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How can vanillin improve the performance of lignocellulosic biomass conversion in an immobilized laccase microreactor system?

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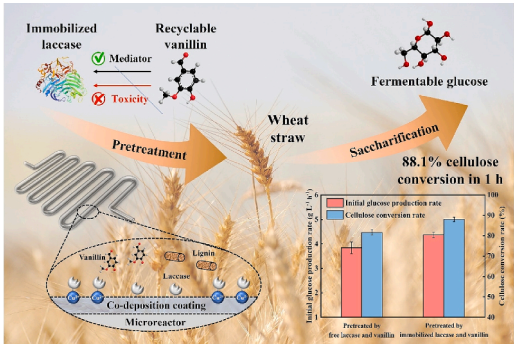
HIGHLIGHTS

- A microreactor system using immobilized laccase and vanillin was first proposed.
- A high immobilization efficiency of laccase reached 87.0% in 30 min.
- The storage stability of immobilized laccase was 210.0% higher than free laccase.
- 6% (w/w) vanillin improved lignin degradation without impairing laccase activity.
- The cellulose conversion rate of pretreated wheat straw reached 88.1% in 1 h.

GRAPHICAL ABSTRACT



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ABSTRACT

Gentle and effective pretreatment is necessary to produce clean lignocellulosic biomass-based fuels. Herein, inspired by the efficient lignin degradation in the foregut of termites, the microreactor system using immobilized laccase and recoverable vanillin was proposed. Firstly, the co-deposition coating of dopamine, hydrogen peroxide and copper sulfate was constructed for laccase immobilization and a high immobilization efficiency of 87.0% was obtained in 30 min. After storage for 10 days, 82.2% activity was maintained in the laccase-loaded microreactor, which is 210.0% higher than free laccase. In addition, 6% (w/w) vanillin can improve lignin degradation in the laccase-loaded microreactor without impairing laccase activity, leading to a 47.3% increment in cellulose accessibility. Finally, a high cellulose conversion rate of 88.1% can be achieved in 1 h with glucose productivity of 2.62 g L^{-1} h^{-1}. These demonstrated that the appropriate addition of vanillin can synergize with immobilized laccase to enhance the conversion of lignocellulosic biomass.

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1. Introduction

The massive burning of fossil fuels releases many greenhouse gases and atmospheric pollutants, which is a huge challenge for global environmental governance (Feng et al., 2022; Wang et al., 2022). While renewable biomass-based fuels can be regarded as an alternative resource to traditional fossil fuels, which is conducive to achieving sustainable development and carbon neutrality (Liu et al., 2023). Lignocellulosic biomass, as the most abundant carbon–neutral resource in the world, can be converted into biofuels through mild enzymatic saccharification and fermentation (Babu et al., 2022; Deng et al., 2022). However, due to its complex cross-linked structure, the external lignin forms a barrier to the polysaccharide, making it difficult to degrade the internal cellulose to fermentable glucose (Lee et al., 2022).

Recently, various techniques have been utilized to pretreat lignocellulosic biomass and enhance glucose production, including acid, alkali, organic solvent, hydrothermal treatment and so on (Chen et al., 2022a; Sun et al., 2022). However, their applicability may be limited by the necessary high temperature and pressure conditions and the probable formation of inhibitors. Conversely, biological pretreatment was expected to have broad development prospects due to its mild conditions, low energy input and environmental friendliness (Wu et al., 2022).

Laccase can oxidize phenolic polymer and produce water, which has widely been applied in lignocellulose pretreatment (Jiang et al., 2020). Meanwhile, immobilization techniques have also been used to enhance the stability and reuse of laccase. Chen et al. (2022b) prepared Fe_3O_4@SiO_2-NH_2 nanoparticles as the carrier to immobilize laccase for 2,4,6-trichlorophenol degradation, the degradation efficiency reached 99.8% in 6 h. However, the glutaraldehyde-based enzyme immobilization method requires more than 30 h, which is time-consuming. Considering that the covalent binding process needs a long time and causes a certain degree of enzyme activity loss, Habimana et al. (2022) constructed zeolitic imidazolate framework (ZIF-8) coated carbon nanotubes to immobilize laccase via metal affinity adsorption. After 1 h of adsorption, immobilized laccase enabled over 95.0% removal of different dyes in 25 min. However, the preparation process for ZIF-8 is complex and requires high pressure conditions. Thus, a simple, rapid and efficient laccase immobilization method is urgently needed.

In addition, for the substrate that contains non-phenolic units (such as lignin), the degradation effect brought by laccase alone is limited. So the laccase-mediator system (LMS) was selected to further enhance the catalytic efficiency of laccase (Deng et al., 2019). Suman et al. (2022) found that the saccharification rate of jute sticks biomass pretreated by laccase (100 U g^{-1}) was enhanced by 11.9% with control. After adding hydroxybenzotriazole (HOBT) as the mediator, the enhancement rate can be further increased to 18.9%. Furthermore, in order to reuse laccase and enhance the catalytic reaction simultaneously, a two-stage laccase-immobilized microreactor was proposed by Lloret et al. (2013). In the first stage, the mediator (ABTS) was oxidized to $ABTS^+$ by immobilized laccase, and the $ABTS^+$ can effectively remove estrogenic compounds in the second stage. However, non-recyclable ABTS may cause reagent waste and other potential contaminations, which also makes this process more expensive (Qiu et al., 2021). Thus, Shan et al. (2022) proposed a strategy to co-immobilize laccase and ABTS on the geopolymer microspheres. In this case, the degradation rate of Congo red dye was 19.6% higher than free LMS. Although co-immobilization of laccase and mediator is beneficial for enhancing catalytic reactions in liquid-phase systems, it may be less effective for solid-phase catalysis. Therefore, it is necessary to create a novel laccase-mediator system that is appropriate for lignin degradation.

In nature, termite is the species with the highest system integration capable of utilizing polysaccharides and digesting partial lignin simultaneously (Xia et al., 2022). The lignin degradation is mainly attributed to lignin-degrading enzymes (e.g. laccase) secreted from the salivary glands of termites (Ali et al., 2022). The mixed lignocellulosic biomass and laccase will be passed into the foregut of termite and reacted for hours. In this process, vanillin is produced due to the degradation of lignin G-type units (Ali et al., 2022). While vanillin is a natural mediator that can enhance the removal efficiency of pesticides by laccase (Kupski et al., 2019). Therefore, it is reasonable to presume that vanillin can also improve the degradation of lignin by laccase. In addition, the termite gut exhibits fabulous mass transfer experience due to its small diameter (less than1 mm) and high specific surface area (Xia et al., 2022). Therefore, the foregut of termite can be considered as a microreactor accompanied by the catalysis of laccase and mediator (vanillin) for lignin degradation. However, it is not clear how vanillin promotes lignin conversion in the immobilized laccase microreactor.

In this study, a functional coating was constructed on the inner surface of the microreactor using co-deposition of dopamine, hydrogen peroxide and copper sulfate. Laccase was immobilized into the microreactor by covalent binding and metal affinity adsorption quickly and efficiently. In addition, vanillin was added to the microreactor loaded with laccase to enhance lignin degradation and modification. Finally, the laccase-vanillin system in the microreactor was demonstrated to be an effective way for lignocellulosic conversion and its expanded production can be achieved by using multiple microreactors of the same size, with the reaction conducted in each reactor remaining the same at any scale (Tamborini et al., 2018).

2. Materials and methods

2.1. Chemicals

Laccase with an activity of 0.9 U mg^{-1}, cellulase Cellic CTec2 with an activity of 200 FPU mL^{-1} and vanillin were purchased from Sigma-Aldrich (United States). Citric acid, sodium citrate, Tris, 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and dopamine hydrochloride (DA) were purchased from Aladdin Industrial Inc (China). Copper sulfate ($CuSO_4$) and hydrogen peroxide (H_2O_2) were obtained from Chengdu Chron Chemicals Co. Ltd (China). Methylene blue was purchased from Beijing Merda Technology Co. Ltd (China). All the chemicals used were used as received.

2.2. Preparation of the microreactor loaded with laccase

A polytetrafluoroethylene (PTFE) capillary with an inner diameter of 600 μm was selected as the microreactor (Lin et al., 2022). After cleaning with deionized water, the mixed solution of DA, H_2O_2 and $CuSO_4$ (Tris buffer, 50 mM, pH 8.5) was passed into the microreactor and kept for 30 min to form a co-deposition coating. The concentration of DA and H_2O_2 were set as 2 mg mL^{-1} and 4.9 mM respectively. The microreactor was further rinsed with deionized water and baked at 60 °C for 30 min. Thereafter, the laccase solution (0.05 M of citric acid buffer, pH 5.0) was infused and incubated for 30 min in the microreactor at room temperature. To remove the residual free laccase, the microreactor was flushed using the citric acid buffer.

2.3. Determination of laccase activity

The laccase activity was measured according to the oxidation of ABTS (Shan et al., 2022). For free laccase, 1 mL laccase solution was reacted with 1 mL ABTS solution (0.5 mM) for 5 min. For immobilized laccase, 0.5 mM ABTS solution was infused and incubated in the microreactor loaded with laccase for 5 min. The absorbance of the reaction product was measured at 420 nm using an ultraviolet spectrophotometer (T6, Beijing Persee Technologies, China). To indicate the enzyme loading capacity, immobilized activity was chosen as an indicator, which can be calculated by Eq. (1). Besides, immobilization efficiency can be used to characterize whether the enzyme binding is effective or not (Sheldon and van Pelt, 2013), which was defined by Eq. (2).

$$Immobilized\ \ activity = A_i - A_f \quad (1)$$

$$Immobilization\ \ efficiency = \left(\frac{A_i - A_f}{A_o}\right) \times 100\% \quad (2)$$

where A_i is the activity of the laccase solution before immobilization (μmol min^{-1}), A_f is the activity of the laccase solution after immobilization (μmol min^{-1}) and A_o is the activity cobserved in the microreactor loaded with laccase (μmol min^{-1}).

2.4. Catalytic performance of free and immobilized laccase

The effects of temperature (30–50 °C) and pH (2.5–5.0) on the activity of free and immobilized laccase were investigated as reported before (Lin et al., 2022). And several microreactors loaded with laccase were prepared and stored at 4 °C for various days to determine the storage stability. The reusability performance of the microreactor loaded with laccase was tested by the ABTS conversion experiment every 10 min.

To test the effect of vanillin toxicity on laccase activity, 1 mL vanillin solution was first mixed with 1 mL laccase solution for 1 h. After that, 1 mL ABTS solution was added to the mixed solution and reacted at 35 °C for 5 min. Furthermore, to verify the role of vanillin as a mediator, 1 mL of each vanillin, laccase and ABTS were mixed and reacted directly at 35 °C for 5 min to test the laccase activity. The activity obtained without vanillin addition was set to 100% and the relative activity under other conditions was converted on this basis.

2.5. Degradation and modification of lignin in the microreactor loaded with laccase

The wheat straw used in the experiment was obtained from Henan Province, China and it was pretreated by ball milling for 24 h before catalysis (Xia et al., 2022). The 5% (w/w) wheat straw solution (0.05 M of citric acid buffer, pH 4.0) was first pumped into the microreactor loaded with laccase and incubated for 12 h at 40 °C. In addition, 2–10% (w/w) vanillin was added to the substrate solution as the mediator. The lignin content was measured as reported (Sheng et al., 2021).

2.6. Recovery of vanillin using adsorption resin SP 700

Adsorption resin SP 700 was purchased from Mitsubishi Chemical Corporation (Japan). 0.1 g adsorption resin was kept in the filter bag (1 cm × 1 cm) and put into 1 mL lignocellulosic slurry containing vanillin, and absorbed at 170 rpm for 60 min. The vanillin concentration was measured by absorbance at 278 nm (Cañadas et al., 2021). After adsorption, the adsorption resin was put into 1 mL of water (or ethanol) for the desorption for 30 min. The adsorption efficiency was defined as the ratio of adsorption capacity to the initial vanillin content while the desorption efficiency referred to the ratio of desorbed quantity to adsorbed quantity.

2.7. Saccharification of wheat straw using cellulase

After pretreatment, the solution was discharged from the microreactor and collected, then centrifuged at 8000 rpm for 10 min and rinsed twice. The residual wheat straw was reconfigured to a 1% (w/w) concentration (0.05 M of citric acid buffer, pH 5.0) in the tube. Then the cellulase (30 FPU g^{-1}) was added to the tube for cellulose hydrolysis. The glucose concentration was determined using high performance liquid chromatography (HPLC) (Thermo Ultimate 3000, United States).

2.8. Characterization

The inner surface chemical compositions of the microreactors were characterized by an X-ray photoelectron spectrometer (XPS) (ESCALAB 250Xi, Thermo, USA). The distribution of copper element on the co-deposition coating was measured by the Electron probe microanalyzer (EPMA) (JXA-8230, JEOL, Japan). The chemical structures of wheat straw and microreactors were examined by a Fourier transform infrared spectrometer (FTIR) (Nicolet iN10, Thermo, USA). Cellulose accessibility was determined as reported before (Xia et al., 2022).

3. Results and discussion

3.1. Optimization of immobilization parameters

In the inner surface of the microreactor, a co-deposition coating of DA, $CuSO_4$ and H_2O_2 was constructed for laccase immobilization. The reaction between $CuSO_4$ and H_2O_2 produces reactive oxygen species (ROS), which can accelerate the oxidative polymerization of dopamine to form the coating of polydopamine.(Zhang et al., 2016) In this process, some copper ions would also be chelated to the surface of the polydopamine coating, which is conducive to laccase immobilization. Thus, the effect of initial copper ion concentration was first investigated (Fig. 1). As revealed in Fig. 1a, as the initial concentration of copper ion rose, when the initial copper ion concentration increased from 10 mM to 25 mM, the immobilized activity of laccase was increased from 5.3 μmol min^{-1} to 9.5 μmol min^{-1}. This is because the amount of ROS increased as the copper ion concentration increased, inducing the formation of uniform and stable polydopamine coating. The amino groups of the laccase can be combined with the quinone functional groups in polydopamine through Schiff-base formation. On the other hand, as the initial copper ion concentration increased, more copper ions could be attached to the coating stably, which is conducive to laccase immobilization through metal affinity adsorption. These allowed the coating formed with an initial copper ion concentration of 25 mM to have a better ability to bind enzymes, thus increasing the immobilized activity.

However, when further increasing the copper ion concentration from 25 to 35 mM, the immobilized activity of laccase was decreased slightly from 9.5 to 8.4 μmol min^{-1}. This is because although the excessive ROS content can oxidize dopamine rapidly, the deposition efficiency is not high. Many dopamine aggregates precipitated out of the deposition solution rather than binding to the microreactor surface to form a stable polydopamine coating (Zhang et al., 2016), which is also not favorable for the uniform distribution of copper ions on the coating. At an initial copper ion concentration of 25 mM, the copper distribution on the polydopamine coating was relatively uniform (see supplementary material). However, when the initial copper ion concentration was increased to 35 mM, the copper became unevenly distributed on the coating and copper aggregation was observed in some areas, making the laccase immobilized by metal affinity adsorption be agglomerated on the coating and resulting in a decrease in the immobilized activity of laccase.

In addition, when the initial copper ion concentration was increased from 10 mM to 25 mM, the immobilization efficiency of laccase increased rapidly from 19.2% and reached the highest (74.9%). This is related to changes in the enzyme immobilization mechanism. At a low initial copper ion concentration, covalent binding was dominant during immobilization. As copper ion content increased, the main immobilization mechanism gradually shifted to metal affinity adsorption, which enables enzymes to retain higher activity. However, continuing to increase the initial copper ion concentration to 35 mM, the immobilization efficiency was slightly decreased to 64.4%. This is associated with uneven copper distribution, leading to partial coverage in the active sites of laccase.

As shown in Fig. 1b, at a low copper ion concentration of 10 mM, the conversion rate of ABTS in the laccase-loaded microreactor was only 7.1% in 5 min. When the copper ion concentration was up to 25 mM, the conversion rate was enhanced by 6.1 times and reached 50.4%. When further increasing the initial copper ion concentration to 35 mM, the conversion rate of ABTS decreased slightly to 38.1%. Thus, the optimal

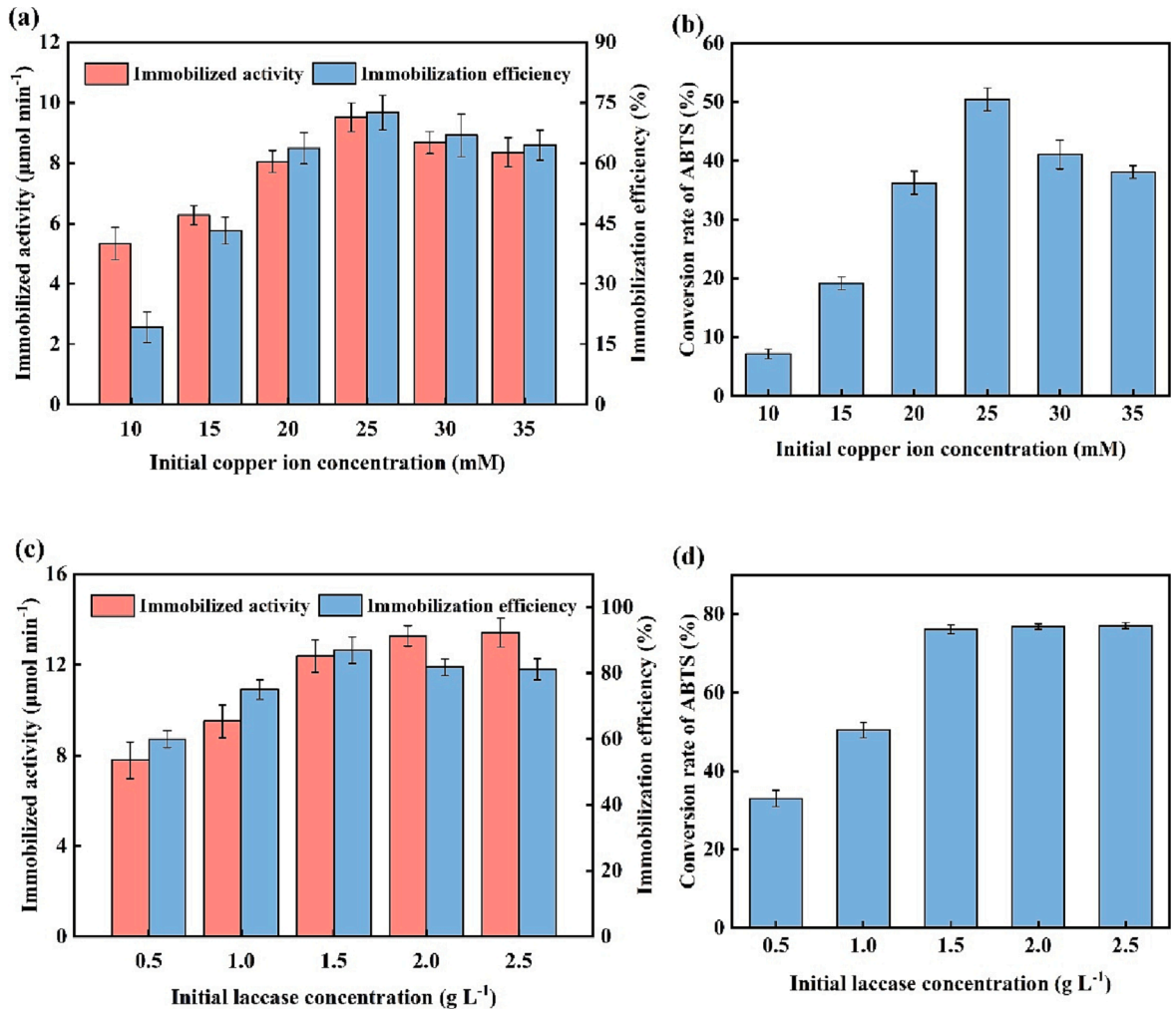

Fig. 1. Effect of initial copper ion concentration on the (a) immobilized activity, immobilization efficiency and (b) conversion rate of ABTS. Effect of initial laccase concentration on the (c) immobilized activity, immobilization efficiency and (d) conversion rate of ABTS.

copper ion concentration of 25 mM was selected for the subsequent experiments.

The effect of initial laccase concentration was also conducted. As revealed in Fig, 1c, with the initial laccase concentration rising from 0.5 to 2.0 g L^{-1}, the immobilized activity increased rapidly from 7.8 to 13.3 μmol min^{-1}. When further increasing the laccase concentration to 2.5 g L^{-1}, this value reached 13.4 μmol min^{-1} only. This is due to the limited enzyme binding sites on the coating, making the enzyme loading cannot be consistently increased. Conversely, excessive initial laccase concentration may cause agglomeration between enzyme molecules on the coating, leading to a decrease in immobilization efficiency (Ghodake et al., 2018). Therefore, the efficiency at an initial enzyme concentration of 2.5 g L^{-1} (81.1%) was lower than the initial enzyme concentration of 1.5 g L^{-1} (87.0%). Furthermore, when the laccase concentration was increased from 0.5 to 1.5 g L^{-1}, the conversion rate was increased from 33.0% to 76.2% (Fig. 1d). When the laccase concentration was further increased to 2.5 g L^{-1}, the conversion rate of ABTS reached 77.1% only. Hence, considering enzyme inputs, the optimal laccase concentration of 1.5 g L^{-1} was chosen for the subsequent experiments.

3.2. Characterization of the laccase-loaded microreactor

Microreactor with coating was constructed by the co-deposition of DA, $CuSO_4$ and H_2O_2. The XPS spectra of the pristine microreactor and microreactor with coating are revealed (see supplementary material). The C1s peak was observed at 284.9 and 292.4 eV. A high peak intensity at 292.4 eV can be found in the pristine microreactor, which was associated with CF_2–CF_2 of the PTFE capillary. Compared with the pristine microreactor, the peak intensity at 292.4 eV was decreased while the peak intensity at 284.9 eV was increased in the microreactor with coating. This is because along with the formation of the coating, the original PTFE material was covered, causing a decrease in peak intensity at 292.4 eV. In addition, the carbon elements in dopamine can be observed at 284.9 eV (Zhang et al., 2016). These phenomena can confirm the successful formation of the co-deposition coating.

In addition, for the pristine microreactor, there is no significant peak between 930.0 and 970.0 eV (see supplementary material). While in the microreactor with coating, two peaks located at 935.2 and 953.6 eV were appeared, which are attributed to Cu $2p_{2/3}$ and Cu $2p_{1/2}$ of copper ion (Li et al., 2020). And the copper elements on the microreactor with coating reached 3.03% (see supplementary material). This illustrates that the copper ions have been chelated to the surface of the microreactor, which can be used for subsequent laccase immobilization.

The FTIR spectra of the microreactor with coating and the laccase-loaded microreactor are obtained (see supplementary material). Compared with the microreactor with coating, the additional peaks at 1402 cm^{-1} and 1589 cm^{-1} were due to the stretching vibration of the C-H bond and amide II bands, indicating the successful loading of laccase through amine bonding (Lin et al., 2022). In addition, the vibration band at 3000–3700 cm^{-1} is attributed to asymmetric and symmetric NH_2 stretching vibrations during laccase immobilization through Schiff-base reaction (Lin et al., 2022). Last but not least, the new peak that appeared

1702 cm^{-1} was attributed to the formation of Lewis adduct through the dative bond between amino acid residues in laccase and copper ions on the coating (Zhang & Hay, 2020), showing that laccase was successfully immobilized onto the coating by metal affinity adsorption. All of these findings show that the microreactor loaded with laccase was constructed successfully.

3.3. Enzymatic properties of microreactor loaded with laccase and free laccase

The effect of temperature and pH values on the activity of free and immobilized laccase was investigated (see supplementary material). The free laccase showed the highest activity at 35 °C. However, due to the enhanced structural stability of the laccase during immobilization, the optimal operating temperature of immobilized laccase was up to 40 °C. Besides, in an acidic environment with a pH ranging from 2.5 to 4.0, more than 94.8% of immobilized laccase activity can be maintained, which is significantly better than free laccase. Thus, the operating conditions of 40 °C and pH 4.0 were used for the subsequent application of the microreactor loaded with laccase.

Stability is the dominant factor to determine whether enzymatic catalysis can be applied on a large scale, as shown in Fig. 2. Firstly, in the presence of 25% ethanol and methanol, the activity of free laccase dropped sharply to about 50.0% (Fig. 2a). This is because the organic solvent would not only remove the tightly bound water layer near the enzyme, which is not suitable for the substrate-enzyme contact but also cause some damage to the enzyme protein structure (Li et al., 2020). While immobilization is an effective way to maintain enzyme conformation. In the presence of 25% ethanol and methanol, the activity of immobilized laccase was 64.5% and 66.1%, which is 26.3% and 22.9% higher than that of free laccase. A similar phenomenon can be observed in the ethylene glycol system, indicating that immobilization can effectively mitigates the adverse effects of organic solvents on enzymes.

The effect of ethanol concentration on laccase activity was further investigated in Fig. 2b. With the concentration of ethanol increased from 10% to 30%, the relative activity of free laccase decreased from 74.2% to 51.9%. However, for immobilized laccase, 85.4% of activity can be retained in the condition of 10% ethanol. And 62.5% activity can be observed under 30% ethanol concentration, which is 20.3% higher than free laccase. This suggests that the microreactor loaded with laccase is suitable for catalytic systems that require trace amounts of ethanol as the solvent.

As shown in Fig. 2c, the activity of free and immobilized laccase both decreased as the storage time increased. After storage for 4 days, the activity of free laccase decreased rapidly to 45.8%, and this value further declined to 26.5% when the storage time was extended to 10 days. This is a common phenomenon that the denaturation of laccase molecules would occur when enzymes are not kept in their native environment for a long time. Differently, 85.2% of activity can be maintained in the laccase-loaded microreactor after 4 days of storage, which is 86.0% higher than free laccase. Furthermore, 82.2% activity can still be observed after 10 days of storage, which is 2.1 times higher than free laccase. This is because immobilization facilitates the stabilization of enzyme conformation, enabling it to maintain higher activity in some harsh circumstances.

The reuse effect of the microreactor loaded with laccase was tested and shown in Fig. 2d. During the first 5 cycles of reuse, there is almost no activity loss. When increasing the reuse times to 10, there is a decrease in the relative activity of the microreactor loaded with laccase, which may be due to the slight enzyme leakage and inactivation during prolonged

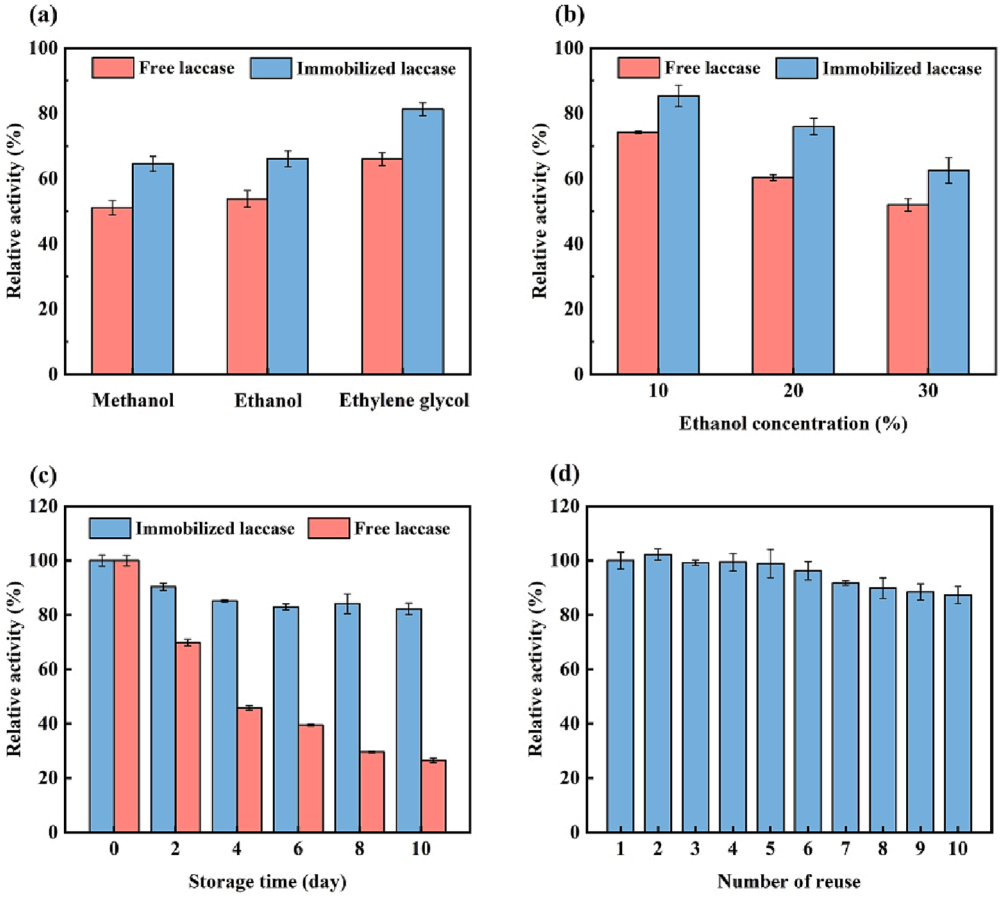

Fig. 2. Stability of immobilized and free laccase under (a) different organic solvents, (b) different ethanol concentrations and (c) a period of storage. (d) Reuse effect of the microreactor loaded with laccase.

operation. But 87.3% of initial activity can still be observed. This phenomenon indicates that the microreactor loaded with laccase has great reusability, thereby helping to reduce production costs.

3.4. Degradation and modification of lignin using immobilized laccase and vanillin

The microreactor loaded with laccase was applied to the pretreatment of wheat straw (Fig. 3), mainly the degradation and modification of lignin. 5% (w/w) wheat straw slurry first filled the microreactor and then reacted with immobilized laccase in batches at 40 °C. While in the presence of vanillin, the immobilized laccase reacted preferentially with vanillin, oxidizing it to phenoxyl radicals to react with the lignin (Jeon et al., 2008). Therefore, the effect of vanillin content on lignin conversion was investigated.

As shown in Fig. 4a, the lignin content of untreated wheat straw was 23.9%, this value was decreased to 22.6% after reaction in the microreactor loaded with laccase for 12 h. This is because laccase can oxidize the phenolic unit of lignin to produce water by using molecular oxygen as the final electron acceptor (Deng et al., 2019). To further improve the lignin degradation efficiency per unit time, the soluble vanillin was added to this system as a mediator. As the vanillin concentration increased from 2% (w/w) to 6% (w/w), the lignin content declined constantly and reached 21.5%. On one hand, the addition of vanillin can be a mediator to improve the oxidation–reduction potential of laccase, enabling laccase to degrade the non-phenolic units of lignin more efficiently. On the other hand, the contact between the straw particles and the surface loaded with laccase through diffusion is relatively inefficient (Fig. 3). While with the addition of vanillin, the laccase can oxidize vanillin firstly, phenoxyl radicals produced by the oxidation of vanillin can be diffused more readily into the wheat straw. This solid–liquid contact between lignin and soluble mediator is more effective than solid–solid contact between lignin and immobilized laccase, leading to a better lignin degradation effect.

However, further increasing the vanillin concentration can not continue to reduce the lignin content. When further increasing the vanillin concentration to 10% (w/w), the lignin content was 22.6%, which is the same as when vanillin is not added. These indicated that excessive vanillin concentration is not conducive to enhancing the catalysis, which may be associated with the laccase activity loss caused by the toxicity of vanillin. In the case that laccase reacted with vanillin firstly for 1 h, with the increment in vanillin concentration, the relative activity of laccase kept stable first and then started to decline. When the vanillin concentration was set as 3.0 g L^{-1}, a high relative activity of 99.1% can still be observed (see supplementary material). But the relative activity was decreased continuously to 85.8% at a vanillin concentration of 7.5 g L^{-1}. This suggests that excessive vanillin does cause a decrease in enzyme activity, which is detrimental to the degradation and modification of lignin.

In addition, the laccase, vanillin and ABTS were reacted together for 5 min to calculate the enzyme activity under such conditions (see supplementary material). As the concentration of vanillin increased, the relative activity of free laccase increased first and then decreased. When the vanillin concentration was increased from 0 to 3.0 g L^{-1}, the relative activity of laccase was increased continually and reached the highest (111.4%). This is associated with the higher oxidation–reduction potential of laccase in the presence of vanillin as a mediator, resulting in a better apparent activity. However, when further increasing the vanillin concentration to 5.0 g L^{-1}, the relative activity was slightly decreased to 101.5%. This suggests a synergistic effect between immobilized laccase and vanillin. Vanillin can act as a mediator to enhance the catalytic process by laccase, but excessive vanillin addition would cause damage to the laccase protein structure, thus reducing laccase activity and catalytic effect. For the substrate concentration of 50 g L^{-1} wheat straw in the microreactor, when the vanillin content was set as 6% (w/w), the lignin degradation rate reached the highest. In such a situation, the vanillin concentration in the system was precisely 3.0 g L^{-1}, which is the same concentration of vanillin that caused the initial laccase inactivation. This phenomenon demonstrated that the vanillin addition of 3.0 g L^{-1} can best exploit its synergistic effect with immobilized laccase.

Moreover, the adsorption characteristics of wheat straw on methylene blue were determined in Fig. 4b to measure cellulose accessibility. For all samples, the adsorption capacity of methylene blue increased rapidly in the initial 10 min and achieved equilibrium at 120 min. In the initial 6 min, the adsorption rate of methylene blue by raw wheat straw catalyzed was only 1.20 mg g^{-1} min^{-1}. This value for wheat straw treated by immobilized laccase was increased by 48.9% and reached 1.79 mg g^{-1} min^{-1}. Moreover, for the wheat straw treated by immobilized laccase and 6% (w/w) vanillin, the adsorption rate of methylene blue was 1.99 mg g^{-1} min^{-1}, which is 66.1% higher than raw wheat straw. In addition, the maximum adsorption capacity of wheat straw treated with immobilized laccase and 6% (w/w) vanillin was up to 32.7 mg g^{-1}, which is 12.4% and 47.3% higher than wheat straw treated with immobilized laccase and raw wheat straw. This indicates that the laccase-vanillin system effectively enhanced the cellulose accessibility of wheat straw, which is expected to enhance the subsequent saccharification efficiency.

The FTIR spectra of wheat straw under different treatments were revealed in Fig. 4c. After treated with immobilized laccase or immobilized laccase (La) and vanillin (Va), peak intensity change can be observed at 1400–1650 cm^{-1} and 3100–3700 cm^{-1}. The decreased peak intensity at 1425 cm^{-1}, 1512 cm^{-1} and 1648 cm^{-1} were associated with aromatic skeletal vibrations, C–O stretching and C = C stretching (Zhang et al., 2018). These changes all indicated the broken of lignin aromatic ring structure. In addition, the peak intensity at 3400 cm^{-1}, which indicates hydroxyl content, was also reduced after being treated with immobilized laccase. This is due to the degradation of phenolic units in lignin by laccase, leading to the decline in the content of phenolic

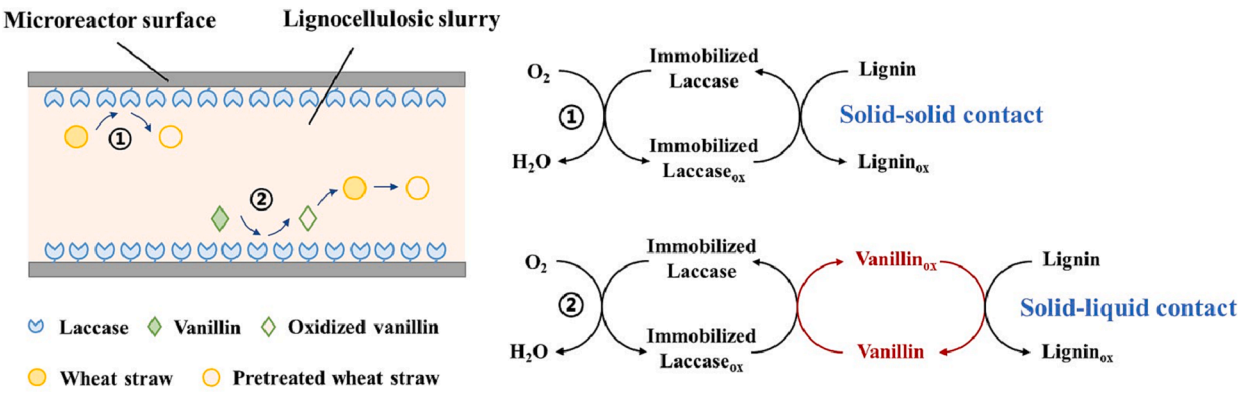

Fig. 3. The degradation pathway of lignin in the microreactor loaded with laccase under the condition of adding vanillin as a mediator.

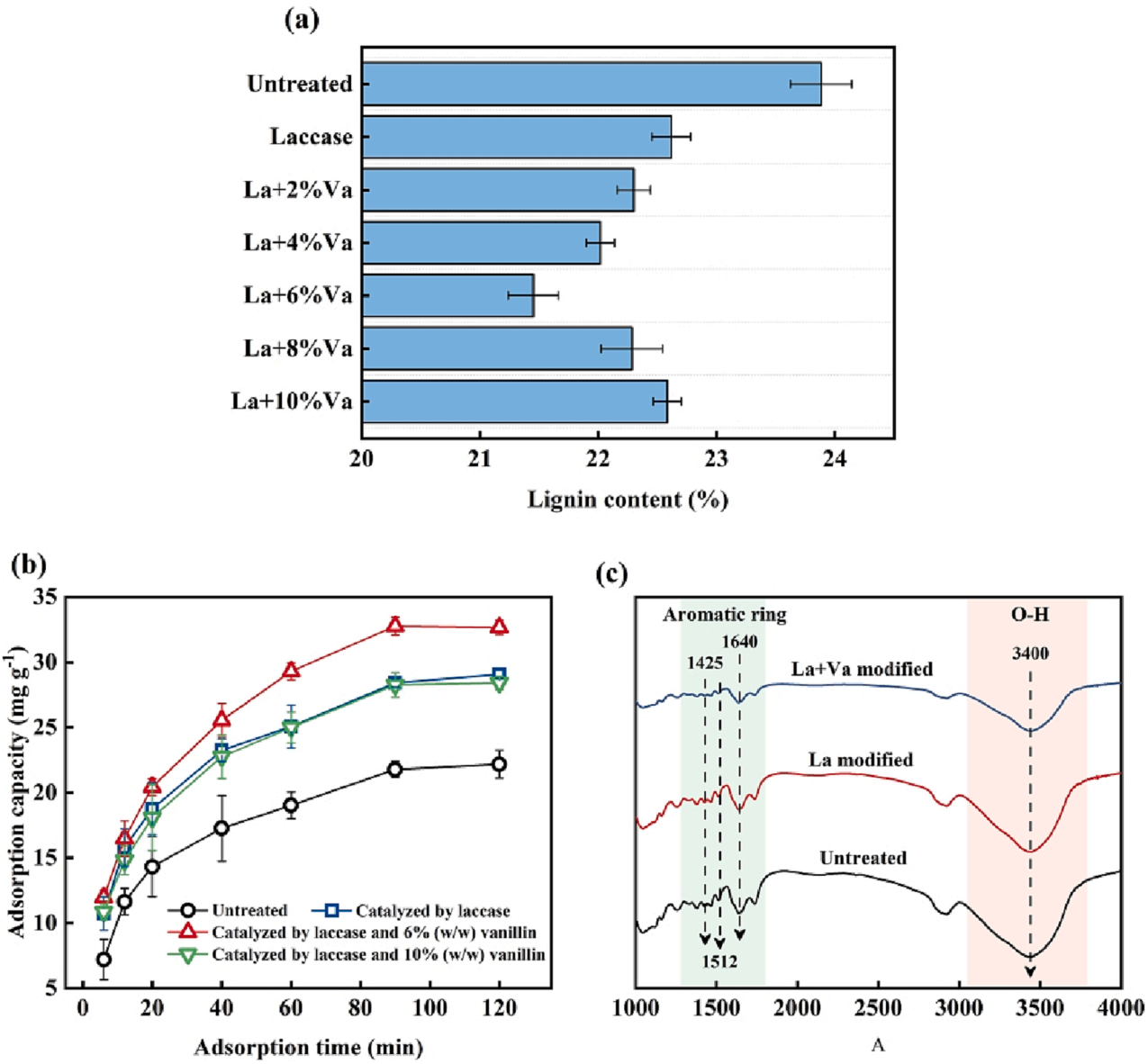

Fig. 4. Effect of different treatment conditions on (a) lignin content and (b) cellulose accessibility of wheat straw. (c) FTIR spectra of wheat straw under different treatments.

hydroxyl groups (Deng et al., 2019). The addition of vanillin can promote this process, thus causing more reduction in peak intensity at 3400 cm^{-1}. It has been reported that the presence of phenolic hydroxyl group would boost the formation of hydrogen bonding, and condensed aromatic rings can enhance hydrophobic interactions (Sun et al., 2016). This will lead to the non-productive adsorption of cellulase and other catalytic enzymes on lignin, which is harmful to subsequent enzymatic saccharification. After the lignin modification with immobilized laccase and vanillin, the aromatic ring structure and phenolic hydroxyl were partly destroyed and removed, which helps to reduce the non-productive adsorption of enzymes and improves the saccharification efficiency. Overall, catalyzing wheat straw by the synergistic interaction of immobilized laccase and vanillin can reduce the lignin content, destroy the cross-linked structure of biomass and alleviate non-productive adsorption, which can be regarded as an effective tool for enhancing subsequent saccharification.

3.5. *Effect of lignin degradation and modification on subsequent enzymatic saccharification*

The 1% (w/w) wheat straw with different pretreatments were reacted with cellulase (30 FPU g^{-1}) for 1 h, as shown in Fig. 5. For the untreated wheat straw, the cellulose conversion rate reached 74.6% in 1 h with the initial glucose productivity of 3.50 g L^{-1}. After being treated by free laccase, the conversion rate was increased to 77.3% and the initial glucose productivity reached 3.68 g L^{-1}. The catalytic effect of wheat straw pretreated with immobilized laccase was slightly better than that using free laccase, the conversion rate reached 79.6% and the initial glucose productivity was 3.82 g L^{-1}. This is because free laccase tends to be easily inactivated even when operating at proper pH and temperature conditions, while due to the increased stability, the immobilized laccase can perform better in the same catalytic time, but the enhancement rate of saccharification (9.3%) is still limited.

For wheat straw treated with immobilized laccase in synergy with vanillin, the efficiency of enzymatic saccharification was further improved. As revealed in Fig. 5b, with the increase of vanillin content during wheat straw treatment, the glucose productivity and cellulose

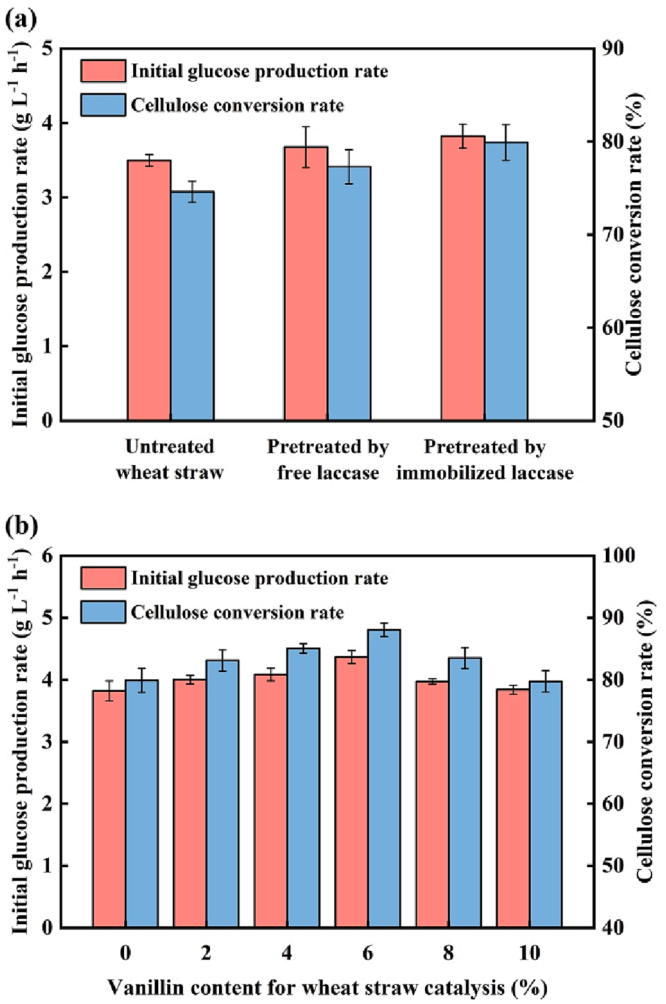

Fig. 5. (a) Effect of different treatment conditions on glucose production. (b) Effect of vanillin concentration during pretreatment of wheat straw on glucose production.

conversion rate were increased first and then decrease. For the wheat straw treated with immobilized laccase and 6% (w/w) vanillin, the highest glucose productivity of 4.37 g L^{-1} h^{-1} can be obtained, which is 24.9% higher than raw wheat straw. Meanwhile, the highest cellulose conversion rate of 88.1% can be observed in the wheat straw treated with immobilized laccase and 6% (w/w) vanillin, which is 18.0% higher than the raw wheat straw. At the same time, the catalytic effect of immobilized laccase and vanillin is also better than that of free laccase and vanillin, with a cellulose conversion rate of 81.6% and an initial glucose productivity of 3.84 g L^{-1} h^{-1} (see supplementary material). This is because immobilization can enhance enzyme stability under extreme conditions, leading to better laccase activity retention during long-time usage. The microreactor loaded with laccase can be successfully constructed within 1.5 h and showed better catalytic performance than free laccase, demonstrating great application potential.

The hydrolysis effect of other lignocellulosic biomass pretreated by laccase was investigated and compared. Rencoret et al. (2017) selected free laccase and 1-hydroxybenzotriazole (HBT) to pretreat sugarcane straw. After further alkaline peroxide extraction process, the cellulose digestibility reached a high level of 93.0% in 72 h. Suman et al. (2022) found that increasing the concentration of laccase and mediator in the pretreatment process can effectively promote subsequent saccharification. For the jute sticks pretreated by laccase (100 U g^{-1}) and 10% (w/w) HOBT, the cellulose conversion rate can reach 79.8% in a shorter time of 24 h, with a glucose productivity of 0.37 g L^{-1} h^{-1}. However, the high enzyme and mediator dosage made the process less economical. The above process also required the assistance of alkali and sodium hydroxide after laccase-mediator treatment, making the procedure more complex and less gentle. Thus, Gou et al. (2020) constructed the modified magnetite laccase immobilized nanoparticles to pretreat corn stover biowaste. For the corn stover pretreated by laccase for 72 h, the cellulose conversion rate reached 38.4% after 72 h. A glucose productivity of 0.10 g L^{-1} h^{-1} and the specific glucose productivity of 3.33 mg L^{-1} h^{-1} FPU^{-1} can be achieved. Sánchez-Ramírez et al. (2018) also used the laccase-loaded superparamagnetic nanoparticles for agave pretreatment. However, due to the diffusion difficulties between immobilized laccase and agave granules, the pretreatment performance using immobilized laccase is not as good as that of free laccase. The cellulose conversion rate in 24 h only reached 35.7%, which is 43.6% lower than the agave pretreated by free laccase. In this case, a glucose productivity of 0.07 g $L^{-1}h^{-1}$ and the specific glucose productivity of 4.67 mg L^{-1} h^{-1} FPU^{-1} can be obtained.

For the catalytic system using immobilized laccase and vanillin in the microreactor proposed in this study, the conversion rate reached 88.1% in 1 h with a glucose productivity of 2.61 g L^{-1} h^{-1}. Meanwhile, the specific glucose productivity reached 87.3 mg L^{-1} h^{-1} FPU^{-1}, which is 10 times higher than the hydrolysis systems mentioned above (3.33–4.67 mg L^{-1} h^{-1} FPU^{-1}). This is because the proposed laccase immobilization method can greatly maintain the laccase activity and keep the laccase stable in operation. In addition, the appropriate concentration of added vanillin can effectively enhance the degradation of non-phenolic units in lignin by laccase, causing structural damage and enhanced cellulose accessibility of wheat straw. In addition, the wheat straw after ball milling was mainly in the range of 1–10 μm and has a high specific surface area, it can also maintain uniformity during the reaction. These are all conducive to the subsequent saccharification of wheat straw using cellulase, resulting in a great performance. Meanwhile, considering that phenolic compounds would affect the catalytic activity of cellulase (Ladeira Azar et al., 2018), the adsorbent resin SP 700 was applied to recover the additional vanillin in the lignocellulosic slurry effectively, with a recovery rate of 90.2% within 60 min (see supplementary material), so it does not inhibit the subsequent enzymatic saccharification (vanillin concentration less than 0.3 g L^{-1}). Unfortunately, the desorption rate of vanillin in 30 min was only 7.4%. However, when using 30% (v/v) ethanol as adsorbed solvent, the adsorption efficiency of vanillin reached 42.0%, which is 5.71 times the desorption efficiency in an aqueous solution. While the addition of ethanol may affect the laccase activity, a milder method for vanillin desorption still needs to be further explored.

Overall, pretreating wheat straw using immobilized laccase and recoverable vanillin in the microreactor can be considered an effective way to improve saccharification efficiency. And combining the laccase-loaded microreactor with the two-stage microreactor loaded with complex enzymes (Xia et al., 2022) to build a cascade system may make the lignocellulose conversion process more integrated and economical.

4. Conclusion

The catalytic system using immobilized laccase and recoverable vanillin was proposed to degrade and modify lignin in a microreactor for the first time. Firstly, laccase was successfully immobilized in the microreactor with the immobilization efficiency of 87.0% in 30 min. The immobilized laccase can be reused easily and showed great stability. Under the synergistic effect of immobilized laccase and 6% (w/w) vanillin, the cellulose accessibility of wheat straw was increased by 47.3%. The cellulose conversion rate of pretreated wheat straw reached 88.1% in 1 h, indicating that system using immobilized laccase and recoverable vanillin can greatly enhance the lignocellulose conversion.

CRediT authorship contribution statement

Kai Lin: Investigation, Methodology, Formal analysis, Writing – original draft. **Ao Xia:** Conceptualization, Writing – review & editing. **Yun Huang:** Investigation. **Xianqing Zhu:** Formal analysis. **Xun Zhu:** Resources, Writing – review & editing. **Kaiyong Cai:** Writing – review & editing. **Zidong Wei:** Writing – review & editing. **Qiang Liao:** Conceptualization, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.128775.

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