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Soluble Lignin as a Multifunctional Enhancer in Microbial Fermentation

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ABSTRACT: Transforming lignocellulose into high-value biochemicals via microbial fermentation is critical for valorizing agricultural/forestry residues and advancing sustainable biomanufacturing. However, fermentation efficiency is hindered by environmental stressors. Herein, soluble lignin was prepared by alkaline dissolution and dialysis of enzymatic hydrolysis lignin and its effects on the above three fermentations were systematically investigated. Results showed that soluble lignin buffered fermentation pH via carboxyl protonation, scavenged ROS through phenolic hydroxyl groups to alleviate microbial inactivation, and chelated heavy metals (e.g., Pb^{2+}, Cd^{2+}) via phenolic hydroxyl/carboxyl coordination to reduce toxicity. Notably, it exhibited pH-responsive recyclability: dissolving at neutral pH (initial fermentation) and precipitating at acidic pH (fermentation end), enabling centrifugal recovery and reuse for ≥10 cycles with retained performance. This study demonstrates that soluble lignin universally enhances multiple microbial fermentations via physicochemical synergies, providing a theoretical basis for optimizing lignocellulose-based fermentation and new avenues for high-value lignin utilization in biomanufacturing.

KEYWORDS: *soluble lignin, microbial fermentation, multifunctional enhancer, biochemical production, pH-responsive recyclability*

1. INTRODUCTION

Plant biomass, such as crop straw, wood, and grass, is the most abundant renewable resource on Earth, widely distributed in many regions of the world.[1] Through chemical or enzymatic fractionation, the primary components of plant biomass—cellulose and hemicellulose—can be efficiently converted into fermentable sugars, which serve as substrates for microorganisms to produce a wide range of high-value biochemicals, including biofuels (e.g., ethanol), food additives (e.g., lactic acid), and essential amino acids (e.g., glutamic acid).[2−5] This bioconversion process not only enables the efficient utilization of agricultural and forestry residues but also promotes sustainable biomanufacturing, reducing reliance on fossil resources and supporting global circular economy goals.[6−8] However, microbial fermentation efficiency in lignocellulose-based processes is frequently constrained by multiple environmental stressors, which vary slightly across different fermentation systems but share core challenges.[9−11]

Fluctuations in pH, driven by organic acid accumulation or substrate metabolism, disrupt enzyme function and membrane integrity—critical for microorganisms like *Saccharomyces cerevisiae*, *Lactic acid bacteria*, and *Corynebacterium glutamicum*, each with distinct pH optima.[12] Conventional pH adjustment methods include exogenous acid−base titration.[13] However, chemical buffers can modify ionic strength and are susceptible to introducing exogenous microorganisms. Innovative strategies encompass the incorporation of buffer solutions or the utilization of pH-responsive polymers to dynamically sustain optimal pH levels without chemical intervention.[14] Additionally, reactive oxygen species (ROS) generated during metabolism induce oxidative damage, while heavy metal ions (e.g., Pb^{2+}, Cd^{2+}) from biomass feedstocks further inhibit microbial growth.[15,16] Compounding these issues, end-product inhibition—where ethanol, lactic acid, or glutamic acid accumulates to toxic levels—limits fermentation yields and scalability. Researchers have improved the fermentation efficiency of yeast by culturing heat-resistant strains or using nano material-based scavengers.[17,18] While these studies have partially alleviated the negative effects on efficiency caused by fermentation system fluctuations, existing solutions exhibit notable limitations, such as thermotolerant strains necessitating extended production cycles, and nanomaterial additives can elevate downstream separation costs.[19,20] Consequently, the requirement for multifunctional additives capable of concurrently addressing multiple stressors through synergistic mechanisms remains to be resolved.

Lignin, a major constituent of lignocellulose, has long been regarded as a fermentation inhibitor due to its recalcitrant structure[21−23] and degradation-derived phenolic compounds, which disrupt microbial membranes.[24,25] As a result, extra efforts were made to isolate/remove lignin in existing practices during plant biomass bioconversion, and the residue lignin from enzymatic processing was treated as a waste, resulting in underutilization.[26,27] Here, we propose to repurpose lignin as a fermentation aid to significantly improve the fermentation

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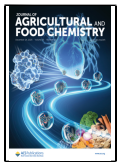

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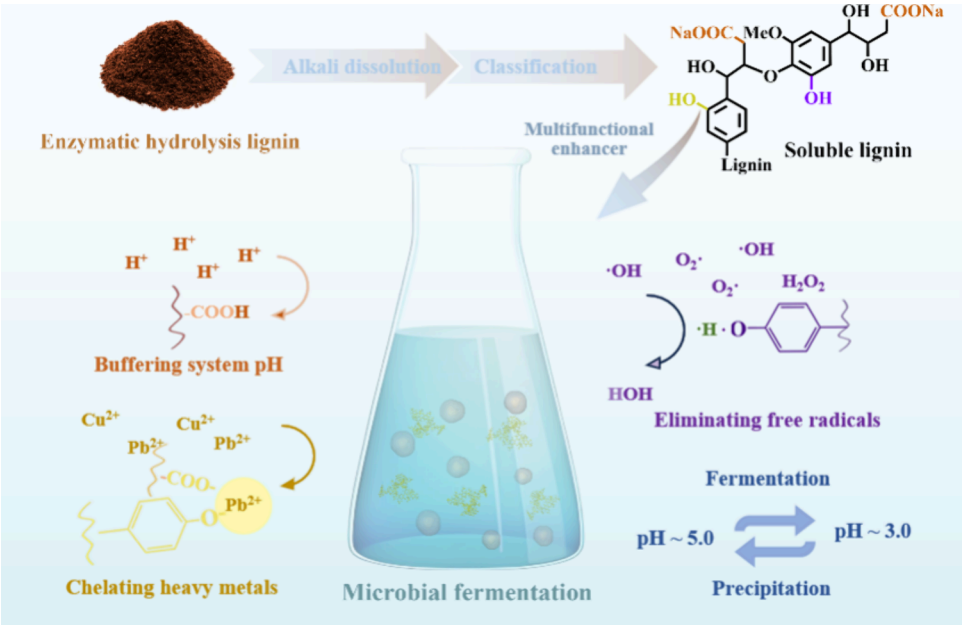

Figure 1. Schematic diagram of soluble lignin as a microbial fermentation enhancer.

processes as well as lignin utilization. There is insufficient evidence to confirm the inhibitory effect of insoluble lignin on microorganism performance, primarily due to its limited interaction with microorganisms. Although the monophenols produced by lignin degradation can impair microorganism activity by altering cell membrane permeability and subsequently affecting intracellular enzyme activity, high molecular weight lignin exhibits poor cellular transportability. Furthermore, lignin with enhanced hydrophilicity struggles to integrate into the phospholipid bilayer of the cell membrane.[28] Unfortunately, systematic research on this subject remains limited. Recent studies have demonstrated that lignin can positively affect the fermentation environment for microorganisms and enhance yield. For instance, amphiphilic lignin molecules obtained through moderate modification can form nanomicelle structures that exhibit substantial encapsulation effects on hydrophobic inhibitors.[29,30] The phenolic hydroxyl groups on the aromatic ring provide lignin with exceptional free radical scavenging and antioxidant capabilities, surpassing conventional antioxidants.[29,31] Moreover, the weak acidic groups within its molecular structure enable ionized lignin to act as a pH buffer.[32] These findings suggest that lignin with an appropriate molecular weight and functional group configuration can serve as an enhancer to promote microbial fermentation.

In this study, high molecular weight soluble lignin obtained by dialysis of predissolved enzymatic hydrolysis residue lignin was used to investigate the multifunctional role of lignin across three major microbial fermentation systems (ethanol, lactic acid, glutamic acid production) (Figure 1). By evaluating its impact on pH stability, oxidative stress reduction, metal detoxification, and product inhibition, we aim to validate soluble lignin as a universal enhancer. Furthermore, we explore its pH-responsive recyclability—dissolution at neutral pH (fermentation initiation) and precipitation at acidic pH (fermentation termination)—to enable cost-effective reuse. This work aims to systematically demonstrate that soluble lignin can universally improve microbial fermentation performance through physicochemical synergies, providing a novel approach to optimize lignocellulose bioconversion and advance high-value utilization of lignin in biomanufacturing.

2. MATERIALS AND METHODS

2.1. Materials. Anhydrous glucose (≥99.0%, AR), NH_4Cl (≥99.5%, AR), KH_2PO_4 (≥99.0%, AR), $MgSO_4·7H_2O$ (≥99.0%, AR), $MnSO_4·4H_2O$ (≥98.0%, AR), NaOH (≥98.0%, GR), $FeSO_4·7H_2O$ (≥99.0%, AR), $(NH_4)_2SO_4$ (≥99.0%, AR), anhydrous ethanol, 1,1-diphenyl-2-picrylhydrazide (DPPH, HPLC grade), MRS medium (premixed powder), beef extract (biological reagent, BR), peptone (≥98.0%, BR), and yeast extract (≥98.0%, BR) were procured from Shanghai Macklin Biochemical Technology Co., Ltd. (Shanghai, China). Enzymatic hydrolysis residue lignin was obtained from Shandong Longli Biotechnology Co., Ltd. (Dezhou, China). The *Saccharomyces cerevisiae* was sourced from Angel Yeast Co., Ltd. (Yichang, China). *Lactic acid bacteria* and *Corynebacterium glutamicum* were purchased from the Shanghai Preservation Microbial Center (Shanghai, China).

2.2. Bioethanol Fermentation. To prepare the YPD culture medium, 50 mL of deionized water, 1 g of anhydrous glucose, 1 g of peptone, and 0.5 g of yeast extract were combined in a 150 mL conical flask and sterilized at 121 °C for 20 min. Subsequently, 3.3 g of yeast was weighed and added to a conical flask, where it was activated at 34 °C and 150 rpm for 60 min. For the fermentation system, 50 mL of deionized water, 100 g/L glucose, 1 g/L NH_4Cl, 1 g/L KH_2PO_4, and 0.3 g/L $MgSO_4·7H_2O$ were added to a 150 mL conical flask and sterilized at 121 °C for 20 min. Finally, 2.5 mL of activated yeast was introduced into the fermentation system for fermentation. No buffer solution was added during fermentation. Samples were collected at 4 h intervals over the total 24-h fermentation period. The fermentation broth was centrifuged, and the supernatant was analyzed for glucose and ethanol concentrations using a biosensing analyzer (SBA-40ES, Jinan Yanhe Biotechnology Co., Ltd.). All experiments were conducted in triplicate, and the average values were recorded.

2.3. Lactic Acid and Glutamic Acid Fermentation. *Lactic acid bacteria* was inoculated in MRS medium (containing glucose 20 g/L, peptone 10 g/L, beef extract 10 g/L, yeast extract 5 g/L, diammonium citrate 2 g/L, sodium acetate 5 g/L, $MgSO_4·7H_2O$ 0.1 g/L, $MnSO_4·4H_2O$ 0.05 g/L, pH 6.3), and incubated at 37 °C for 24 h to prepare the seed culture. The seed culture was transferred to fermentation medium (glucose 100 g/L, yeast extract 5 g/L, $(NH_4)_2HPO_4$ 2 g/L, KH_2PO_4, $MgSO_4·7H_2O$ 0.2 g/L) at 5% (v/v)

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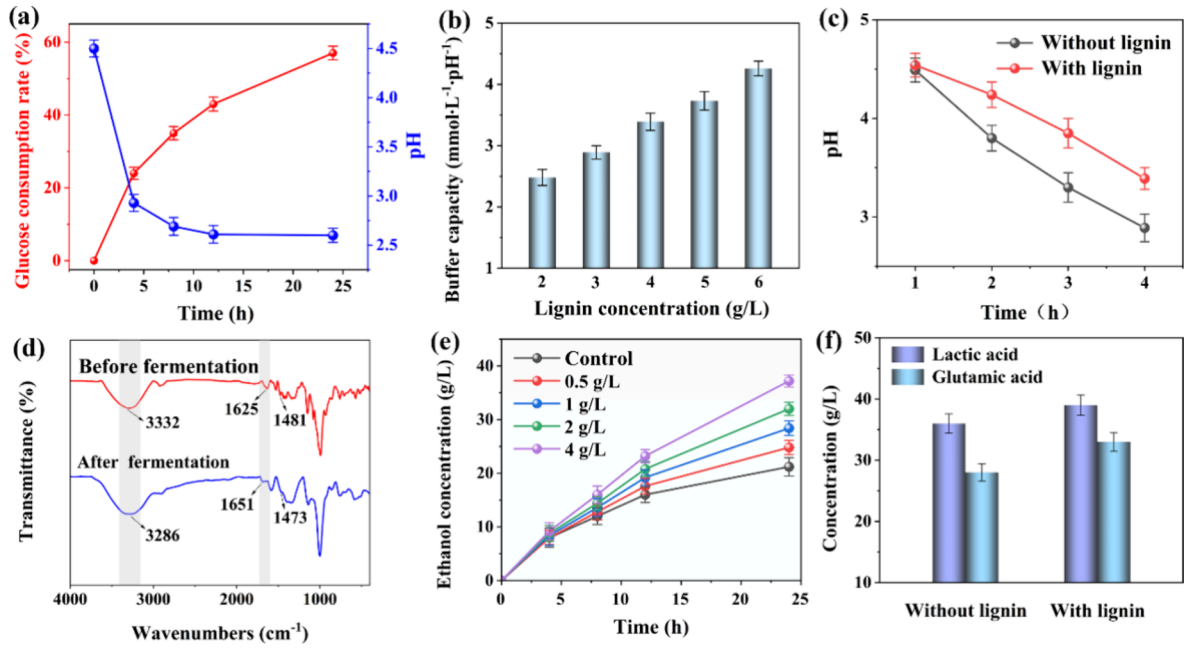

Figure 2. (a) Variations in glucose consumption rate and pH value of the system as a function of fermentation time. (b) Buffer capacity of the system under varying lignin concentrations. (c) Variation of pH with time in the early stage of the fermentation system. (d) Infrared spectra of lignin before and after fermentation. (e) Ethanol concentration variation with fermentation time under different lignin concentrations in the system. (f) Comparison of lactic acid and glutamic acid concentrations before and after adding lignin.

inoculation rate. The fermentation system was then purged with N_2 to maintain microaerobic conditions (dissolved oxygen <5%) and incubated at 36 °C for 48 h with constant stirring at 100 rpm (Constant temperature shaker, IKAKS400, IKA Works Guangzhou).

Corynebacterium glutamicum was inoculated in seed culture medium (containing glucose 20 g/L, corn steep liquor 20 g/L, $(NH_4)_2SO_4$ 5 g/L, KH_2PO_4 1 g/L, $MgSO_4{\cdot}7H_2O$ 0.5 g/L, pH 7.0), and incubated at 32 °C with agitation at 200 rpm for 16 h. The seed culture was transferred to the production medium (glucose 100 g/L, $(NH_4)_2SO_4$ 30 g/L, corn steep liquor 5 g/L, biotin concentration 1.0 μg/L, KH_2PO_4 1.5 g/L, $MgSO_4{\cdot}7H_2O$ 0.6 g/L, $FeSO_4{\cdot}7H_2O$ 0.01 g/L, $MnSO_4{\cdot}4H_2O$ 0.01 g/L) at 5% (v/v) inoculation rate. The initial pH was adjusted to 7.0 with ammonia−water, and the fermentation was conducted at 32 °Cfor 48 h. During fermentation, the stirring speed was maintained between 600 rpm, and oxygen was supplied to maintain dissolved oxygen at 30% saturation. The fermentation broth was centrifuged, and the supernatant was analyzed for Lactic acid and glutamic acid concentrations using a biosensing analyzer (SBA-40ES, Jinan Yanhe Biotechnology Co., Ltd.). The analysis of fermentation results is performed by comparing the diluted fermentation broth with standard samples, based on the core principle that the detection value (current signal) of the instrument is proportional to the concentration of the target substance. All experiments were conducted in triplicate, and the average values were recorded.

2.4. Treatment and Characteristics of Lignin. The lignin used in this study is enzymatic hydrolysis lignin (EHL) derived from corn cobs (a representative graminaceous biomass), which is classified as grass lignin predominantly composed of H-type structural units. Its preparation followed a three-step protocol: corn cobs were first pretreated and enzymatically hydrolyzed to collect insoluble residues, which were then extracted and acidified to obtain crude EHL. 16g of EHL and 184 mL of ultrapure water were added to beakers, adjusted to pH 12 with NaOH, and thoroughly stirred. Ultrasonic treatment for 1 h (power 300W, frequency 40 kHz), then filtered through a 0.45 μm membrane. The filtrate was loaded into a 500 Da dialysis bag and dialyzed in ultrapure water for 48 h at room temperature, with the external solution replaced every 6 h. Postdialysis, the solution was subjected to rotary evaporation at 60 °C to yield black soluble lignin powder. The lignin utilized in subsequent experiments refers to this soluble lignin. The number-averaged molecular weight and weight-average molecular weight of lignin, as determined by gel permeation chromatography (Shodex, OHpak SB-803), were 1729 and 3598 Da, respectively. The carboxyl group content measured by an established test method was found to be 2.01 mmol/g.[33] The phenolic hydroxyl content of lignin was quantified to be 2.68 mmol/g using the Lowry method.[34]

2.5. Determination of Buffer Capacity. Various quantities of lignin were added to a 1 L fermentation system to achieve concentrations of 2, 3, 4, 5, and 6 g/L, respectively. The corresponding pH values were recorded as pH_1. A 0.2 mol/L hydrochloric acid solution was gradually added dropwise while stirring until the pH value reached 2.5. The volume of the solution added was recorded as V. The calculation formula for buffer capacity β is

$$\beta = \Delta n/\Delta \mathrm{pH} = 0.1 \times V/(\mathrm{pH}_1 - 2.5)$$

2.6. Testing of Yeast Cell Survival Rate. 0.5 mL of yeast fermentation broth and 0.5 mL of 0.1% alkaline methylene blue staining solution were combined in a centrifuge tube and incubated for 5 min at 34 °C. Subsequently, a small volume of the mixed solution was transferred to a cell-counting chamber and observed microscopically. The viability of yeast cells was determined based on color differentiation between live and dead cells, followed by a calculation of the survival rate μ:

$$\mu = \text{Number of living cells/Total Cells} \times 100\%$$

2.7. Free Radical Scavenging Experiment. DPPH radicals were employed as a stable model to simulate biological free radicals. A 0.1 mmol/L DPPH solution was prepared using anhydrous ethanol. For the blank group, 1 mL of anhydrous ethanol and 1 mL of DPPH solution were added. For the test group, 1 mL of lignin solution at varying concentrations and 1 mL of DPPH solution were combined. The reaction mixtures were gently agitated and incubated at room temperature for 30 min to ensure a complete reaction with DPPH radicals. DPPH radicals exhibit a deep purple hue in organic solvents and possess a characteristic absorption peak at 517 nm. The presence of a free radical scavenger leads to a reaction with DPPH radicals, neutralizing their unpaired electrons and resulting in a lighter coloration, thereby reducing the absorbance at 517 nm. It is

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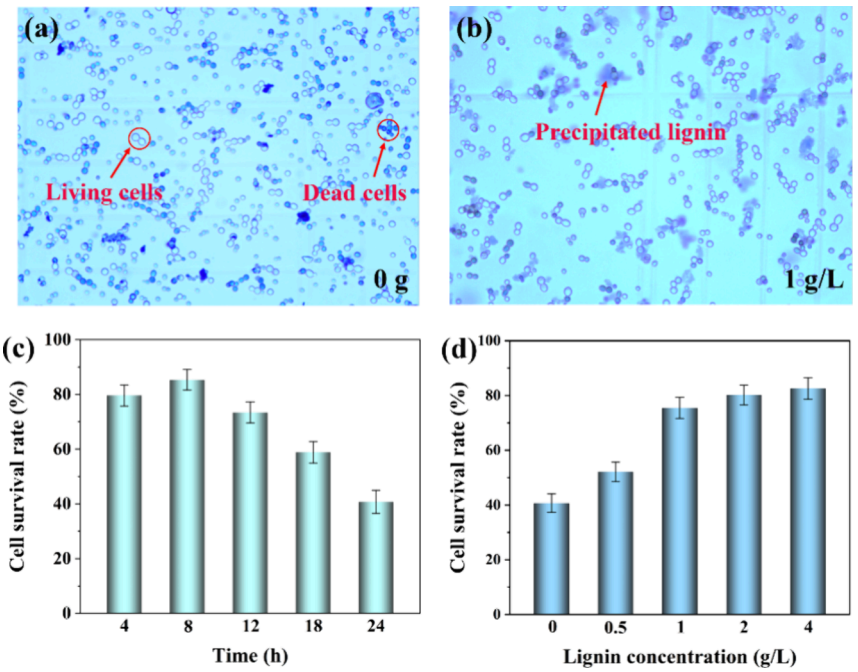

Figure 3. (a, b) Cell images after fermentation with 0 and 1 g/L lignin for 24 h. (c) Survival rate of cells at different time points. (d) Survival rate of cells after 24 h of fermentation with different concentrations of lignin added.

imperative to note that when utilizing a UV−visible spectrophotometer (Shimadzu Corporation, UV-2600i), the intrinsic absorbance of lignin must be accounted for. The absorbance of the blank group is denoted as A_0, and the absorbance of the test group is denoted as A_1. The formula for calculating the scavenging rate Δ is:

$$\Delta = \frac{(A_0 - A_1)}{A_0} \times 100\%$$

To directly ascertain the reduction of intracellular ROS, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), a cell-permeable probe, was employed. This probe is hydrolyzed by yeast intracellular esterases into ROS-oxidizable 2′,7′-dichlorodihydrofluorescein (DCFH), wherein the fluorescence intensity of DCF is positively correlated with intracellular ROS levels. The assay was conducted within a 40 °C high-temperature stress model, wherein two experimental groups were established: a control group, wherein yeast fermentation was performed at 40 °C in the absence of soluble lignin, and a lignin group, wherein yeast fermentation was executed at 40 °C in the presence of 2 g/L soluble lignin. At the 24-h mark of fermentation, yeast cells were harvested, rinsed, and subsequently loaded with 10 μmol/L DCFH-DA. These cells were then incubated in the dark at 37 °C for 30 min before being analyzed using laser confocal microscopy (ZEISS, LSM 900).

2.8. Heavy Metal Ion Complexation Experiment. Introduce heavy metal ions into the fermentation system before the fermentation process, incorporating 0 and 2 g/L of soluble lignin, respectively. Following a 24-h fermentation period, centrifuge the fermentation broth and collect the supernatant for analysis.

2.9. Experiment on the Recycling of Soluble Lignin. The system containing soluble lignin demonstrates pronounced stratification postfermentation. Upon centrifugation of the fermentation broth, solid material is observed in the lower layer of the test tube. After sterilizing the solid material, it can be reintegrated into the fermentation system for cyclic experimentation.

3. RESULTS AND DISCUSSION

3.1. Buffering Capacity of Soluble Lignin. The stability of the pH value exerts a decisive influence on the fermentation efficiency within the complex process of yeast fermentation for ethanol production. As fermentation progresses, yeast cells metabolize sugars into pyruvic acid via the glycolysis pathway, which subsequently undergoes further metabolic transformations to yield ethanol and carbon dioxide. Throughout this process, organic acids-including acetic acid and lactic acid-accumulate as byproducts within the fermentation system, resulting in a gradual decline in the pH of the system. As depicted in Figure 2a, during the initial fermentation phase, the pH value of the system is typically maintained between 4.3 and 4.5. However, the pH of the system decreased rapidly within 4 h after the start of fermentation. After 24 h of fermentation, the pH value will decrease to 2.3−2.5, indicating that the pH value will decrease by about 2 units. This abrupt pH decline can impede the activity of various critical enzymes within yeast cells, thereby diminishing the efficiency of sugar uptake and utilization by yeast. Moreover, unstable pH conditions may foster the proliferation of acidophilic microorganisms, which compete with yeast for nutrients and further hinder the ethanol fermentation process.

Lignin molecules possess weakly acidic functional groups, such as phenolic hydroxyl and carboxyl groups, which impart a natural buffering capacity to lignin. As illustrated in Figure 2b, with the gradual increase of lignin content, the buffering capacity of the system also increases accordingly. When the pH of the fermentation system decreases, the ionized carboxyl groups in lignin molecules can accept protons, thereby mitigating the rapid decline in pH. Notably, during the initial phases of fermentation, lignin exerts the most pronounced buffering effect against the rapid decline in pH induced by the accumulation of organic acid byproducts (Figure 2c). To elucidate the structural basis of this buffering capacity, the FTIR spectral analysis in Figure 2d elucidates the structural differences of soluble lignin before and after fermentation. Both before and after fermentation, lignin exhibited distinct absorption peaks at 1481 and 1473 cm^{-1}, corresponding to the characteristic C═C skeletal stretching vibrations in lignin.[29] Postfermentation, the maximum absorption peak associated

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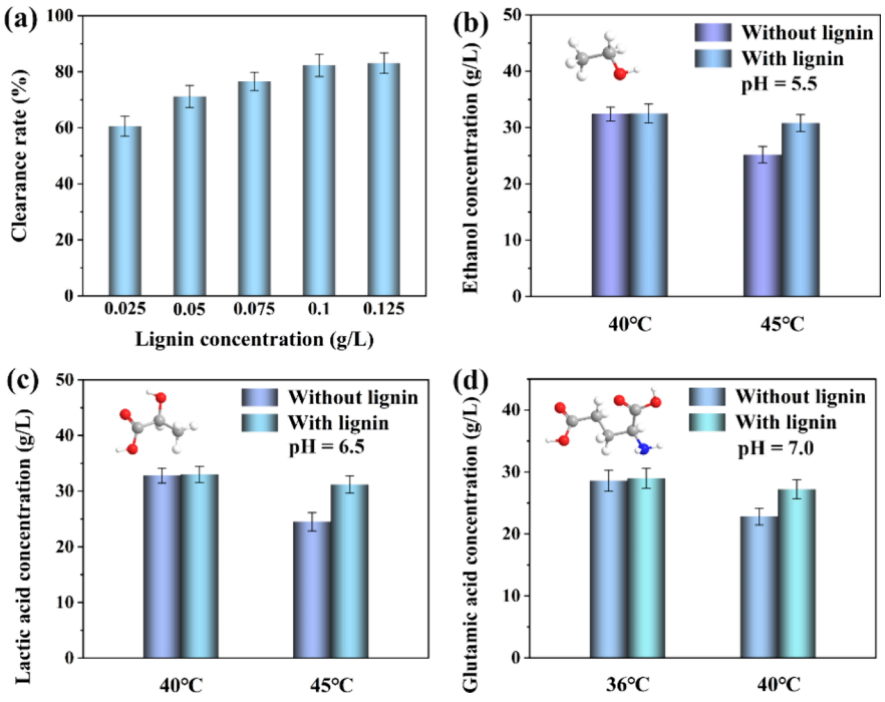

Figure 4. (a) Impact of lignin at varying concentrations on free radical scavenging activity. (b–d) Comparison of ethanol, lactic acid, and glutamic acid products before and after adding lignin to a buffer system at high temperatures.

with lignin hydroxyl O–H stretching vibrations shifted from 3332 to 3286 cm^{-1}. This shift resulted from the protonation of the phenolic and carboxyl groups of lignin during fermentation, leading to increased conjugation of hydroxyl groups and a corresponding red shift in the absorption peak. Concurrently, the protonation of carboxyl groups postfermentation caused the COO^- antisymmetric stretching vibration to transition from 1625 to 1651 cm^{-1}, indicative of C=O stretching.[27] Collectively, these spectral changes confirm that the fermentation process indeed induces protonation of lignin carboxyl groups, and may also involve protonation of certain phenolic hydroxyl groups. This buffering mechanism mediated by high molecular weight soluble lignin differs from the inhibitory effects of lignin fragments reported by Gu et al.,[35] who found that such fragments disrupt yeast membranes rather than stabilizing the fermentation pH. Additionally, elemental analysis demonstrates that the nitrogen (N) content of the soluble lignin is substantially lower than the N content supplemented before fermentation (Table S2), thereby ruling out the potential influence of N from soluble lignin on the fermentation-enhancing effect. Consistently, the fermentation outcomes of soluble lignin derived from three distinct raw materials indicate that the capacity of lignin to enhance fermentation is broadly efficacious across diverse biomass types (Figure S1). With respect to fermentation performance, Figure 2e shows that while lignin cannot entirely prevent the deceleration of the fermentation rate caused by the decline in the pH of the system, the ethanol concentration continues to rise with increasing lignin content at a given fermentation time. Beyond ethanol fermentation, the addition of lignin during the fermentation of lactic acid and glutamic acid was beneficial to the maintenance of microbial activity. As shown in Figure 2f, after adding 2 g/L lignin for 48h, even if a large amount of H^+ is accumulated in the fermentation system, the concentration of lactic acid and glutamic acid in the product will still increase.

Throughout the fermentation process, microorganisms demonstrated distinct growth patterns under different pH conditions. Taking yeast cells as an example, in the initial fermentation phase, when the pH of the system was maintained within the range of 4.3–4.5, yeast cell growth was optimal, with cell concentration increasing rapidly as fermentation progressed. As depicted in Figure 3c, after approximately 8 h of fermentation, the cell viability began to decline. Concurrently, the continuous production of organic acids led to a rapid decrease in the pH of the system, which in turn reduced cell viability. This phenomenon is primarily attributed to the fact that lower pH levels can impair the structural and functional integrity of yeast cell membranes, altering membrane permeability and disrupting intracellular ion balance. Viable yeast cells exhibit strong reducing activity, enabling them to effectively reduce methylene blue within the cell to a colorless form, whereas nonviable cells exhibit diminished or absent reducing activity. Leveraging this characteristic, viable and nonviable cells can be distinguished via staining differences, and yeast cell viability can be quantified using a hemocytometer. As shown in Figure 3a, after 24 h of fermentation, yeast cell viability decreased to 40.7%. In stark contrast, the addition of 1 g/L lignin to the system elevated yeast cell viability to 75.5% (Figure 3b). Further increases in lignin concentration significantly enhanced the buffering capacity of the system, thereby further improving yeast cell viability (Figure 3d). The elevated cell viability directly contributed to an increase in ethanol concentration, a trend consistent with the data presented in Figure 2d.

3.2. Effect of Soluble Lignin on Scavenging Free Radicals. The production of free radicals can significantly disrupt the normal metabolic processes of cells, thereby impairing their functional integrity. When the membrane permeability of yeast cells increases or cellular lysis occurs, it can result in the leakage of ROS to the extracellular environment, potentially causing detrimental effects.[36,37]

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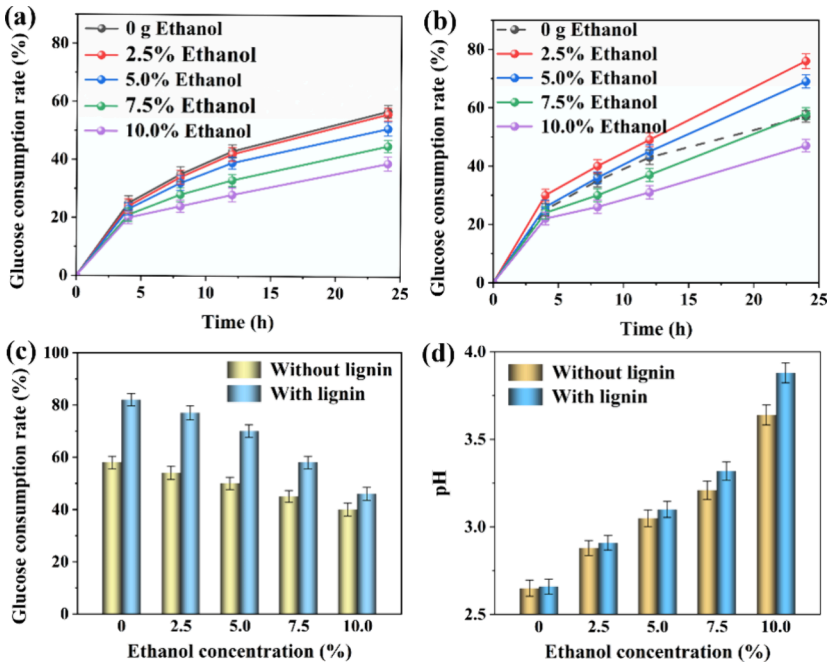

Figure 5. (a) Temporal variation of glucose consumption rate following the addition of different ethanol concentrations. (b) Changes in glucose consumption rate over time after introducing varying ethanol concentrations at a lignin concentration of 2 g/L. (c) Glucose consumption rate after 24 h of fermentation under different ethanol concentrations. (d) pH value of the system after 24 h of fermentation under varying ethanol concentrations.

Lignin can effectively mitigate ROS, primarily due to the phenolic hydroxyl groups in its molecular structure, which can form stable phenolic oxygen radicals through hydrogen donation. This process reduces the reactivity of free radicals and enhances the overall antioxidant capacity of the system.[38] To further investigate the antioxidant efficacy of lignin within the fermentation system, DPPH free radicals were selected as the model substrates for the experiment.[39] As depicted in Figure 4a, the scavenging rate of DPPH free radicals progressively increases with the gradual elevation of lignin concentration, reaching its maximum at 0.1 g/L. Even at lower concentrations, lignin demonstrates substantial clearance effects, indicating its significant antioxidant-promoting effects on yeast fermentation systems and its ability to effectively mitigate oxidative stress-induced damage to yeast cells. Moreover, after 24 h of fermentation at 40 °C, laser confocal microscopy showed strong diffuse DCF fluorescence in the control group (yeast fermentation without soluble lignin) but weak fluorescence in the group with 2 g/L soluble lignin (Figure S3). This result confirms soluble lignin effectively reduces intracellular ROS in yeast under high-temperature stress.

As shown in Figure S4, compared to fermentation conducted at 34 °C, there was a marked reduction in the ethanol concentration produced during fermentation at 40 °C. This phenomenon is primarily attributed to the fact that elevated temperatures intensify the thermal motion of biomolecules within yeast cells, thereby compromising molecular structural stability. Such conditions can readily induce oxidative damage and impair normal cellular growth and proliferation. Figure S5 illustrates that ethanol production is predominantly concentrated within the initial 12 h of fermentation. Notably, in the absence of lignin, minimal ethanol is generated during the 12−24-h fermentation period, suggesting an extremely low yeast cell survival rate under 40 °C conditions during this interval. The addition of lignin, however, results in increased ethanol concentrations across all fermentation periods. Higher lignin concentrations correlate with stronger free radical scavenging capabilities and more pronounced improvements in fermentation efficiency. To eliminate the confounding effects of pH fluctuations across different temperatures, the experiment maintained the system pH at 5.5 through buffering with acetic acid and sodium acetate (Figure 4b). The mitigation of yeast cell inactivation caused by system acidification led to a 238% increase (from 13.6 to 32.4 g/L) in the fermentation rate of yeast at 40 °C compared to the unbuffered system. The addition of lignin did not exhibit a significant synergistic effect, as the pH-buffering properties of lignin were overshadowed by the buffer solution. The optimal pH improved the high-temperature tolerance of yeast. As depicted in Figure 4b, when the temperature was further increased to 45 °C, the ethanol concentration in the system without lignin supplementation declined sharply, whereas the ethanol concentration in the system with 2 g/L lignin supplementation only marginally decreased (from 32.5 to 30.8 g/L). This indicates that lignin can enhance the resistance of the yeast to high temperatures, likely by scavenging excess ROS generated under such conditions through its polyphenolic structure. Similarly, as shown in Figure 4c,d, in the fermentation system involving lactic acid and glutamic acid, the pH was adjusted to the optimal value using an acetic acid-sodium acetate buffer solution, and the fermentation temperature was also increased. After lignin was added to the system, the concentration of the fermentation products increased significantly.4

In addition to the aforementioned factors, the fermentation product can also substantially impede the normal growth and reproductive activities of microorganisms. To further investigate the influence of product concentration on the fermentation process, varying concentrations of ethanol were

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J. Agric. Food Chem. 2025, 73, 32700−32709

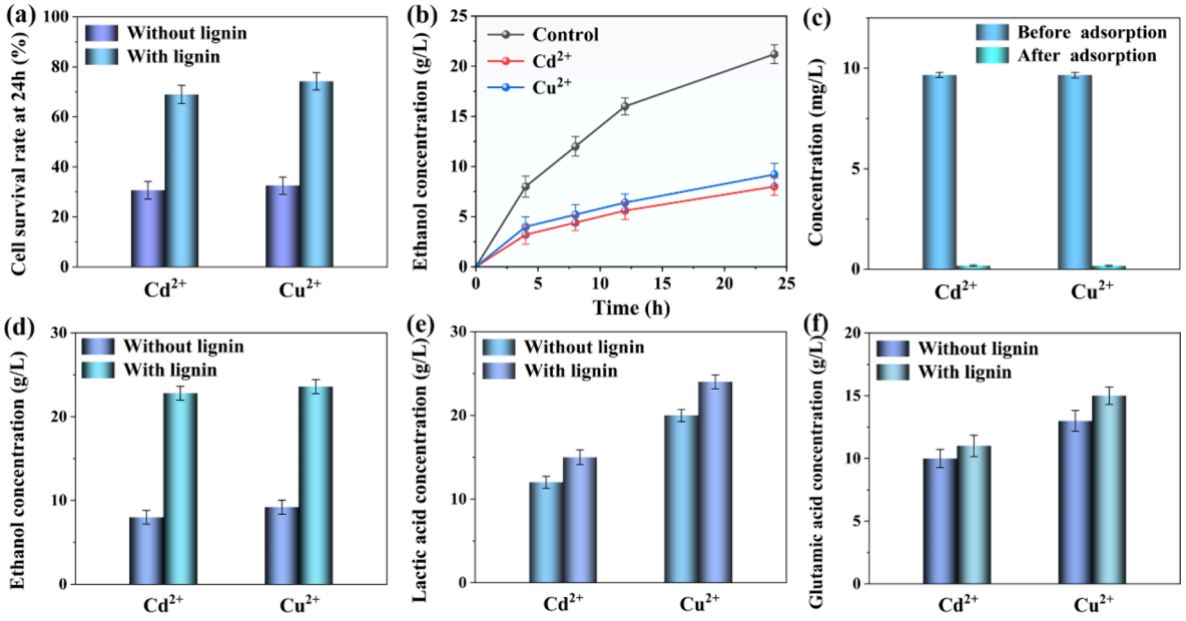

Figure 6. (a) Cell survival rate after 24 h of fermentation with the addition of heavy metals. (b) Variation in ethanol concentration with fermentation time following the addition of different heavy metals. (c) Impact of lignin addition on the concentration of heavy metal ions in the supernatant of the fermentation system. (d–f) Influence of lignin addition in the presence of heavy metal ions on the concentrations of ethanol, lactic acid, and glutamic acid in fermentation products.

introduced into the ethanol fermentation system, and the fermentation outcomes were meticulously analyzed. The findings revealed that as the ethanol concentration progressively increased, the substrate glucose consumption rate exhibited a marked decline (Figure 5a). This phenomenon is primarily attributed to ethanol integrating into the phospholipid bilayer of the yeast cell membrane,[40] thereby disrupting its normal functionality and inhibiting cellular growth and fermentation capacity.[41] The results indicated that when the lignin concentration in the system reached 2 g/L, even with the addition of up to 7.5% ethanol, the glucose consumption rate after 24 h of fermentation surpassed that of the control system devoid of lignin and ethanol, as depicted by the dashed line in Figure 5b. Figure 5c further demonstrates that the introduction of varying lignin concentrations into the initial fermentation system significantly enhances the fermentation performance of yeast, particularly when ethanol is present. Typically, a higher glucose consumption rate correlates with an increased accumulation of acidic metabolites, which generally results in a decline in the pH value of the system. However, as illustrated in Figure 5d, after 24 h of fermentation, the pH value of the system with added lignin was comparable to or even exceeded that of the system without lignin. This observation suggests that soluble lignin can effectively neutralize certain acidic hydrogen ions within the system, mitigating the reduction in pH.

3.3. Adsorption Effect of Soluble Lignin on Heavy Metals. The presence of heavy metal ions in the fermentation broth poses a significant challenge that cannot be overlooked during the production of ethanol via yeast fermentation. These heavy metal ions primarily originated from the absorption and accumulation of various heavy metal elements from environmental media, such as soil and water, during the plant growing period.[42] Heavy metal ions exhibit extreme toxicity to yeast cells, resulting in substantial adverse effects on cell proliferation, metabolic processes, and fermentation efficiency.[42,43] As depicted in Figure 6a, in a system supplemented with heavy metal ions, the mean viability of yeast cells after 24 h of fermentation was merely 30.1%. Conversely, the addition of 2 g/L lignin markedly enhanced the cell viability to 71.6%, indicating that lignin effectively mitigates the detrimental impact of heavy metals on yeast cells. As illustrated in Figure 6b, the introduction of three distinct heavy metal ions into the fermentation system yielded a significantly lower ethanol concentration after 24 h compared to the control group without heavy metals. This underscores the pronounced inhibitory effects of heavy metal ions on the yeast fermentation process, which necessitate the implementation of effective measures to eliminate or reduce their concentration in the fermentation broth to ensure the normal growth and metabolic activity of yeast cells.

Lignin molecules possess diverse functional groups, including phenolic hydroxyl, carboxyl, and carbonyl groups, which can form stable chemical bonds with heavy metal ions in solution through mechanisms such as complexation,[44] chelation, and ion exchange, thereby reducing the concentration of heavy metal ions in the solution.[45] Specifically, during the chelation process, the oxygen atoms in oxygen-containing groups utilize their lone pair electrons as electron donors to form robust coordination bonds with heavy metal ions, thereby achieving efficient adsorption of these ions. Additionally, in acidic conditions, carboxyl groups undergo ionization, releasing hydrogen ions that subsequently participate in ion exchange reactions with heavy metal ions, leading to their firm adsorption onto the lignin surface. Figure 6c presents the analytical results of heavy metal content in the supernatant after 24 h of fermentation: the average removal efficiency of heavy metal ions reached 98.3% following the addition of lignin to the fermentation system. This effectively prevents the inhibitory effects of heavy metal ions on yeast cells, thereby ensuring the stability and continuity of the fermentation process. As shown in Figure 6d, in fermentation

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J. Agric. Food Chem. 2025, 73, 32700−32709

systems containing different heavy metal ions, the addition of lignin significantly elevated the ethanol concentration within 24 h, reaching approximately 2.5 times that of the control group without lignin. This confirms that lignin can effectively alleviate the inhibitory effects of heavy metal ions on the fermentation system. Additionally, as illustrated in Figure 6d,f, the average concentrations of lactic acid and glutamic acid increased by 61.2 and 36.8%, respectively, following a 48 h fermentation period after the addition of 2 g/L lignin. This phenomenon occurs primarily because during the fermentation of glucose to produce lactic acid and glutamic acid, the strong complexation capacity of the metabolites (lactate and glutamate) with heavy metals, combined with the morphological alterations of lignin induced by pH fluctuations in the system, collectively result in diminished heavy metal adsorption efficiency of lignin. Notably, the inhibitory effect of glutamic acid fermentation on lignin adsorption is more pronounced due to the efficient chelation properties of amino acids, whereas the inhibition from lactic acid fermentation primarily stems from lactate competition, which is comparatively less significant.6

3.4. pH-Responsive Recovery of Soluble Lignin. The variation of the solubility of lignin with pH can be utilized to recover lignin. Figure 7a illustrates the distinct states before and after the addition of lignin powder to the fermentation system. Before fermentation, the pH of the system was approximately 5.0, allowing lignin to dissolve effectively and disperse uniformly. After fermentation, the pH decreased to about 3.0, causing lignin to precipitate due to reduced electrostatic repulsion between protonated acidic groups. Through simple centrifugal separation, the precipitated lignin can be recovered and recycled. As depicted in Figure 7b, lignin in the fermentation system underwent 10 experimental cycles, with each fermentation lasting 24 h. The dashed line in the figure represents glucose consumption after 24 h of fermentation without lignin addition. The experimental results demonstrate that the recovered lignin can also significantly enhance the fermentation rate of yeast. After 10 cycles of testing, the ethanol concentration in the product remained at 84.3% of the initial fermentation level (as shown in Figure 7c). To further validate the long-term structural stability of soluble lignin—an essential prerequisite for its repeated application—its structure was characterized before and after 10 cycles: FTIR analysis revealed no deviation in the aromatic ring skeleton peaks and stable O−H stretching peaks of phenolic/carboxylic hydroxyl groups (Figure S6); GPC results indicated minimal fluctuations in molecular weight and polydispersity index (PDI); and quantification of functional groups demonstrated negligible changes in key active sites, namely carboxyl and phenolic hydroxyl groups (Table S3). This indicates that leveraging the pH-responsive properties of lignin enables multiple cycles of reuse, thereby reducing the overall process cost.

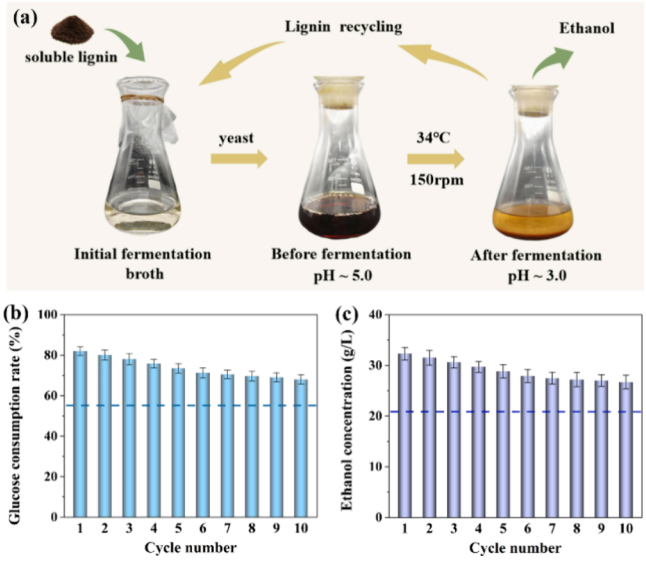

Figure 7. (a) Physical images before and after lignin fermentation. (b, c) Glucose consumption and ethanol concentration during the lignin recycling process.

Collectively, this study demonstrates that soluble lignin derived from corn cobs dilute acid-pretreated enzymatic hydrolysis lignin functions as a multifunctional enhancer in microbial fermentation, rather than the traditional inhibitor it was once deemed. Its synergistic effects of pH buffering, ROS scavenging, and metal chelation collectively mitigate fermentation stressors, with the most pronounced promotional impact observed on *Saccharomyces cerevisiae* metabolism. This finding overturns the conventional perception of lignin as a fermentation hindrance and highlights its untapped value in biomanufacturing. Beyond the immediate results, this work establishes a cost-effective strategy for lignin valorization—converting an abundant industrial byproduct into a recyclable, high-performance fermentation additive—that aligns with the sustainability goals of lignocellulose-based bioproduction.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.5c10457.

Ethanol concentration after 24 h fermentation with lignin (2 g/L) from different raw materials (Figure S1); molecular weight (Mn, Mw), PDI, and functional group content (carboxyl and phenolic hydroxyl) and concentration of four types of lignin (Table S1); elemental analysis (N, C, H, S) of four types of lignin (Table S2); variation of glucose consumption rate with fermentation time under different lignin concentrations (Figure S2); DCF fluorescence images of yeast after 24 h fermentation at 40 °C (Figure S3); ethanol concentration after 24 h fermentation at 34 and 40 °C under different lignin concentrations (Figure S4); temporal variation of ethanol concentration in the fermentation system at 40 °C (Figure S5); FTIR spectra of soluble lignin before and after 10 cycles of fermentation (Figure S6); and Mn, Mw, PDI, carboxyl content, and phenolic hydroxyl content and concentration of soluble lignin before and after 10 fermentation cycles (Table S3) (PDF)

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Author Contributions

C.C., J.X., and Y.C. supervised the project. Z.S. conceived the idea and wrote the manuscript. Z.S., C.Z., and Y.W. performed the materials fabrication, characterization, and carried out the data analysis. C.Z., H.L., and J.Y.Z. assisted in the experimental work and data analysis. All authors discussed the results and commented on the manuscript.

Notes

The authors declare no competing financial interest.

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