability of lignocellulosic biorefineries. Our findings suggest that the addition of AA enhanced the performance of enzymes responsible for lignin degradation in terms of enzyme activity, degradation rate, lignin structural change, and product mapping. Label-free differential proteomics analysis yielded crucial insights that can guide the development of novel strategies to further enhance the enzymatic breakdown of lignin by microorganisms. Overall, this study has provided a successful strategy and valuable starting point for improving the activity of lignin-degrading enzymes in fungus.

Abbreviations

AA	Ascorbic acid
ER	Endoplasmic reticulum
Lac	Laccase
MnP	Manganese peroxidase
PDA	Potato dextrose agar
Mw	Weight-averaged molecular weight
Mn	Number-averaged molecular weight
FT-IR	Fourier-transform infrared spectroscopy
GC–MS	Gas chromatography-mass spectrometry
GPC	Gel permeation chromatography
PDI	Protein disulfide-isomerase
PPI	Peptidyl-prolyl *cis–trans* isomerase
DHA	Dehydroascorbic acid
GSH	Glutathione

Supplementary Information

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Additional file 1.

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Author contributions

APL, WMW and SQG contributed equally to this work. APL: Conceptualization, Investigation, Writing–original draft, Writing–review & editing. WMW: Investigation, Visualization. SQG: Conceptualization, Writing–review & editing. CZL: Methodology, Writing–review & editing. XYW: Visualization. QF: Conceptualization, Funding acquisition, Writing–review & editing, Supervision.

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Availability of data and materials

Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

The authors declare no competing interests.

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References

1. Gao X, Ma R, Liu Z, Wang S, Wu Y, Song G. Hydrodeoxygenation of lignin-derived phenols into cycloalkanes by atomically dispersed Pt-polyoxometalate catalysts. Appl Catal B: Environ Energy. 2024;352: 124059.
2. Usmani Z, Sharma M, Diwan D, Tripathi M, Whale E, Jayakody LN, Moreau B, Thakur VK, Tuohy M, Gupta VK. Valorization of sugar beet pulp to value-added products: a review. Bioresour Technol. 2022;346: 126580.
3. Zhang B, Meng Q, Liu H, Han B. Catalytic conversion of lignin into valuable chemicals: Full utilization of aromatic nuclei and side chains. Acc Chem Res. 2023;56(24):3558–71.
4. Werner AZ, Eltis LD. Tandem chemocatalysis and biological funneling to valorize lignin. Trends Biotechnol. 2023;41(3):270–2.
5. Rath S, Pradhan D, Du H, Mohapatra S, Thatoi H. Transforming lignin into value-added products: perspectives on lignin chemistry, lignin-based

biocomposites, and pathways for augmenting ligninolytic enzyme production. Adv Compos Hybrid Ma. 2024;7:27.
6. Wang J, Li L, Xu H, Zhang Y, Liu Y, Zhang F, Shen G, Yan L, Wang W, Tang H, et al. Construction of a fungal consortium for effective degradation of rice straw lignin and potential application in bio-pulping. Bioresour Technol. 2022;344(Pt B): 126168.
7. Cui T, Yuan B, Guo H, Tian H, Wang W, Ma Y, Li C, Fei Q. Enhanced lignin biodegradation by consortium of white rot fungi: microbial synergistic effects and product mapping. Biotechnol Biofuels. 2021;14(1):162.
8. Wang L, Luo X, Pan Y, Zheng Z, Yin R, Tian X, Zhang L. Mechanism of laccase induction *via* emodin in *Trametes versicolor*. Front Bioeng Biotechnol. 2021;9: 653800.
9. Sies H, Mailloux RJ, Jakob U. Fundamentals of redox regulation in biology. Nat Rev Mol Cell Bio. 2024. https://doi.org/10.1038/s41580-024-00730-2.
10. Wang L, Wang C. Oxidative protein folding fidelity and redoxtasis in the endoplasmic reticulum. Trends Biochem Sci. 2023;48(1):40–52.
11. Singh AK, Iqbal HMN, Cardullo N, Muccilli V, Fernandez-Lucas J, Schmidt JE, Jesionowski T, Bilal M. Structural insights, biocatalytic characteristics, and application prospects of lignin-modifying enzymes for sustainable biotechnology. Int J Biol Macromol. 2023;242(Pt 3): 124968.
12. Paul M, Pandey NK, Banerjee A, Shroti GK, Tomer P, Gazara RK, Thatoi H, Bhaskar T, Hazra S, Ghosh D. An insight into omics analysis and metabolic pathway engineering of lignin-degrading enzymes for enhanced lignin valorization. Bioresour Technol. 2023;379: 129045.
13. Liu N, Wang R, Gao S, Zhang R, Fan F, Ma Y, Luo X, Ding D, Wu W. High-performance piezo-electrocatalytic sensing of ascorbic acid with nanostructured wurtzite zinc oxide. Adv Mater. 2021;33(51): e2105697.
14. Ali SS, Ahsan H, Zia MK, Siddiqui T, Khan FH. Understanding oxidants and antioxidants: classical team with new players. J Food Biochem. 2020;44(3): e13145.
15. Pozzer D, Invernizzi RW, Blaauw B, Cantoni O, Zito E. Ascorbic acid route to the endoplasmic reticulum: function and role in disease. Antioxid Redox Sign. 2021;34(11):845–55.
16. Szarka A, Lorincz T. The role of ascorbate in protein folding. Protoplasma. 2014;251(3):489–97.
17. Mandl J, Szarka A, Banhegyi G. Vitamin C: update on physiology and pharmacology. Br J Pharmacol. 2009;157(7):1097–110.
18. Verma AK, Chettri D, Verma AK, Selvaraj M, Assiri MA. Bioprospecting lignin for biorefinery: emerging innovations and strategies in microbial technology. Biomass Bioenerg. 2024;181: 107052.
19. Ladeira Ázar RIS, Morgan T, dos Santos ACF, de Aquino XE, Ladisch MR, Guimarães VM. Deactivation and activation of lignocellulose degrading enzymes in the presence of laccase. Enzyme Microb Technol. 2018;109:25–30.
20. Bermek H, Yazıcı H, Öztürk H, Tamerler C, Jung H, Li K, Brown KM, Ding H, Xu F. Purification and characterization of manganese peroxidase from wood-degrading fungus *Trichophyton rubrum* LSK-27. Enzyme Microb Technol. 2004;35(1):87–92.
21. Ramirez DA, Munoz SV, Atehortua L, Michel FC Jr. Effects of different wavelengths of light on lignin peroxidase production by the white-rot fungi *Phanerochaete chrysosporium* grown in submerged cultures. Bioresour Technol. 2010;101(23):9213–20.
22. Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker D. Determination of structural carbohydrates and lignin in biomass. NREL/TP-510–42618. 2012.
23. Raj A, Krishna Reddy MM, Chandra R. Identification of low molecular weight aromatic compounds by gas chromatography–mass spectrometry (GC–MS) from kraft lignin degradation by three *Bacillus* sp. Int Biodeter Biodegr. 2007;59(4):292–6.
24. Zhu D, Zhang P, Xie C, Zhang W, Sun J, Qian W-J, Yang B. Biodegradation of alkaline lignin by Bacillus ligniniphilus L1. Biotechnol Biofuels. 2017;10(1):44.
25. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nature Biotechnol. 2008;26:1367–72.
26. Zheng MZ, Chen WX, Zhao YX, Fang Q, Wang LG, Tian SY, Shi YG, Chen JS. Ascorbic acid potentiates photodynamic inactivation mediated by octyl gallate and blue light for rapid eradication of planktonic bacteria and biofilms. Food Chem. 2024;448: 139073.
27. Przekwas J, Wiktorczyk N, Budzynska A, Walecka-Zacharska E, Gospodarek-Komkowska E. Ascorbic acid changes growth of food-borne pathogens in the early stage of biofilm formation. Microorganisms. 2020;8(4):553.
28. Cock IE, Rayan P. Ascorbic acid potentiates the Giardia duodenalis growth inhibitory activity of pure *Terminalia ferdinandiana* Exell compounds. Parasitol Res. 2020;119(3):1125–37.
29. Hou X, Shen W, Huang X, Ai Z, Zhang L. Ascorbic acid enhanced activation of oxygen by ferrous iron: a case of aerobic degradation of rhodamine B. J Hazard Mater. 2016;308:67–74.
30. Zhou S, Yu Y, Zhang W, Meng X, Luo J, Deng L, Shi Z, Crittenden J. Oxidation of microcystin-LR via activation of peroxymonosulfate using ascorbic acid: kinetic modeling and toxicity assessment. Environ Sci Technol. 2018;52(7):4305–12.
31. Chen H, Gao J, Wang Q, Liu Y, Fu X, Guo Y, Wang H, Wang Y, An J. Natural pyrite and ascorbic acid co-enhance periodate activation for inactivation of antibiotic resistant bacteria and inhibition of resistance genes transmission: A green disinfection process dominated by singlet oxygen. J Hazard Mater. 2024;477: 135217.
32. Droge W. Free radicals in the physiological control of cell function. Physiol Rev. 2002;82:47–95.
33. Di Meo S, Venditti P. Evolution of the knowledge of free radicals and other oxidants. Oxid Med Cell Longev. 2020;2020:1–32.
34. Wang G, Haringa C, Noorman H, Chu J, Zhuang Y. Developing a computational framework to advance bioprocess scale-up. Trends Biotechnol. 2020;38(8):846–56.
35. Bensid A, El Abed N, Houicher A, Regenstein JM, Özogul F. Antioxidant and antimicrobial preservatives: properties, mechanism of action and applications in food – a review. Crit Rev Food Sci. 2020;62(11):2985–3001.
36. Huchzermeyer B, Menghani E, Khardia P, Shilu A. Metabolic pathway of natural antioxidants, antioxidant enzymes and ros providence. Antioxidants. 2022;11(4):761.
37. Chandra P, Sharma RK, Arora DS. Antioxidant compounds from microbial sources: a review. Food Res Int. 2020;129: 108849.
38. Okumura M, Kadokura H, Inaba K. Structures and functions of protein disulfide isomerase family members involved in proteostasis in the endoplasmic reticulum. Free Radic Biol Med. 2015;83:314–22.
39. Schönbrunner ER, Schmid FX. Peptidyl-prolyl *cis-trans* isomerase improves the efficiency of protein disulfide isomerase as a catalyst of protein folding. P Natl A Sci. 1992;89(10):4510–3.
40. Arrigoni O, Tullio MCD. Ascorbic acid: much more than just an antioxidant. Biochim Biophys Acta. 2002;1569:1–9.
41. Buettner GR, Jurkiewicz BA. Catalytic metals, ascorbate and free radicals: combinations to avoid. Radiat Res. 1996;145(5):532–41.
42. Ma B, Wang R, Chen B, Liu W, Zhou S, Li X, Gong J, Shang S, Li Y, Xu D, et al. Insights into the effect of protein glycosylation on carbohydrate substrate binding. Int J Biol Macromol. 2023;235: 123833.
43. Zhao ZM, Liu ZH, Zhang T, Meng R, Gong Z, Li Y, Hu J, Ragauskas AJ, Li BZ, Yuan YJ. Unleashing the capacity of *Rhodococcus* for converting lignin into lipids. Biotechnol Adv. 2024;70: 108274.
44. Singhania RR, Patel AK, Raj T, Chen CW, Ponnusamy VK, Tahir N, Kim SH, Dong C-D. Lignin valorisation via enzymes: a sustainable approach. Fuel. 2022;311: 122608.
45. Kurebayashi K, Nakazawa T, Shivani HY, Kawauchi M, Sakamoto M, Honda Y. Visualizing organelles with recombinant fluorescent proteins in the white-rot fungus Pleurotus ostreatus. Fungal Biol-UK. 2023;127(10–11):1336–44.

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