

Synthesizing Magnetic Support for Laccase Immobilization for the Purification of Pre-hydrolysis Liquor

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Laccase immobilization is a promising method for the purification of pre-hydrolysis liquor (PHL) for large-scale applications based on recyclability and reusability. In this work, magnetic supports made of Fe₃O₄/SiO₂ were synthesized by a sol-gel method and coated with silica. Then, glutaraldehyde was used as a linker between the support and laccase for immobilization. The immobilized laccase was evaluated by purifying the PHL from a kraft-based dissolving pulp production process. The results showed that the optimal immobilization conditions were a molar TEOS/Fe₃O₄ ratio of 0.6, glutaraldehyde concentration of 6%, laccase concentration of 0.4 mL/mg, and time of 3 h, which led to 71% laccase immobilization. The concentration of total sugar in the PHL increased from 71% to 75%, while the lignin content decreased from 15% to 10% after the immobilized laccase treatment. The separation advantage of the magnetic support enhanced the utility of the laccase.

Keywords: Magnetic support; Laccase; Immobilization; Recovery

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INTRODUCTION

Fractionation of lignocellulose is a promising strategy to utilize natural biological resources for the production of diversified products, in addition to pulp and paper (van Heiningen 2006; Fan *et al.* 2017). Kraft-based dissolving pulp production fits well into this strategy by separating hemicellulose, lignin, and cellulose into different streams (Wang *et al.* 2014a). Aside from the high-grade dissolving pulp and energy generated from recovery furnaces, the hemicellulose in pre-hydrolysis liquor (PHL) is not well utilized for generating value-added products because of the presence of lignin. The chemical and physical properties of lignin severely hinder the efficiency of the removal technique because of a high content of phenolic hydroxyl groups and overlapping of the molecular weight distribution with oligosaccharides (Wang *et al.* 2015).

The use of laccase to transform the chemical and physical properties of lignin is effective for many reasons, such as a high selectivity, biocompatibility, energy savings, and environmental compatibility (Gouveia *et al.* 2013). Laccase contains copper atoms, which display a wide range of reactivities with orthodiphenols, paradiphenols, aminophenols, polyphenols, polyamines, lignin, and some inorganic ions (Wang *et al.*

2012; Fu *et al.* 2013; Wang *et al.* 2014a). Laccase has many applications, such as in bio-transformation, medicine, food, and wastewater (Deng *et al.* 2008; Mattinen *et al.* 2008; van de Pas *et al.* 2011). However, the cost of laccase is prohibitively high considering the low recovery and stability.

Fortunately, the immobilization of laccase onto insoluble supports provides an alternative way to recycle and reuse this material. Many types of supports have been employed, such as chitosan, nanoparticles, alginate, nylon, and activated carbon (Zhu *et al.* 2007). Xiao *et al.* (2006) immobilized laccase onto amine-terminated magnetic supports to increase the stability. Lu *et al.* (2007) used laccase immobilized by alginate-chitosan microcapsules for dye de-colorization and reported that the loading efficiency and immobilization yield of the immobilized laccase were 88.1% and 46.9%, respectively. Wang *et al.* (2010) found that the use of functional Cu^{2+} -chelated large-pore magnetic mesoporous silica nanoparticles led to a laccase recovery activity of 81.5% at a laccase concentration of 0.1 mg/mL. The activity of lipases was increased when it was immobilized on alkyl silane-modified magnetic nanoparticles (Wang *et al.* 2012). Xu *et al.* (2013) used chitosan poly-composite nano-fibrous membranes to immobilize laccase for 2,4-dichlorophenol removal, which resulted in a removal of 87.6%. There is a current and urgent demand to develop an effective magnetic support for laccase immobilization, especially to remove lignin from PHL in kraft-based dissolving pulp production.

The objective of this study was to characterize the magnetic support features of $\text{Fe}_3\text{O}_4/\text{SiO}_2$ and evaluate its performance in PHL purification. Various tetraethoxysilane (TEOS)/ Fe_3O_4 mole ratios and glutaraldehyde concentrations were employed to optimize the laccase immobilization ratio. The stability of the immobilized laccase was tested over a range of pH values and temperatures. A kinetic study of the immobilized laccase was performed. Finally, the immobilized laccase and recycled laccase was used to purify PHL.

EXPERIMENTAL

Materials

The PHL was collected from a kraft-based dissolving pulp production line from the Shandong Sun Paper Industry Company (Yanzhou, China). Laccase from *Trametes versicolor* was purchased from Sigma-Aldrich (Shanghai, China). Iron chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), ferrous chloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$), isopropanol, TEOS, acetic acid, and sodium acetate were all of analytical grade and purchased from Kelvin Chemical Company (Jinan, China). The chemical (3-Aminopropyl) triethoxysilane (APTES) was purchased from Aladdin Industrial Corporation (Shanghai, China). Deionized (DI) water was used for all of the experiments.

Preparation of the $\text{Fe}_3\text{O}_4/\text{SiO}_2$ magnetic support

The Fe_3O_4 was synthesized by a chemical precipitation method (Kang *et al.* 1996). In 100 mL of DI water, 2.16 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 0.796 g of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ were dissolved in an oil bath at 80 °C and 1000 rpm under an N_2 atmosphere. Then, 10 mL of ammonia were added to the flask, and the conditions were kept constant for 1 h. The resulting Fe_3O_4 particles were collected using an external magnet and washed with 50 mL of DI water. The $\text{Fe}_3\text{O}_4/\text{SiO}_2$ was fabricated according to a modified sol-gel method (Park *et al.* 2005). Initially, a certain amount of Fe_3O_4 particles was dissolved in isopropanol, and the pH was adjusted to 11 using ammonium hydroxide. The TEOS was added to the solution

and reacted for 12 h at 25 °C and a shaking speed of 150 rpm. The mole ratios of TEOS/Fe₃O₄ were 0.4, 0.6, 0.8, and 1.0. The resulting Fe₃O₄/SiO₂ was separated with an external magnet and washed with 50 mL of DI water.

Modification of the Fe₃O₄/SiO₂ magnetic support

Amine modification of the Fe₃O₄/SiO₂ composite was performed according to Huang *et al.* (2005). One gram of Fe₃O₄/SiO₂ composite was dispersed in 100 mL of ethanol. Then, 2 mL of APTES and 10 mL of DI water were added to the solution. The reaction was performed at 25 °C and 150 rpm for 12 h. Once completed, the composite was separated with an external magnet and washed with 50 mL of DI water to remove any excess APTES.

Laccase immobilization

The Fe₃O₄/SiO₂-NH₂ composite was crosslinked with glutaraldehyde solution at 25 °C and 150 rpm for 3 h. The glutaraldehyde concentration was set at 4%, 6%, 8%, and 10%. Then, the excess glutaraldehyde was washed with DI water in the presence of an external magnet. Subsequently, a certain amount of laccase was dissolved in the DI water, and the resulting magnetic support was added and kept at 25 °C and 150 rpm for 3 h. Once immobilization was completed, the immobilized laccase was separated with an external magnet and washed with DI water until no protein was detected in the eluate.

Methods

Fourier transform infrared (FTIR) spectroscopy was performed using a Nexus 670 instrument (Thermo Nicolet, Waltham, MA, USA). Each spectrum was recorded over a frequency range of 500 cm⁻¹ to 4000 cm⁻¹ using a potassium bromide (KBr) disc. The KBr was previously oven-dried to reduce the interference of water (Wang *et al.* 2014b).

The laccase activity of the immobilized and free laccase was determined by FTIR as described by Mansfield (2002) at 420 nm ($\epsilon = 36000 \text{ M}^{-1}\text{cm}^{-1}$), a pH of 4.5, and 20 °C with 0.5 mM ABTS as the substrate. One activity unit was defined as the amount of enzyme that oxidized 1 μmol of ABTS per min. The laccase immobilization efficiency was calculated using Eq. 1,

$$E (\%) = (A_t - A_f) / A_t \times 100\% \quad (1)$$

where E is the laccase immobilization efficiency (%), A_t is the total activity of the laccase added to the immobilization process (U), and A_f is the activity of the free laccase after immobilization (U).

The kinetic parameters of the Michaelis-Menten equation for the free and immobilized laccase were determined by measuring the initial rates of the reaction at a constant temperature and pH (Wang *et al.* 2010). The kinetic constants (K_m and V_{max}) were obtained by fitting the data to the Michaelis-Menten equation using non-linear regression.

For the laccase treatment of the PHL, 10 mL of PHL was added to a 100-mL flask. Then, 1 U/mL of the immobilized laccase was added to the flask, and the reaction occurred in an incubator shaker (ZWY-240, Zhicheng, Shanghai, China) for 2 h. The temperature and pH were 40 °C and 3.6, respectively. The lignin content of the PHL was analyzed by ultraviolet-visible spectrophotometry (UV-2550, Shimadzu, Shanghai, China) at 205 nm. The concentration of the sugar in the PHL was determined with an ion chromatography unit (IC5000+, Thermo Fisher Scientific, Inc., Sunnyvale, USA)

equipped with a Dionex CarboPacTMPA 20 column (Sunnyvale, USA). The total sugar was determined after acid hydrolysis of the PHL at 121 °C for 1 h to convert the oligo-sugars to mono-sugars. The surface area was calculated by the standard Brunauer-Emmett-Teller (BET) method, based on the adsorption data over the relative pressure (p/p_0) range of 0.06 to 0.18.

RESULTS AND DISCUSSION

Scheme of the Synthesis and Immobilization Procedure

Figure 1 shows the scheme of the magnetic support preparation and laccase immobilization process. Initially, the magnetite microspheres were synthesized from $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ through a chemical precipitation method. The magnetite microspheres were coated with a silica layer through a sol-gel approach using TEOS to obtain the magnetic support composites, which were designated as $\text{Fe}_3\text{O}_4/\text{SiO}_2$. The calculated BET surface area was 335 m^2/g . The magnetic support composites were modified by APTES for amino group installation, which could react with glutaraldehyde to form a Schiff-base linkage and provide a terminal aldehyde, and was designated as $\text{Fe}_3\text{O}_4/\text{SiO}_2\text{-NH}_2$. Finally, the laccase was immobilized onto the $\text{Fe}_3\text{O}_4/\text{SiO}_2\text{-NH}_2$ composites through Schiff-base linkages by glutaraldehyde.

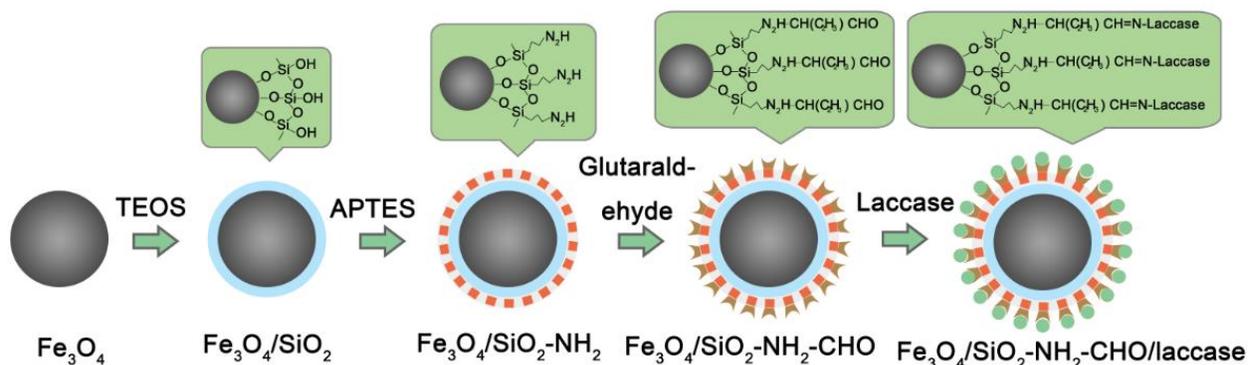


Fig. 1. Scheme for immobilizing laccase onto the $\text{Fe}_3\text{O}_4/\text{SiO}_2$ magnetic support

FTIR Spectra of the Synthesized Substrates

Figure 2 shows the FTIR spectra for the three prepared substrates. All of the chemical identification was done according to the literature (Hu *et al.* 2012; Chang and Tang 2014). The peaks at 1634 cm^{-1} and 3428 cm^{-1} corresponded to bending vibrations and stretching vibrations of hydroxyls, respectively, for all three substrates. The peaks at 800 cm^{-1} and 1084 cm^{-1} were attributed to Si-O-Si symmetrical stretching vibration and antisymmetric stretching vibration, respectively, in the $\text{Fe}_3\text{O}_4/\text{SiO}_2$ and $\text{Fe}_3\text{O}_4/\text{SiO}_2\text{-NH}_2$ spectra, which confirmed that SiO_2 was successfully coated onto the composites. The bending vibration of Si-OH was found at 967 cm^{-1} . The hydroxyls on the surface of the Fe_3O_4 reacted with SiO_2 during the preparation of $\text{Fe}_3\text{O}_4/\text{SiO}_2$, which caused the decrease in the hydroxyl stretching vibration peak at 3428 cm^{-1} in the $\text{Fe}_3\text{O}_4/\text{SiO}_2$ spectrum. The 1579 cm^{-1} peak in the $\text{Fe}_3\text{O}_4/\text{SiO}_2\text{-NH}_2$ spectrum was attributed to the bending vibration of N-H in the $-\text{NH}_2$. This confirmed the linkage of amino groups onto the surface of the SiO_2 layer.

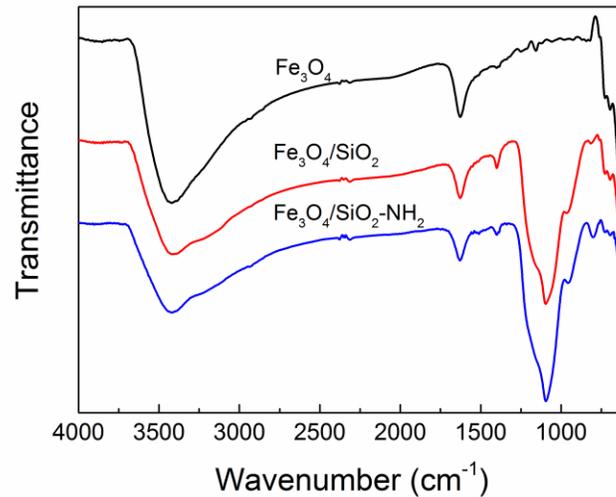


Fig. 2. FTIR spectra of the prepared Fe_3O_4 , $\text{Fe}_3\text{O}_4/\text{SiO}_2$, and $\text{Fe}_3\text{O}_4/\text{SiO}_2\text{-NH}_2$ composites

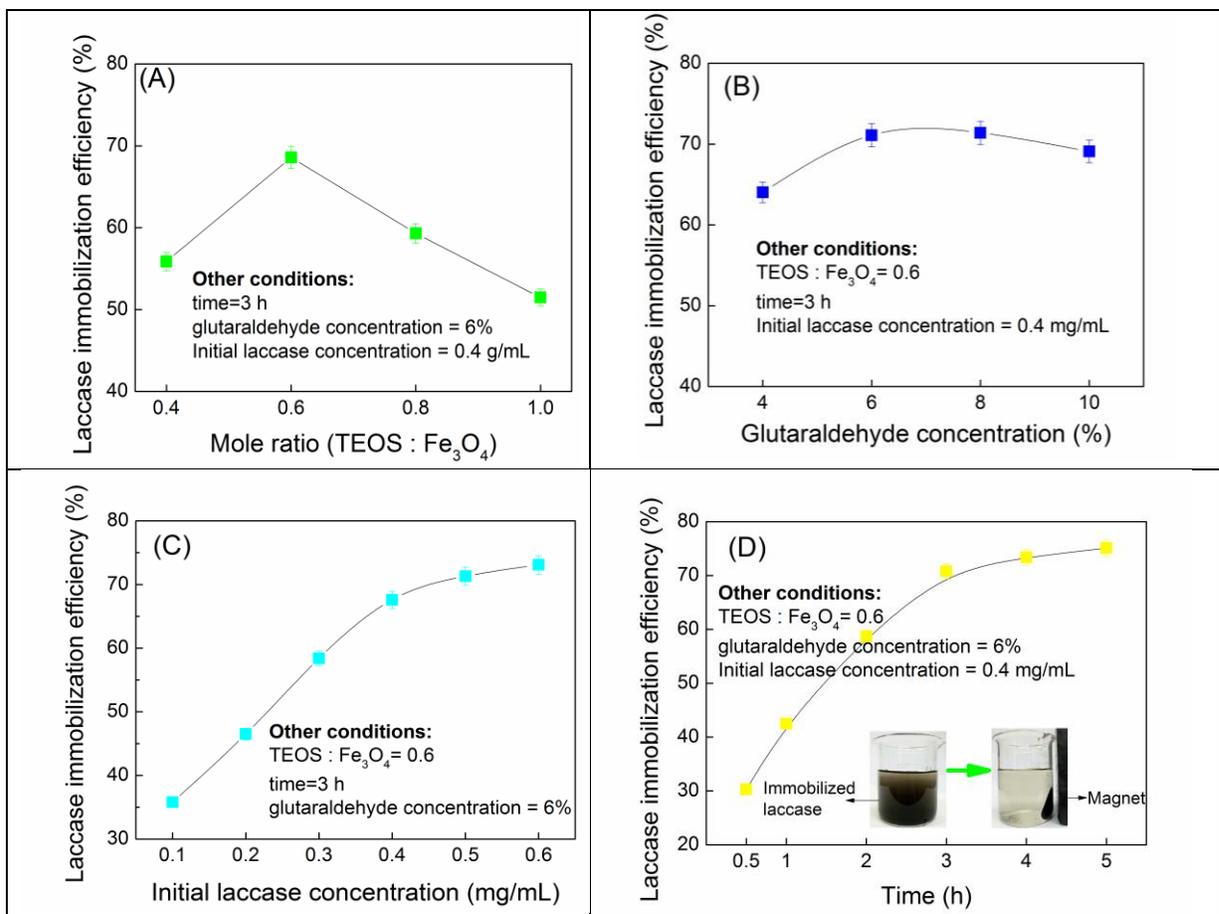


Fig. 3. Effect of the (A) TEOS/ Fe_3O_4 mole ratio, (B) glutaraldehyde concentration, (C) initial laccase concentration, and (D) time (inserted picture shows the removal of immobilized laccase with a magnet) on the laccase immobilization efficiency

Optimized Conditions for Laccase Immobilization

The effects of various parameters on the laccase immobilization efficiency are shown in Fig. 3. Figure 3A shows that the laccase immobilization efficiency increased initially, and then dropped sharply at mole ratios above 0.6. Chen *et al.* (2005) found that the magnetic particle diameter increased sharply at first and then decreased by varying the mole ratio of TEOS and Fe₃O₄ in the range of 0.2 to 0.8 when characterization immune-magnetic nuclei. The immobilization efficiency of the laccase increased initially, and then decreased with an increasing glutaraldehyde concentration (Fig. 3B). The maximum immobilization efficiency was 71% with a glutaraldehyde concentration of 6%. This was because of an optimal amount of aldehyde groups being present on the surface of the magnetic composites. For example, when the amount of aldehyde groups was low, the stability of the immobilized laccase was poor. In contrast, with a high amount of aldehyde groups, a loss of laccase activity for the deactivation effect on the enzyme protein was evident. Figure 3C shows that the laccase immobilization efficiency increased sharply at first, and then it reached a plateau when the initial laccase concentration was higher than 0.4 mg/mL. This may have been caused by the saturation of immobilization sites. Figure 3D indicates that 4 h was the optimum time to reach the maximum laccase immobilization efficiency (71%). It should be noted that in the picture inserted in Fig. 3D, which shows the immobilized laccase was easily recovered from the liquid phase by an external magnet, the separation process was so quick that this material has great potential in industrial applications. Similarly, Zhu *et al.* (2007) also found that the immobilization process reached plateau at a certain enzyme concentration and time when using mesoporous silica spheres to immobilize laccase.

Relative Activity of the Immobilized Laccase

The relative activities of the free and immobilized laccase at different pH values and temperatures are shown in Fig. 4. The free and immobilized laccase exhibited a maximum relative activity at a pH of 4. The immobilized laccase showed a relatively higher activity in comparison with the free laccase over the pH range of 2 to 10. Figure 4B shows a maximum relative activity at 40 °C.

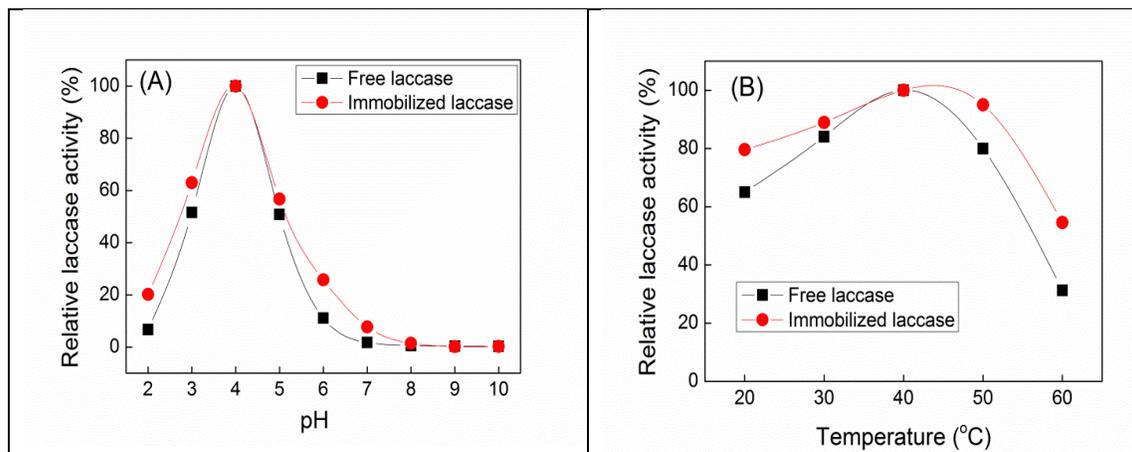


Fig. 4. Effect of the (A) pH and (B) temperature on the relative activity of the free and immobilized laccase

As the temperature increased, both laccases showed a decrease in activity. The immobilized laccase retained a higher activity over the temperature range of 20 °C to 60 °C compared with the free laccase. The improved stability of the immobilized laccase was also confirmed by Wang *et al.* (2010) when using magnetic mesoporous silica nanoparticles for laccase immobilization. The relatively higher pH stability and broader temperature for the immobilized laccase increases its application potential.

Kinetic Study of the Immobilized Laccase

A kinetic study based on the Michaelis-Menten equation was performed. Figure 5 shows that the K_m of the immobilized laccase was 0.87 mmol/L, which was approximately two times higher than that of the free laccase. The K_m indicates the accessibility between the enzyme and substrate. If it is lower, accessibility is more favorable. Immobilization reduces the active sites of laccase, which decreases the laccase accessibility. Liu *et al.* (2012) found a greatly increased K_m for immobilized lipase in comparison with free lipase during the treatment of white-water from paper mills. In contrast, the V_{max} of the immobilized laccase was higher than that of the free laccase, which may have been caused by the effective adsorption of substrates onto the immobilized laccase.

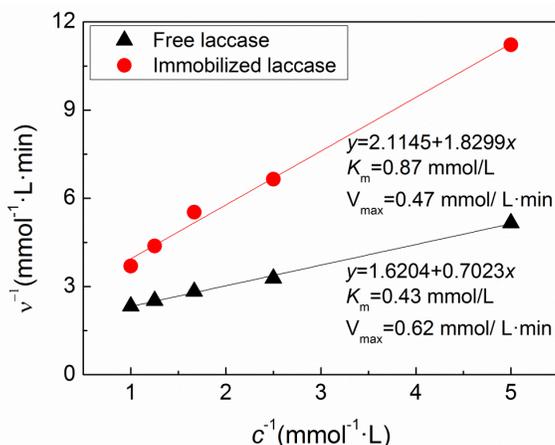


Fig. 5. Lineweaver-Burk plot of the free and immobilized laccase

Evaluation of the Immobilized Laccase

The immobilized laccase was used to purify the PHL from kraft-based dissolving pulp production. Figure 6 shows that the total sugar was the major component of the PHL and accounted for 71% of all of the organic material. The lignin and acetic acid contents were 15% and 13%, respectively. The purification of the sugar composition, such as xylitol and xylooligosaccharide, is a prerequisite for down-stream utilization. It should be noted that furfural was not included in Fig. 6 because of the low content (< 1 g/L). After the fresh immobilized laccase treatment of the PHL, the total sugar content increased to 75% and the lignin content decreased to 10%, while the acetic acid was only marginally affected. Interestingly, it was found that the sample that was treated with recycled laccase showed comparable results; the contents of the total sugar and lignin were 74% and 11%, respectively. Considering the easy recovery process, the immobilized laccase showed a great potential in large-scale applications for lignin removal from PHL. However, the total sugar purity of the treated PHL still needs to be increased. Therefore, other sequential approaches, such as activated carbon adsorption and resin treatment (Shen *et*

al. 2013; Chen *et al.* 2017), should be integrated into this process to further remove inhibitors.

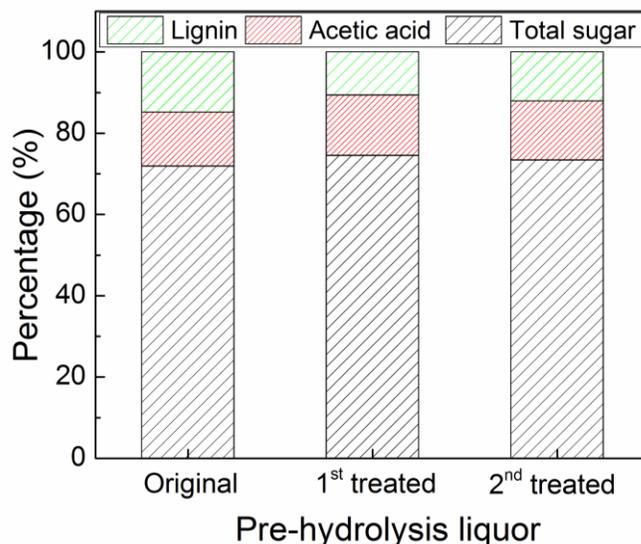


Fig. 6. Composition of the PHL before and after the immobilized laccase treatment; fresh immobilized laccase was used with the 1st treated sample and recycled immobilized laccase was used with the 2nd treated sample; other conditions were 1 U/mL, 2 h, 40 °C, and a pH of 3.6

CONCLUSIONS

1. Magnetic Fe₃O₄/SiO₂ supports were synthesized through a sol-gel method, which could immobilize the laccase efficiently *via* Schiff-base linkages using glutaraldehyde. The optimal laccase immobilization conditions were a TEOS/Fe₃O₄ mole ratio of 0.6 and a glutaraldehyde concentration of 6%. These conditions led to 71% laccase immobilization.
2. The pH and thermal stabilities of the immobilized laccase were improved to some extent compared with the free laccase. The K_m was 0.87 mmol/L for the immobilized laccase, which was higher than that of the free laccase (0.43 mmol/L).
3. The total sugar content in the PHL increased from 71% to 75% after the immobilized laccase treatment. The immobilized laccase displayed rapid separation with an external magnetic field, and thus showed great potential in terms of the purification of PHL with easy recyclability and reusability.

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