A first report on competitive inhibition of laccase enzyme by lignin degradation intermediates

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Abstract

Laccases have been widely explored for their ligninolytic capability in bioethanol production and bioremediation of industrial effluents. However, low reaction rates have posed a major challenge to commercialization of such processes. This study reports the first evidence of laccase inhibition by two types of lignin degradation intermediates – fungal solubilized lignin and alkali treated lignin, thus offering a highly plausible explanation for low reaction rates due to build up of inhibitors during the actual process. Reversed-phase high-performance liquid chromatography revealed the presence of similar polar compounds in both lignin samples. A detailed kinetic study on laccase, using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) as substrate was used to calculate the Michaelis constant (K_m) and maximum reaction rate (V_{max}). With an increase in the concentration of lignin degradation intermediates, V_{max} remained nearly constant, while K_m increased from 1.3 to 4.0 times that of pure laccase, revealing that the inhibition was competitive in nature. The kinetic studies reported here and the insight gained into the nature of inhibition can help design process strategies to mitigate this effect and improve overall process efficiency. This work is applicable to processes that employ laccase for delignification of biomass - such as second-generation biofuels processes, as well as for industrial effluent treatment in paper and pulp industries.

Keywords: Laccase activity, fungal solubilized lignin, alkali treated lignin, competitive enzyme inhibition.

Introduction

Laccases (EC 1.10.3.2) are a group of multi-copper oxidase enzymes, that have recently been explored for various applications such as delignification of biomass for biofuel production, bio-bleaching in the paper and pulp industry, bioremediation of industrial effluents, as well as in surface modification of wood fibers (Couto and Herrera 2006; Brijwani et al. 2010; Cannatelli and Ragauskas 2017; Agrawal et al. 2018). Laccases have been found in several lignin-degrading microorganisms such as ascomycetes, basidiomycetes, and deuteromycetes (Shraddha et al. 2011; Plácido and Capareda 2015). They play an important role in the biodegradation of lignin by breaking down the phenolic residues and are also capable of degrading the non-phenolic residues in the presence of small mediator molecules (Wong 2009; Roth and Spiess 2015). However, low rates of delignification and considerably long processing times have been major challenges in microbial and enzymatic delignification processes that have prevented scale-up and commercialization (Plácido and Capareda 2015).

In our lab, we compared the extent of lignin degradation in sugarcane bagasse (~25% lignin) and cocopeat (~50% lignin) using two microorganisms - *Neurospora discreta*, a locally isolated fungus, and *Phanerochaete chrysosporium*, a well-known white rot fungus (Pamidipati and Ahmed 2017). In both cases, the extent of delignification was found to be less than 50%, and the activity of laccase dropped to levels too low to be detected towards the end of the process. Literature also reports studies with other white rot fungi wherein laccase activities decrease as lignin degradation proceeds (Kerem et al. 1992; Gupte et al. 2007).

In order to improve reaction rates, several studies have been conducted on the effect of inducers or cofactors on activities of lignin-degrading enzymes (Palmieri et al. 2000; Wang et al. 2016). However, little is known about the reasons for low enzyme activities during biomass degradation. While laccase inhibition due to the presence of mercury ions (Juárez-Gómez et al. 2018), pH and chloride ions (Raseda et al. 2014), sulfhydryl organic compounds (Johannes and Majcherczyk 2000), free radicals (Kurniawati and Nicell

2009) as well as certain bulky organic compounds such as medicarpin (Bertrand et al. 2017; Cannatelli and Ragauskas 2017) have been reported, in-process lignin degradation intermediates have never been explored as potential inhibitors of laccase. Reports of water-soluble high molar mass intermediates of lignin, called acid precipitable polymeric lignin (APPL) during delignification of woody biomass by bacterial laccase exist (Borrmeyer and Crawford 1985; Fisher and Fong 2014; Singh et al. 2017), but not in the context of laccase inhibition. As laccases are extracellular enzymes, such water-soluble lignin degradation intermediates could potentially cause inhibition, thereby explaining the decrease in enzyme activity as the process proceeds.

The present study was designed to address this gap in the literature and to explain the decreased laccase activities seen in our lab. The study was based on the hypothesis that intermediates of lignin generated during lignin degradation inhibit laccase activity, thereby causing decreased delignification rates. This hypothesis was tested using lignin degradation intermediates from two sources: (1) lignin degradation products generated during fungal pre-treatment and (2) commercially available lignin generated by alkaline pre-treatment of lignocellulosic biomass. These were chosen to represent lignin degradation products obtained during microbial as well as chemical pre-treatment methods. The nature of inhibition was also evaluated in this study using the Michaelis-Menten model. This is a first report on the inhibition of laccase by lignin degradation intermediates and provides a plausible explanation for the low rates of delignification seen in actual processes that utilize laccase.

Materials and Methods

Laccase Activity

Activity of laccase (source: *Trametes versicolor*, Sigma-Aldrich, Product No. 51639) was determined by measuring the oxidation kinetics of ABTS (Make: Himedia, Product No: 9720). Among all substrates of laccase, including guaiacol, 2,6-dimethoxyphenol and syringaldazine (Tinoco et al. 2001; Lorenzo et al. 2005), ABTS is one of the most popular substrates due to the robustness of its assay and stability of the

product formed. As the reaction with ABTS is much faster compared to actual delignification, the use of ABTS as a substrate also enables the study of lignin degradation products as potential inhibitors. Due to these reasons, ABTS was used as the substrate for the present study. The concentration of the green-blue cation radical formed from oxidation of ABTS was calculated from the spectral time scans noted at 420 nm and correlated to the enzyme activity using the extinction coefficient (ε_{420}) 36000 L/mol per cm. As the optimal pH range for laccase enzyme from *T. versicolor* is between pH 4.5 to 5, 100 mmol/L citrate buffer (pH 5) was used for the study (Cardinal-Watkins and Nicell 2011). One unit of enzyme activity was defined as the amount of enzyme forming 1 µmol of product per min at standard assay conditions (U/L) (Saito et al. 2003; Wang et al. 2016). ABTS concentrations were varied between 0.03 mmol/L - 0.3 mmol/L.

Laccase Inhibition Studies

Laccase activity was measured in the presence of varying concentrations of lignin degradation intermediates, which included both fungal solubilized lignin and alkali lignin samples described above. The final concentrations of the inhibitors were between 80-800 mg/L. For each concentration, to 0.6 mL of 15 mg/L pure laccase enzyme made in 100 mmol/L pH 5 citrate buffer, 0.3 mL of inhibitor sample was added and equilibrated at room temperature overnight. For controls, 0.3 mL of deionized water was added instead of the liquid supernatant. To this mixture, 0.3 mL of pH 5 citrate buffer and 0.6 mL of varying concentrations of ABTS substrate was added and the increase in absorbance was noted at 420 nm. As lignin is known to be a free radical scavenger (Dizhbite et al. 2004; García et al. 2012), a separate study was carried out to rule out any interference in the assay due to the interaction between the lignin intermediates and the ABTS cation radical that forms in the laccase assay. For this, alkali treated lignin was added to a pre-formed ABTS cation radical and the absorbance was noted and compared with that of the cation radical itself. Multiple repeats of these two cases showed no significant difference in the absorbance values, indicating that there was no interference in the assay from lignin intermediates.

Fungal solubilized lignin: *Neurospora discreta*, a locally isolated fungal strain reported in our previous paper (Pamidipati and Ahmed 2017) was used for generating solubilized lignin degradation intermediates from cocopeat. Submerged fermentations were set-up using cocopeat, at 10 g/L and 33 g/L biomass loadings using Vogel's minimal media (Vogel 1964). The flasks were aseptically inoculated and incubated for 30 d at 30^{o}C as described previously (Pamidipati and Ahmed 2017). At the end of the fermentation, liquid fractions were filtered, centrifuged and the clear supernatant sample was used as fungal solubilized lignin. The supernatant samples contained negligible amounts of sugars analyzed using 3,5-Dinitrosalicylic acid reagent, indicating that the primary composition of the sample was solubilized lignin. Moreover, the supernatant samples were heat treated to prevent interference from inherent enzyme activity.

Chemically-treated alkali lignin: Commercially available alkali treated lignin (Kraft lignin) obtained by treating lignocellulosic biomass with high alkaline sulphide solutions (Hu et al. 2018), was procured from Sigma-Aldrich (CAS No. 8068-05-1). Alkali lignin was dissolved in 100 mmol/L pH 5 citrate buffer to maintain optimum pH for enzyme activity.

Reversed Phase High-performance liquid chromatography (RP-HPLC)

Both the fungal solubilized lignin as well as the alkali treated lignin samples were analyzed on a C-18 RP-HPLC column using an acetonitrile-water solvent system. The total run time was 35 min with a flow rate of 1 mL/min and a sample injection volume of 25 µL. A gradient elution with the following concentrations of acetonitrile was used to differentiate between polar and non-polar compounds of solubilized lignin: at 0 min— 0 %; 5 min— 3 %; 10 min—6 %; 15 min—10% and 20 min—20 %, 25 min— 40% and 35 min – 80%. The compounds were detected at 254 nm using a photodiode array detector and the data was integrated and analyzed using the Shimadzu Automated Software system. Alkali treated lignin was used as the standard, and a calibration curve was plotted over a concentration range of 1000 mg/L - 5000 mg/L. The inhibitor concentration was calculated from the chromatogram area corresponding to the peak at 2.7 min retention time (RT).

Results and Discussion

Analysis of the lignin samples using a C-18 column on RP-HPLC indicated that both the fungal solubilized lignin and alkali treated lignin contained a major peak at the RT of 2.7 min (Fig 1), indicating the presence of highly polar lignin degradation intermediates in both samples. In addition to this, alkali treated lignin showed another peak at RT of 27 min which was absent in the fungal solubilized lignin sample.

Mass spectra of the two distinct peaks were obtained using LC-MS (Online Resource: Fig S1) and the average molar mass values were calculated. The average molar mass values of the highly polar lignin degradation intermediates at RT 2.7 min in alkali treated lignin and in fungal solubilized lignin were 2110 and 2189 g/mol respectively (Online Resource: Table S1), which were in close agreement with values reported in the literature for alkali treated lignin (Andrianova et al. 2018). The peak corresponding to RT 27 min found only in alkali treated lignin had an average molar mass of 819 g/mol indicating the presence of lignin intermediates that are lower in molar mass as well as polarity compared to the intermediates eluting at RT of 2.7 min.

The effect of these lignin degradation intermediates was evaluated on commercially available pure laccase using ABTS as substrate. At 0.03 mmol/L ABTS, the reaction rates of laccase were found to be lower in the presence of both fungal solubilized lignin and alkali treated lignin, compared to that of pure laccase (Fig 2), indicating enzyme inhibition.

To investigate the nature of inhibition, a kinetic study was carried out by measuring reaction rates of the pure laccase enzyme (v) and the inhibited enzyme (v_{inh}) at varying substrate (S) and inhibitor (I) concentrations as discussed in the Materials and Methods section. Michaelis-Menten model (equations 1 and 2) was used for data-fitting and obtaining kinetic parameters (Shuler and Kargi 2002). Michaelis

constants, K_m & $K_{m,app}$ and maximum reaction rate V_{max} were obtained for the pure and inhibited enzyme from Lineweaver–Burk plots. (Fig 3a and 3b).

$$\frac{1}{v} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}} \quad ---------(1)$$

$$\frac{1}{v_{inh}} = \frac{K_{m,app}}{V_{max,app}}\frac{1}{[S]} + \frac{1}{V_{max,app}} \quad ----(2)$$

For pure laccase, K_m (from the slope of the line in Fig 3) was found to be 75.4 ± 2 µmol/L (39 ± 2 mg/L), similar to the values reported in the literature: K_m was reported to be 59 µmol/L for *T.versicolor* laccase enzyme using ABTS as substrate by (Tinoco et al. 2001) and 38 µmol/L by (Lorenzo et al. 2005). In the presence of increasing concentrations of lignin degradation intermediates, the value of K_m, (denoted by $K_{m,app}$) increased. However, there was no significant difference between V_{max} of pure laccase (59 ± 6 U/L), and the V_{max} in the presence of alkali treated lignin (61 ± 9 U/L) and fungal solubilized lignin (55 ± 10 U/L). The increase in K_m and the nearly constant V_{max} in the presence of lignin degradation intermediates (Table 1) indicate a decrease in affinity of laccase enzyme for the substrate in the presence of the inhibitor, i.e., competitive inhibition. Interestingly, as seen in Fig 3 and Table 1, inhibition by both sources of lignin degradation intermediates i.e., fungal solubilized as well as alkali treated lignin was similar at simlar values of inhibitor concentrations, as calculated from the area under the 2.7 min peak (based on alkali treated lignin as discussed in Materials and Methods). This confirms that the 2.7 min peak, consisting of highly polar constituents, is the primary cause of inhibition. Had there been a contribution from the 27 min RT peak found only in alkali lignin, the extent of inhibition due to alkali lignin would have been much greater as compared to fungal solubilized lignin. The value of the dissociation constant of the enzyme-inhibitor complex K_I, which represents affinity of the enzyme to the inhibitor molecules can be calculated from the inverse of the slope of the plot of $K_{m,app}/K_m$ vs inhibitor concentration (I) (Fig 4). The slopes were

approximately 0.0037 for fungal solubilized lignin and 0.004 for alkali treated lignin, yielding K_I of 270 mg/L and 250 mg/L respectively, indicating similar levels of affinity of laccase for both types of lignin.

Table 1 Inhibitor concentration, kinetic constants and % inhibition for lignin degradation intermediates

Samples	Inhibitor conc'n from HPLC (mg/L)	$K_{m,app}$ (mg/L)	% Inhibition $[(v-v_{inh})/v]$	
			at S = 10 µmol/L	at S = 100 µmol/L
Laccase + Alkali treated lignin-1	86	49 ± 4	20%	10%
Laccase + Fungal solubilized lignin-1	135	51 ± 2	20%	11%
Laccase + Alkali treated lignin-2	164	60 ± 1	33%	14%
Laccase + Fungal solubilized lignin-2	179	64 ± 5	36%	20%
Laccase + Alkali treated lignin-3	362	84 ± 2	52%	29%
Laccase + Fungal solubilized lignin-3	379	83 ± 2	51%	45%
Laccase + Alkali treated lignin-4	486	116 ± 1	64%	42%
Laccase + Fungal solubilized lignin-4	779	169 ± 2	75%	64%

Delignification of biomass by laccase involves multiple steps, and has an added layer of complexity as some of the lignin degradation intermediates can also serve as substrates of laccase. Laccase activity can also be affected by various other factors and process conditions as delignification proceeds. However, the present study reveals that the build-up of lignin-degradation intermediates can further lower the activity of laccase by competitive inhibition, and this information can lead to the design of process strategies to prevent severe inhibition. Furthermore, competitive inhibition is usually overcome by increasing the substrate concentration, thereby increasing the probability of enzyme-substrate binding and decreasing the effect of the inhibitor (Shuler and Kargi 2002). Evidence of this is also seen in Table 1, where the extent of inhibition is lower at higher concentrations of the substrate (ABTS). However, it must be noted that in actual delignification processes, the substrate for laccase is the lignin polymer, and increasing its concentration would lead to an increase in the concentration of the lignin degradation intermediates, thus ironically, compounding the problem of laccase inhibition. Therefore, to overcome inhibition, the build-up of the

lignin degradation intermediates should be prevented in the culture broth by alternate strategies such as intermittent dilution using fresh medium as in a fed-batch culture, or a recycle system to remove the intermediates continuously from the system. The extent of inhibition shown in Table 1 can provide an estimate on the threshold concentrations below which the inhibitor levels should be maintained.

Conclusion

The present study demonstrates the first kinetic evidence of competitive inhibition of laccase by in-process intermediates generated during the degradation of lignin. This result is timely and significant as it provides insight into one of the key reasons for low rates of delignification seen in microbial and enzyme-based processes involving laccases. Moreover, as the inhibition is competitive in nature, specific process strategies can be employed to improve delignification rates by overcoming the inhibition. As the inhibition model tested applied to both fungal solubilized lignin as well as alkali treated lignin, this work is applicable to both second generation biofuel processes utilizing lignocellulosic biomass residues as well as to bioremediation of industrial effluents where laccase enzymes are used. A detailed characterization of the lignin degradation intermediates could provide further insight into the molecular mechanism of laccase inhibition.

Online Resource

Analysis of lignin intermediates by liquid chromatography-mass spectrometry (LC-MS). **Fig. S1** Mass spectra in positive ESI mode of the highly polar lignin intermediates of fungal solubilized and alkali treated lignin samples. **Table S1** Average molar mass values of lignin degradation intermediates

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Conflict of Interest: The authors declare that they have no conflict of interest.

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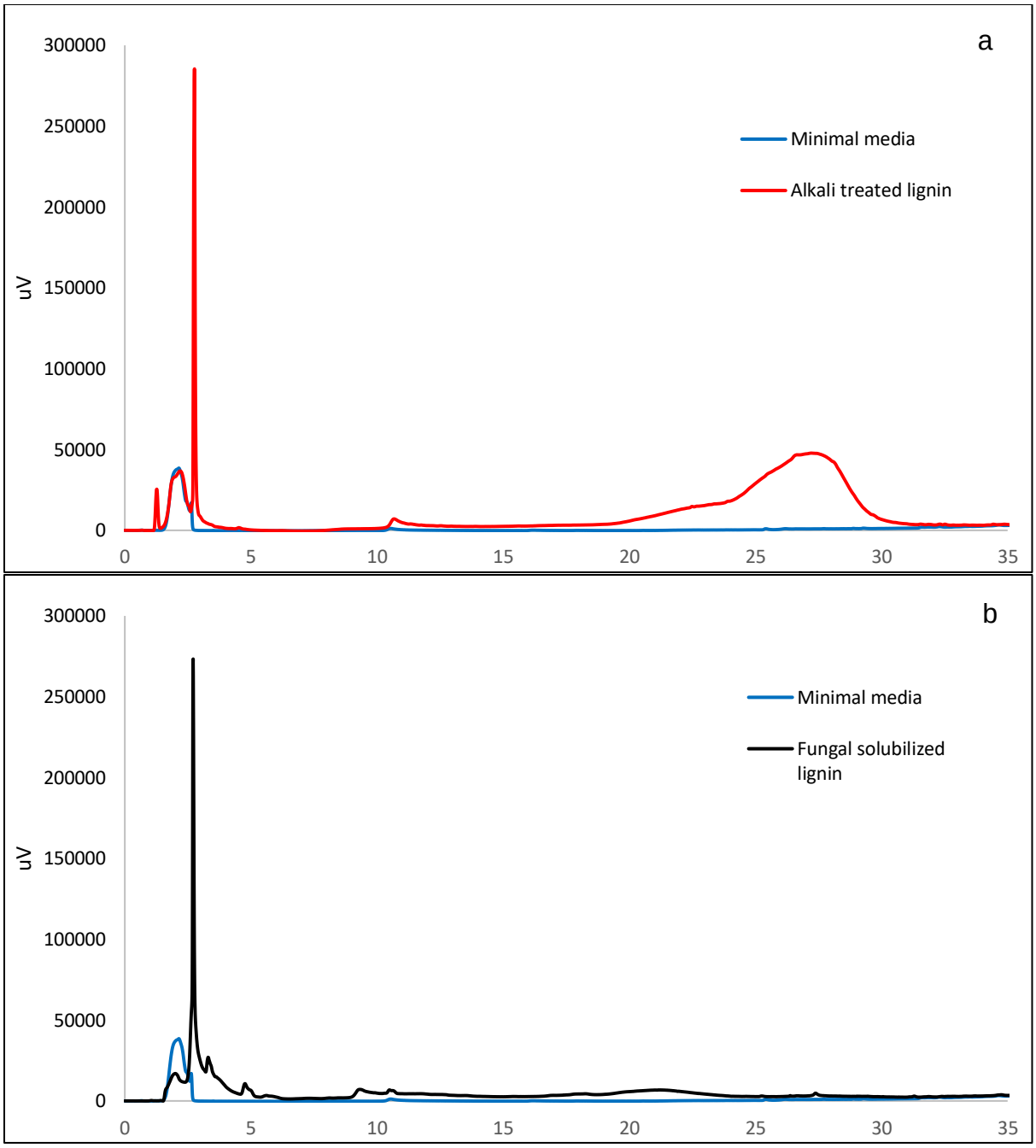
List of Figure Captions

Fig 1 RP-HPLC chromatograms of alkali treated lignin (a) and fungal solubilized lignin (b). The medium in which both samples were prepared was run as a blank (black).

Fig 2 Representative rate plot showing laccase activity with and without lignin degradation intermediates.

Fig 3 Lineweaver–Burk plots of pure laccase enzyme and inhibited laccase enzyme in the presence of varying concentrations of alkali treated lignin (a) and fungal solubilized lignin (b).

Fig 4 Calculation of dissociation constant K_I.



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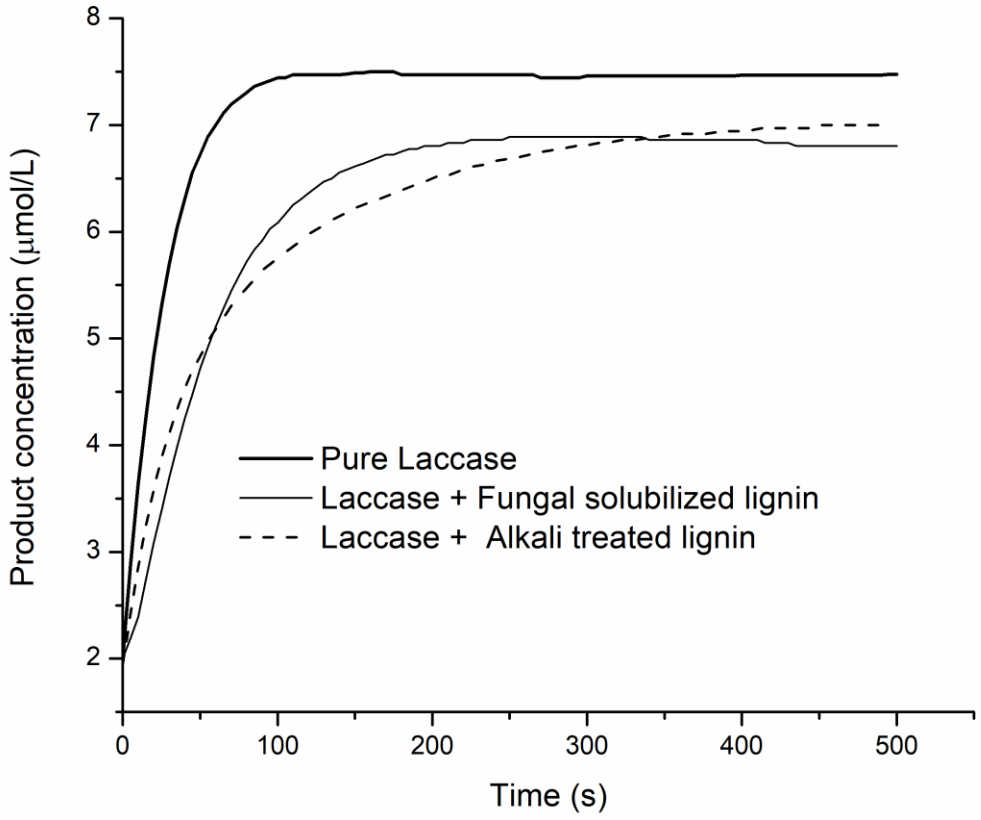

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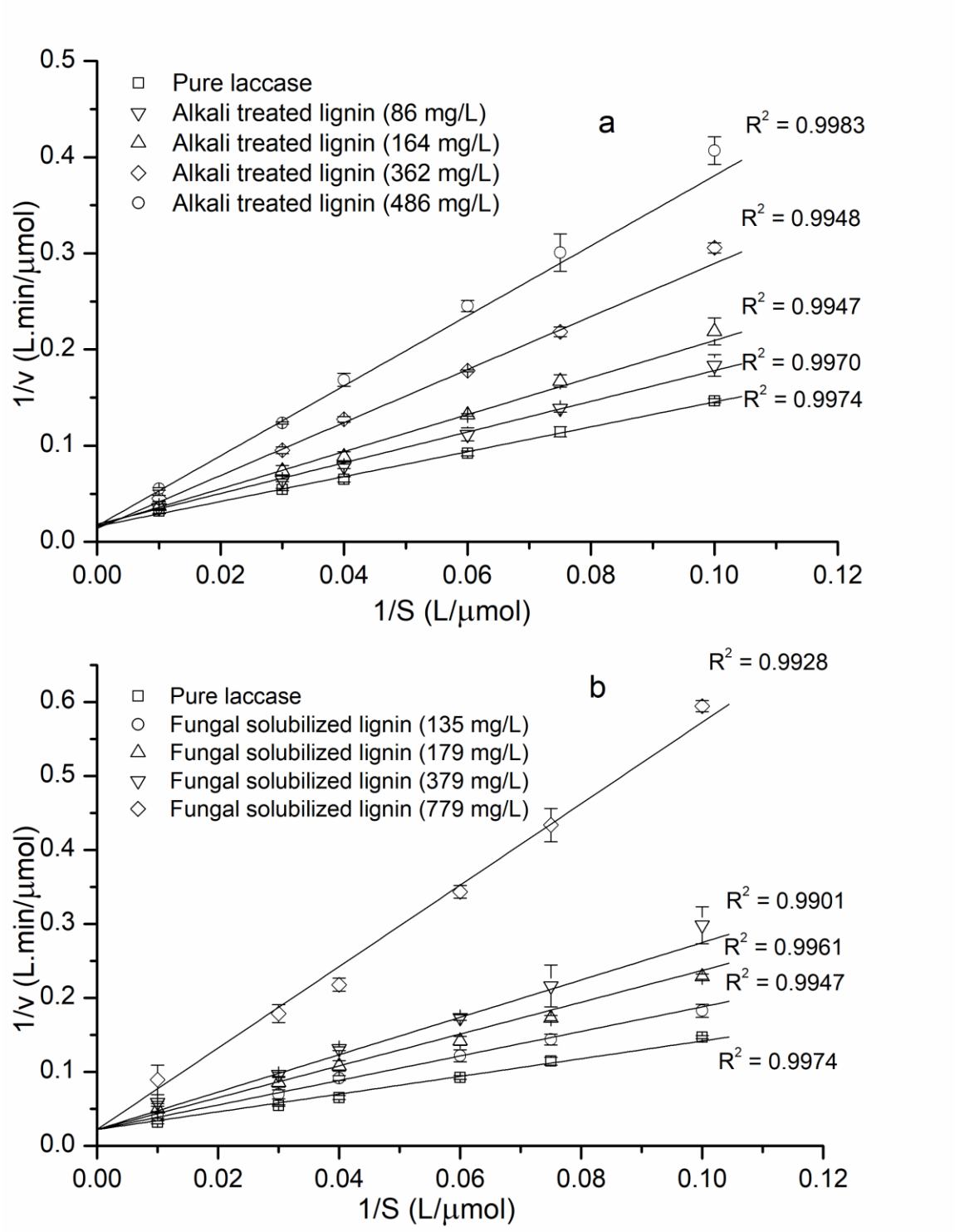

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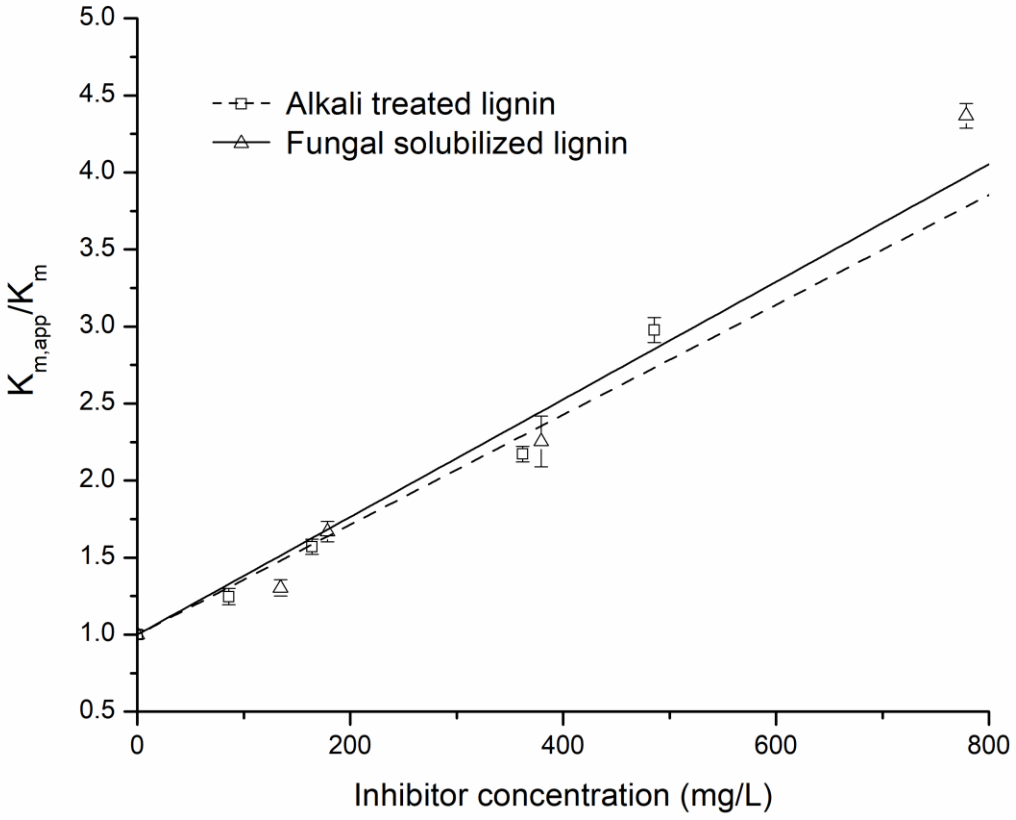

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