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ARTICLE

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Isothermal titration calorimetric assessment of lignin conversion by laccases

Shams T. A. Islam[1] | Jie Zhang[2] | Fabio Tonin[1] | Renske Hinderks[1] | Yanthi N. Deurloo[1] | Vlada B. Urlacher[3] | Peter-Leon Hagedoorn[1]

[1]Department of Biotechnology, Delft University of Technology, Delft, The Netherlands

[2]Chongqing Engineering Research Center for Processing, Storage and Transportation of Characterized Agro-Products, College of Environment and Resources, Chongqing Technology and Business University, Chongqing, China

[3]Institute of Biochemistry, Heinrich-Heine University Düsseldorf, Düsseldorf, Germany

Correspondence
Peter-Leon Hagedoorn, Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629 HZ Delft, The Netherlands.
Email: P.L.Hagedoorn@tudelft.nl

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Abstract

Lignin valorization may offer a sustainable approach to achieve a chemical industry that is not completely dependent on fossil resources for the production of aromatics. However, lignin is a recalcitrant, heterogeneous, and complex polymeric compound for which only very few catalysts can act in a predictable and reproducible manner. Laccase is one of those catalysts and has often been referred to as an ideal "green" catalyst, as it is able to oxidize various linkages within lignin to release aromatic products, with the use of molecular oxygen and formation of water as the only side product. The extent and rate of laccase-catalyzed lignin conversion were measured using the label-free analytical technique isothermal titration calorimetry (ITC). IITC provides the molar enthalpy of the reaction, which reflects the extent of conversion and the time-dependent power trace, which reflects the rate of the reaction. Calorimetric assessment of the lignin conversion brought about by various fungal and bacterial laccases in the absence of mediators showed marked differences in the extent and rate of conversion for the different enzymes. Kraft lignin conversion by *Trametes versicolor* laccase followed Michaelis–Menten kinetics and was characterized by the following thermodynamic and kinetic parameters $\Delta H_{ITC} = -(2.06 \pm 0.06) \cdot 10^3$ kJ mol^{-1}, $K_M = 6.6 \pm 1.2$ μM and $V_{max} = 0.30 \pm 0.02$ U/mg at 25°C and pH 6.5. We envision calorimetric techniques as important tools for the development of enzymatic lignin valorization strategies.

KEYWORDS
enzyme kinetics, isothermal titration calorimetry, laccase, lignin

1 | INTRODUCTION

Biorefineries create lignin waste streams, the amount of which is expected to exceed 200 megatons by 2022 (Bruijnincx et al., 2016). These waste streams are mainly burnt to produce energy and utilized commercially only in a limited manner (2%) although it could serve as a source of valuable aromatics (S. Xie et al., 2016). The chemical industry is currently completely dependent on fossil resources to satisfy the demand for aromatics and the valorization/depolymerization of lignin could offer a sustainable alternative for the future (Roberts & Caserio, 2021). The move to using lignin waste streams could render the biorefineries more economically viable as lignin can

gain up to 10 times in value upon valorization (Nanayakkara et al., 2014).

Lignin's structure is complex as it is a heterogeneous polymer made of aromatic units (Figure 1); *p*-coumaryl, coniferyl, and sinapyl alcohol monomers are coupled together with mostly ether and C-C linkages (de Gonzalo et al., 2016; Den et al., 2018; Venkatesagowda & Dekker, 2020). The resulting lignin polymer constitutes different proportions of p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units. The inherent heterogeneity, arising due to the multiple types of bonds that may form among the monomeric groups, leads to the recalcitrant nature of the lignin (S. Xie et al., 2016).

Only a few biocatalysts act upon lignin in a predictable and reproducible manner (Bruijnincx et al., 2016; Bugg & Rahmanpour, 2015; Cajnko et al., 2021; Christopher et al., 2014; de Gonzalo et al., 2016; Pollegioni et al., 2015; Venkatesagowda & Dekker, 2020). Among the enzymes which act upon lignin, laccases are important in nature to catalyze lignin conversion by plants, fungi, and bacteria (de Gonzalo et al., 2016; Mate & Alcalde, 2017; Pollegioni et al., 2015; Rodgers et al., 2010; Thurston, 1994). Other oxidative enzymes, such as lignin peroxidases (LiPs), manganese peroxidases (MnPs), and versatile peroxidases (VPs), are extracellular and use H_2O_2 as co-substrate whereas laccases use molecular oxygen. LiP generally possesses a very high redox potential of the active site heme cofactor (1.2 V vs. NHE at pH 3.0) compared to the other enzymes (Kersten et al., 1990). This property enables it to catalyze the oxidation of non-phenolic aromatic compounds directly, without the need for a mediator. Laccases from plants are generally involved in polymerization (i.e., production of lignin), and those from fungi and bacteria are generally involved in depolymerization (i.e., degradation of lignin), which reflects their physiological role (Abdel-Hamid et al., 2013; Zakzeski et al., 2010). For this study, the focus was on lignin conversion brought about by fungal and bacterial laccases to add to the body of knowledge regarding lignin depolymerization/valorization in a more economical and sustainable manner.

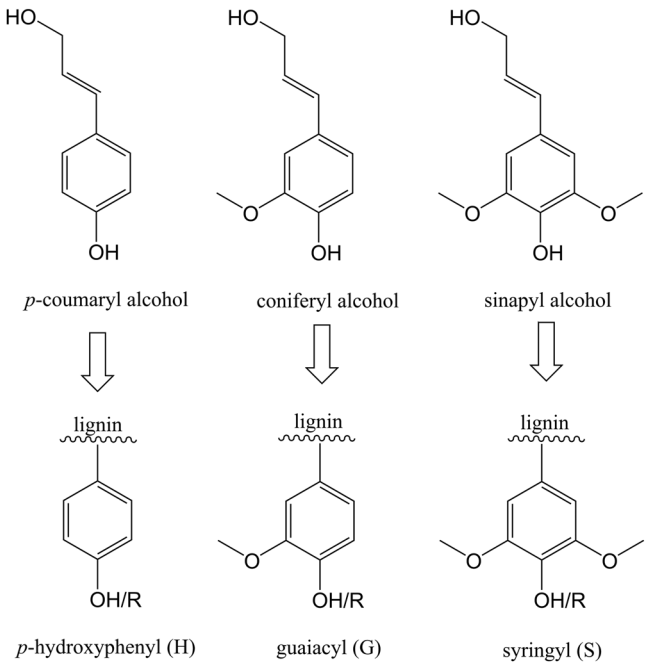

FIGURE 1 The basic monomeric units of lignin. These units are linked to one another to make lignin. The amount of each monomer and the type of linkages among them, depend upon the source of lignin, its treatment, and handling (Bugg & Rahmanpour, 2015)

Oxidation by laccases is brought about with molecular oxygen and low-molecular weight products, aromatic in nature, are formed with water as side product. Laccase possesses moderately high redox potentials of the active site T1 Cu center (0.42–0.79 V) and can oxidize, in principle, only phenolic residues of lignin directly. Thus, in most studies of laccase-catalyzed lignin conversions, mediators have been used to mitigate the challenges associated with the large size of lignin and the high redox potential of the non-phenolic reactions (Cañas & Camarero, 2010; Christopher et al., 2014; Hilgers et al., 2018; Hilgers, Kabel, et al., 2020; Hilgers, van Erven, et al., 2020; Rich et al., 2016; Zhu et al., 2020). Laccase catalyzes the one-electron oxidation of the mediator to cation radicals which subsequently react with the high redox potential groups within bulky lignin. ABTS (2,2′-azino-bis(3-ethybenzothiazoline-6-sulphonic acid)) has been frequently used as a mediator to enhance the substrate scope of the enzyme (Hilgers et al., 2018; Könst et al., 2013). Mediators also prevent re-polymerization of the phenoxy radical products of laccase (Bourbonnais et al., 1995). Most mediators, however, show side-reactions with substrates and products and even inactivate enzymes in certain cases (Christopher et al., 2014; Hilgers et al., 2018). Thus, in this report, lignin conversion by laccases was studied without the addition of mediators.

For measuring the rates of enzymatic reactions with difficult, that is, complex and/or non- (or partially) soluble substrates, such as lignin, many of the conventional assay techniques are not suitable. Generally, the change in color or fluorescence of the substrates or the products are followed in time—the absence of such can be tackled by using labeling, coupled reactions, or chemical analysis. However, labeling or using coupled enzymatic or chemical reactions is not always possible and may require extensive optimization for each reaction. Over the past two decades, several non-spectrophotometric label-free methods to measure enzyme activity have been developed (Das et al., 2002; He et al., 2014; Hennig et al., 2007; Leung et al., 2013; Lu et al., 2014; Orosco et al., 2009; Syahir et al., 2015) and among them, isothermal titration calorimetry (ITC) allows reactions to be followed based upon a universal thermodynamic parameter, the molar enthalpy of the catalyzed reaction, ΔH (Bianconi, 2007; Todd & Gomez, 2001).

In an enzyme-catalyzed reaction, the total heat generated (or consumed) is dependent on many factors, but, if the temperature and pH are well controlled, it is proportional to the total conversion that takes place under the influence of the enzyme and the molar enthalpy of the reaction itself. The ITC set-up contains a sample cell and a reference cell and the temperature difference among them is monitored continually using a thermoelectric device. Any observed difference is corrected by a PID (proportional integral derivative) controller which changes the power supplied to the sample cell to maintain a constant temperature difference, for example, an exothermic reaction in the sample cell is countered by a lowering of the power to compensate for the heat generated. The area

under the curve of the power supply to maintain isothermal conditions for the duration of the experiment, gives information about the total heat released/consumed during the experiment. The molar enthalpy is calculated based on the principles of Wiseman from this area under the curve (Wiseman et al., 1989).

Furthermore, with proper calibrations, the power readings during calorimetric experiments are reflections of the heat flows which, in turn, are directly proportional to the rates of the reaction (Hobbs et al., 2013; Honarmand Ebrahimi et al., 2015; Watt, 1990). However, in practice, the measured heat-flow is not a real-time reflection of the rate of the enzyme. The instrumental response time has to be taken into account, which has not always been properly done in literature (Todd & Gomez, 2001). An empirical solution to this problem is the IrCal method (Honarmand Ebrahimi et al., 2015) which is based on the fact that the initial ITC power data can be used to obtain an apparent initial rate of a reaction. An empirical calibration constant can be obtained for the ITC setup using a standard enzyme-catalyzed reaction carried out both in the ITC device and a conventional assay setup, for example, a spectrophotometer, under the same reaction conditions. This empirical calibration constant can subsequently be used for reporting the rates of other reactions catalyzed by their respective enzymes within the same ITC setup.

Since the rate of an enzyme-catalyzed reaction increases linearly with the enzyme concentration, the ITC power changes that occur upon adding a specific concentration of substrate to different enzyme concentrations also correlate linearly. Under conditions of the same temperature, pH, and enzyme concentration, changes in the rates of the enzyme-catalyzed reactions can be brought about by changing the concentration of the substrate and these would be reflected in the ITC power measurements. As reaction rates brought about by changes in substrate concentration follow appropriate rate laws describing the enzyme kinetics, the power measurements may thus be a means, in principle, of determining the appropriate rate laws (Honarmand Ebrahimi et al., 2015). To summarize, enzyme calorimetry using ITC and IrCal gives us two parameters: (1) the area under the curve—reflecting the total conversion (and equilibrium) and the molar enthalpy of the reaction and (2) the power trace over time which reflects the rate of the reaction and which can be further utilized to determine appropriate kinetic parameters of the enzyme-catalyzed reaction.

Here we present the study, with ITC and IrCal method of analysis, of various bacterial and fungal laccases acting upon lignin, without the use of mediators. ABTS oxidation was used as a reference substrate for laccase as the formation of the blue-green colored $ABTS^{\bullet+}$ radical can be followed by both UV-visible spectrophotometry and ITC (Honarmand Ebrahimi et al., 2015).

2 | EXPERIMENTAL

2.1 | Materials

The substrate lignin (alkali, low sulfonate, ~10 kDa molecular weight (Mw)) and ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) were purchased from Sigma-Aldrich. The fungal enzyme *Trametes versicolor* laccase (65 kDa Mw protein; Mayolo-Deloisa et al., 2015) was also purchased from Sigma-Aldrich while the *Myceliophthora thermophila* laccase (61 kDa Mw protein; Ernst et al., 2018) was obtained from Novozymes. His-tagged *Bacillus subtilis* CotA laccase (59.3 kDa Mw protein) was recombinantly expressed and isolated as previously described (Koschorreck et al., 2017). Alkaline phosphatase and *p*-nitrophenylphosphate were sourced from Bioké and Fluka, respectively. Sodium and potassium phosphate were purchased from Sigma-Aldrich to make buffers by dissolving required amounts in MilliQ purified water (Millipore). Phosphate-buffered saline (PBS) was prepared by dissolving 10 mM NaCl in 100 mM potassium phosphate buffer, pH 5.8.

2.2 | Methods

2.2.1 | Molar enthalpy determination of *T. versicolor* laccase-catalyzed ABTS conversion reaction with ITC

The multi-injection method with a VP-ITC instrument (Malvern) was applied as reported previously (Honarmand Ebrahimi et al., 2015) to measure the molar reaction enthalpy of *T. versicolor* laccase acting upon ABTS ($\Delta H_{r,\ ABTS}$). The sample cell was filled with 0.06 µM *T. versicolor* laccase in PBS buffer pH 5.8 and during the titration experiment, small aliquots of a 5 mM ABTS solution were injected. Experimental parameters were set as follows: temperature 34°C, reference power 12 µcal s^{-1}, initial delay 100 s, stirring speed 502 rpm, high feedback mode, cell concentration 0.06 µM *T. versicolor* laccase in PBS pH 5.8, syringe concentration 5 mM ABTS in PBS pH 5.8, total injections 6, injection volume 14 µl except for the first injection which was 2 µl to serve as the dummy injection. The small initial dummy injection is necessary as the volume of the first injection is not precise due to the concave meniscus at the tip of the needle and diffusion at the liquid-liquid interface. The duration for each injection was set at 28 s and the spacing between injections was 1800 s (30 min). A control experiment without any enzyme, but only the buffer solution, in the cell was carried out to obtain the heat of dilution for the ABTS solution.

2.2.2 | Initial rate measurements of ABTS conversions by *T. versicolor*, measured with UV-visible spectroscopy and ITC

The rate of ABTS oxidation by *T. versicolor* laccase was measured spectrophotometrically (Cary 60, Agilent) at 34°C according to Mishra and Kumar (2007) with some modifications. The assay solution (1.0 ml) contained 850 µl 0.06 µM of *T. versicolor* laccase in PBS pH 5.8 and various amounts of 1.0 mM ABTS (in PBS pH 5.8), with the balance provided by buffer volume adjustments, to give final concentrations from 0 to 125 µM. The absorbance at 420 nm during the first 60 s was measured and the molar extinction coefficient at

420 nm $\varepsilon_{420} = 36{\cdot}10^3\,M^{-1}\,cm^{-1}$ for $ABTS^{\bullet+}$ was used to calculate the activity (Mishra & Kumar, 2007). One unit (U) of laccase activity was defined as the amount of laccase that oxidizes 1 µmol ABTS per min. The specific activities were expressed in units per milligram protein ($U\,mg^{-1}$).

Initial rate measurements of ABTS oxidation by *T. versicolor* laccase were performed with ITC using the IrCal method (Honarmand Ebrahimi et al., 2015). The syringe of the set-up was filled with prepared ABTS solutions of 1, 5, and 20 mM in PBS pH 5.8, and injected in different volumes (3 µl [1 mM]; 3, 6, and 9 µl [5 mM]; 3, 6, 9, and 12 µl [20 mM]) to ensure varying final concentrations of ABTS in the cell at different runs (0, 2, 11, 21, 32, 42, 85, and 127 µM). The durations of injections varied as a result of injection of various volumes. The sample cell was filled with 0.06 µM *T. versicolor* laccase in PBS, pH 5.8. ITC experimental parameters were set as follows: reference power of 1 µcal/s, no feedback mode, temperature 34°C, stirring speed 502 rpm, filter period 2 s, total injections 1, initial delay 100 s. The power traces were monitored for 1200 s (20 min). Data analysis was performed according to the IrCal method as explained in more detail in Figure S1.

The IrCal calibration constant was determined using the hydrolysis reaction of *p*-nitrophenylphosphate (1–210 µM) by 5 nM alkaline phosphatase by both UV-visible spectroscopy and ITC (Figure S2). The calibration constant of 0.059 was used to report the initial rate measurements for ABTS conversions brought about by *T. versicolor* laccase by ITC. Plots of rates with varying concentrations were fitted to find the appropriate rate law using Origin 2019 software.

2.2.3 | Laccase activity measurements with ABTS

The activities of three different laccases (bacterial *B. subtilis* laccase and fungal *T. versicolor* and *M. thermophile* laccases) were measured spectrophotometrically using ABTS as substrate at 25°C according to Mishra and Kumar (2007) using a Cary 60 spectrophotometer (Agilent). The assay solution (1.0 ml) contained 950 µl of 1.0 mM ABTS in 0.1 M sodium phosphate buffer pH 6.5 and 50 µl of 0.20 mg ml^{-1} *T. versicolor* laccase/0.007 mg ml^{-1} *B. subtilis* laccase/0.003 mg ml^{-1} *M. thermophile* laccase. The increase of the absorbance at 420 nm was followed in time for at least 1 min and the activity was determined as described in Section 2.2.2.

2.2.4 | Molar enthalpy determination of lignin conversions by various laccases

The multi-injection method with a VP-ITC instrument (Malvern) was applied as reported previously (Honarmand Ebrahimi et al., 2015) to measure the molar reaction enthalpies of *T. versicolor*, *M. thermophila*, and *B. subtilis* laccases acting upon lignin ($\Delta H_{r,\ lignin}$). An aliquot of 0.5 g L^{-1} lignin solution (in 0.1 M sodium phosphate solution pH 6.5) was introduced in the syringe of the ITC set-up. To add similar enzyme amounts, 0.259 U (ABTS oxidation activity) of each enzyme was used for the molar enthalpy and initial rate measurements with ITC.

Experimental parameters were set as follows: temperature 25°C, reference power 20 µcal s^{-1}, initial delay 100 s, stirring speed 459 rpm, high feedback mode, the cell concentration of 15.57 µM *T. versicolor* laccase or 0.03 µM *B. subtilis* laccase or 0.05 µM *M. thermophila* laccase, each corresponding to 0.259 U ABTS oxidation activity, syringe concentration 0.05 mM lignin (assuming 10 kDa Mw), total injections 6, injection volume 14 µl except for the first injection which was 2 µl to serve as the dummy injection. The duration for each injection was set at 28 s and the spacing between injections was 2700 s (45 min). A control experiment without any enzyme, but the buffer solution, in the cell was carried out to obtain the heat of lignin solution dilution.

2.2.5 | Initial rate measurements of lignin conversions with various laccases

The initial rates of reactions of the various laccases in this study were derived by analyzing data from the enthalpy measurements conducted as outlined in Section 2.2.4, by the IrCal method (Honarmand Ebrahimi et al., 2015). Here, 15.57 µM *T. versicolor*, 0.03 µM *B. subtilis*, and 0.05 µM *M. thermophila* laccases were present in the ITC sample cell during separate experimental runs to which aliquots of 0.5 g/L lignin solution were injected. It should be mentioned that subsequent injections yielded identical power traces during each set, implying that the enzyme quantity was sufficient to bring about the full conversion of the lignin injected each time.

The initial rate measurements were further used to verify the rate law appropriate to the catalysis of lignin by *T. versicolor* laccase. The syringe of the set-up was filled with the prepared lignin solutions of 0.3 and 2 mM which were injected in different volumes (3, 6, and 9 µl [0.3 mM]; 3, 6, 9, and 12 µl [2 mM]) to ensure varying final concentrations of lignin in the cell (0.64, 1.27, 1.91, 4.24, 8.49, 12.7, and 17.0 µM) at different runs. The sample cell was filled with *T. versicolor* laccase solution (0.06 µM, in 0.1 M sodium phosphate, pH 6.5). The feedback mode, the stirring speed, and the reference power were set as mentioned in Section 2.2.4. The initial delay before injection of substrate was set to 600 s and other experimental parameters were set as follows: temperature 25°C, total injections 1. The power traces were monitored for 1200 s (20 min). Analysis of data was performed according to the IrCal method (Figure S1).

3 | RESULTS

3.1 | Validation of the ITC enzyme assays using the conversion of the chromogenic substrate ABTS by *T. versicolor* laccase

To validate the ITC enzyme assay, the oxidation of the chromogenic substrate ATBS by *T. versicolor* laccase was measured calorimetrically

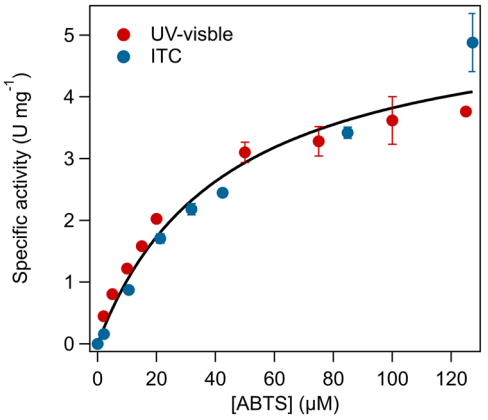

FIGURE 2 Michaelis–Menten fit of *Trametes versicolor* laccase-catalyzed oxidation of ABTS, as measured using UV-visible spectrophotometry (red closed circles) and ITC (blue closed circles). The activities of 0.06 μM of *T. versicolor* laccase with a range of ABTS concentrations (0–125 μM) as measured by both techniques gave a global Michaelis–Menten fit with $K_M = 43.2 \pm 7.9$ μM and $V_{max} = 5.5 \pm 0.4$ U/mg (34°C and pH 5.8)

using the ITC IrCal method and the conventional UV-visible spectrophotometric assay. ABTS is a non-phenolic heterocyclic compound that is known to undergo one-electron oxidation to its cation radical. The activity of *T. versicolor* laccase toward varying concentrations of ABTS as measured by UV-visible spectrophotometry and ITC showed similar Michaelis–Menten kinetics with global fit kinetic parameters $K_M = 43.2 \pm 7.9$ μM and a $V_{max} = 5.5 \pm 0.4$ U/mg (Figure 2). A heat of dilution for ABTS at pH 5.8°C and 34°C $\Delta H_{dil} = +6.96$ kJ mol^{-1} was observed. The observed molar enthalpy change of ABTS conversion by laccase at pH 5.8 and 34°C was $\Delta H_r = -42.2 + 0.9$ kJ/mol. The parameters were obtained by using the IrCal calibration constant of 0.059, which was independently determined (Figure S2). Since the kinetics of ABTS oxidation could be reliably determined using the ITC IrCal method, we shall be able to use the same method to determine the kinetics of oxidation of other substrates as well.

3.2 | Normalizing the activity of the different laccases

In order to measure the amount of heat generated upon their reaction with lignin using ITC, it was imperative that the amount of each laccase was somehow normalized to allow comparison. To add a similar amount of the different laccases in the ITC experiments, ABTS oxidation activity was chosen to normalize the different enzymes. The three different laccases of this study were therefore analyzed for their activities toward ABTS using a conventional spectrophotometric assay under identical experimental conditions (Table 1). The different enzymes exhibited three orders of magnitude differences in specific activity at 25°C. The rate was assumed to be close to V_{max} at an ABTS concentration of 0.95 mM, at least for *T. versicolor* laccase (Figure 2). The nearly 20-fold difference with the rate in Table 1 and the V_{max} in

TABLE 1 The specific activities as measured by oxidation of 0.95 mM ABTS by three laccases

	Laccases		
	T. versicolor	*M. thermophila*	*B. subtilis*
Specific activity (U/mg)[a]	0.186 ± 0.007	56.8 ± 1.2	103.9 ± 0.9

[a]The assay solution (1.0 ml) contained 950 μl of 1.0 mM ABTS in 0.1 M sodium phosphate buffer, pH 6.5 and 50 μl of the enzyme sample. The absorbance at 420 nm was monitored to follow the formation of the product of reaction, $ABTS^{\bullet+}$ at 25°C. One unit (U) of laccase activity was defined as the amount of laccase that oxidizes 1 μmol ABTS per min.

Figure 2 can be explained by suboptimal temperature (25°C instead of 34°C) and suboptimal pH (pH 6.5 instead of 5.8). Based on the results given in Table 1, calculated volumes of the enzymes were taken to reflect 0.259 U ABTS oxidation activity of each enzyme within the ITC sample cell (1.4 ml). So for each laccase, an amount of enzyme was added that can oxidize 0.259 μmol ABTS per minute at 25°C in 0.1 M sodium phosphate buffer pH 6.5.

3.3 | Molar enthalpy determination of lignin conversion by various laccases

The power traces were obtained from the ITC experiments of laccase-catalyzed lignin conversions (Figure 3). The control reaction with buffer solution in the sample cell instead of enzyme only gave a small but sharp endothermic peak and subsequently a small broad exothermic response, which resulted in the heat of dilution for lignin $\Delta H_{dil,\ lignin} = +10.75$ kJ mol^{-1}. Lignin conversion by the different laccases showed reproducible power peaks with each subsequent injection. This shows that the reaction is complete as the power returns to the baseline after each peak and there is no product inhibition as all peaks have the same shape and amplitude. At the time of the injection, the power sharply decreases indicating the occurrence of an exothermic reaction. This is true for all three laccases and the areas under the curves give the total amounts of heat released. The values of ΔH_{ITC} were calculated from the area under the curve by the Wiseman principle (Wiseman et al., 1989). The ΔH_{ITC} of the reaction of *T. versicolor* laccase and other laccases, with lignin and with the multi-injection method, is given in Table 2. *T. versicolor* laccase gives more heat for lignin conversion than *M. thermophila* laccase which in turn gives more heat than *B. subtilis* laccase.

Whatever the precise reaction catalyzed by these enzymes, a measurement is obtained for each one in the form of the molar enthalpy for that reaction $\Delta H_{r,\ lignin}$. Furthermore, each injection and the time provided ensures a completed reaction as indicated by return to the baseline and there are no clear indications of side-reactions or inhibition by product and/or substrates. The number of phenolic OH groups in different commercially available lignin sources has been determined using ^{31}P-NMR. The reported values are between 2.7 and 4.1 mmol phenolic OH per gram lignin. For the lignin source that was used in this study one mole of lignin contains approximately

40 moles of phenolic groups (Li et al., 2018). Assuming the ITC thermograms predominantly reflect the conversion of phenolic groups we can calculate that the traces in Figure 3 reflect the whole or partial conversion of circa 20 µM phenolic groups.

Since 0.259 U ABTS oxidation activity of each laccase was used to generate the runs shown in Figure 3, the difference in the peaks directly reflect differences in rates and overall conversions brought about by them with respect to the non-phenolic model substrate ABTS. This would signify that either the laccases act upon different linkages/groups and/or upon the same groups but to different degrees. Either way, this gives us the means to quantify the extent of laccase-catalyzed lignin conversion in terms of $\Delta H_{r,\ lignin}$. Subsequently, the $\Delta H_{r,\ lignin}$ value can be used to determine the rate of this conversion (Honarmand Ebrahimi et al., 2015).

If the enzymes were indeed acting upon the same type of groups, the values of $\Delta H_{r,\ lignin}$ are explained by the fact that the *B. subtilis* to *M. thermophila* to *T. versicolor* laccases act upon increasing quantities of that type of group per lignin molecule. This is not entirely unexpected as bacterial laccases are known to have a more narrow substrate scope compared to their fungal counterparts. Fungal laccases generally having higher redox potential of the T1 Cu center within its structure which is responsible for the oxidation of the organic substrate. It is generally believed that a higher T1 Cu redox potential correlates with a broader substrate scope for laccases (Mateljak et al., 2019; Xu et al., 1996). An attempt at correlation was undertaken as shown in Table 2, which indeed shows that the laccase with the highest T1 Cu redox potential shows the most extensive laccase conversion. However, for *M. thermophila* and *B. subtilis* laccases which have a relatively close T1 Cu redox potential this correlation did not hold. This can be explained by the fact that the enzymes may not be catalyzing exactly the same reactions and there are differences in substrate binding affinity or substrate accessibility which will affect the rates and overall conversion of lignin observed here.

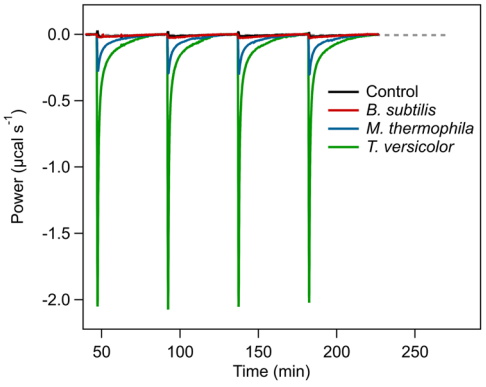

FIGURE 3 The measurement of ΔH_{ITC} of lignin conversion by three different laccases: *Trametes versicolor* (green line), *Myceliophthora thermophila* (blue line), and *Bacillus subtilis* laccase (red line). ITC experimental conditions: temperature 25°C, reference power 20 µcal s^{-1}, high feedback mode, 459 rpm stirring speed, six injections, 14 µl injection volume. The initial delay was set to 100 s to monitor any baseline drift. The syringe was filled with 0.5 g/L lignin in 0.1 M sodium phosphate buffer, pH 6.5, and the sample cell containing 0.259 U of the relevant laccase in the same buffer. The duration of the injection was set at 28 s and spacing between injections was set at 2700 s (45 min). The control experiment without enzyme was carried out to obtain the heat of dilution of lignin in buffer (dotted line)

3.4 | Initial rate measurements of lignin conversions with various laccases

Under identical conditions of 0.259 U ABTS oxidation activity of each of the enzymes reacting with 7 µg lignin per injection (equivalent to 0.495 µM lignin, considering 10 kDa Mw), the initial rates of conversions of lignin were obtained and shown in Table 3. *M. thermophila* laccase exhibited the highest rate under the conditions used, and *T. versicolor* laccase the lowest. However, *T. versicolor* laccase showed the highest extent of lignin conversion. The ratio of the lignin and ABTS conversion rates showed a much smaller variation and a different trend, which is characterized by a roughly three orders of magnitude lower lignin conversion rate as compared to the ABTS conversion rate.

The IrCal measurements of the lignin conversions brought about by *T. versicolor* laccase at different substrate concentrations also followed Michaelis–Menten kinetics (Figure 4). This resulted in a K_M of 6.6 ± 1.2 µM and V_{max} of 0.30 ± 0.02 U/mg, corresponding to a k_{cat} = 0.33 ± 0.02 s^{-1}, at 25°C and pH 6.5. Considering the fact that circa 40 moles of phenolic groups are likely to be contained by one mole of lignin, an apparent K_M for lignin phenolic groups can be estimated to be circa

TABLE 2 The ΔH_{ITC} for three laccases as measured by isothermal titration calorimetry

	Laccases		
	T. versicolor	*M. thermophila*	*B. subtilis*
$\Delta H_{r,\ lignin}$ (kJ mol^{-1})[a]	−2063 ± 60	−760 ± 16	−263 ± 12
Extent of apparent lignin conversion[b]	100 ± 3%	37 ± 2%	13 ± 1%
T1 E_m (mV)[c]	780 ± 3 (Taniguchi et al., 1982)	465 ± 10 (Xu et al., 1996)	525 ± 10 (Durão et al., 2008)

[a]An injection of 0.5 g/L of lignin (in sodium buffer, pH 6.5) was added to a sample cell containing 0.259 U of laccase solution, corresponding to 15.57 µM *T. versisolor*, 0.03 µM *M. thermophila*, or 0.05 µM *B. subtilis* laccase.

[b]Relative extent of conversion as compared to the *T. versicolor* laccase result.

[c]Reported reduction potential of the T1 copper center versus NHE.

TABLE 3 The initial rates of lignin conversion by three laccases obtained using IrCal ITC

	Laccases		
	T. versicolor	*M. thermophila*	*B. subtilis*
Initial rates (U/mg)[a]	$0.2{\cdot}10^{-3}$[b]	$16.7{\cdot}10^{-3}$	$38.7{\cdot}10^{-3}$
	(0.71%)	(43.2%)	(100%)
Ratio rate lignin/ABTS	$1.5{\cdot}10^{-3}$ (100%)	$0.3{\cdot}10^{-3}$ (19.98%)	$0.4{\cdot}10^{-3}$ (25.33%)

[a]Experimental conditions as in Table 2.

[b]Since the enzyme concentration is high compared to the substrate concentration the rate does not follow Michaelis–Menten kinetics under these conditions.

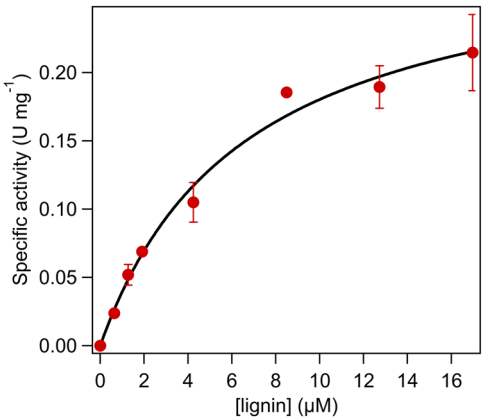

FIGURE 4 Enzyme calorimetry of *Trametes versicolor* laccase with lignin. The rates were measured using the IrCal method. The black line represents a fit to the Michaelis–Menten equation. The lignin concentration is based on a 10 kDa molecular weight

0.3 mM, which reflects a moderate affinity. The reported K_M values for the oxidation of different ortho- and para-substituted phenols by different fungal laccases are in the sub mM to mM range (Xu, 1996). The oxidation of different phenols by *T. versicolor* laccase has been shown to exhibit substrate inhibition and apparent K_M values in the sub mM to mM range depending on the nature and concentration of the different substrates (Canfora et al., 2008).

4 | DISCUSSION

The extent of the recalcitrant nature of lignin varies according to the source (e.g., softwood vs. hardwood, age of wood), treatment, and handling (Bugg & Rahmanpour, 2015; Christopher et al., 2014). It is anticipated that the full potential of lignin can be realized when efficient and viable depolymerization processes are in place to obtain the low molecular weight aromatic components which are valuable and/or which could serve as building blocks after further downstream processing (Bruijnincx et al., 2016; Bugg & Rahmanpour, 2015; Lora, 2008; S. Xie et al., 2016). Although various routes of valorization are available (Cao et al., 2018; Wendisch et al., 2018; S. Xie et al., 2016), enzymatic valorization is desirable since chemical and thermal treatments are generally harsh in comparison to conditions of enzymatic reactions and they lack specificity and efficiency.

Biocalorimetry has been used previously to measure the enzymatic action of laccases, and also to measure the biological degradation of wood (Wadsö et al., 2017). The action of the plant laccase from *Rhus vernicifera* on different small phenolic substrates has been measured using microcalorimetry (Wang et al., 2000; Wong & Yu, 1999; X. Y. Xie et al., 2002). Molar enthalpy values in the range of −40 to −550 kJ mol^{-1} were found, depending on the number of phenolic groups. The oxidation of the lignin model compound syringic acid by *Galerina* sp HC1 and *T. versicolor* laccase was measured using ITC previously (Volkova et al., 2012). A molar enthalpy of −230 kJ mol^{-1} was reported. However, this value is based on an unusually slow conversion of 8 h, after an initial sharp peak of less than 30 min. Since the authors showed independently that the reaction was complete within 30 min we attribute the very slow reaction to subsequent non-catalyzed radical polymerization reactions. According to the authors, the enthalpy of the initial 30 min reaction is 15% of the total, resulting in an apparent molar enthalpy of −35 kJ mol^{-1}.

The conversion of lignosulfonic acid by fungal *Lentinus* sp. nLcc4 laccase was measured using the ITC multi-injection approach (Maestre-Reyna et al., 2015). An apparent molar enthalpy of −105 kJ mol^{-1} was reported. The kinetic parameters for lignosulfonic acid conversion were $k_{cat} = 0.234\ s^{-1}$ and $K_M = 56.7\ \mu M$. Although lignosulfonic acid is a heterogeneous lignin-derived mixture of soluble compounds, the authors have performed docking studies with their lignosulfonic acid substrate, which suggests it has a very defined structure containing one sinapyl and one coniferyl unit and has one phenol group, two methoxy groups, and two sulfonate groups (CAS 8062-15-5).

Quantitative measurements of the reaction kinetics of fungal laccases *T. versicolor* and *Ganoderma lucidum* with monomeric lignin units and a dimeric lignin model compound OH-dilignol was performed using HPLC-MS (Perna et al., 2018). The conversion of OH-dilignol with *T. versicolor* laccase showed Michaelis–Menten kinetics with apparent kinetic parameters $K_M = 12.89\ \mu M$ and $V_{max} = 0.39\ \mu M\ min^{-1}$. Using a different MS approach the kinetics of the conversion of a labeled lignin model substrates by *T. versicolor* laccase was measured. This approach involves nanostructure-initiator mass spectrometry (NIMS), which involves surface immobilization of the labeled substrates and products and laser desorption/ionization MS analysis (Deng et al., 2018). Using this approach it was possible to determine the time-dependent formation of specific dimerization and degradation products.

Electron paramagnetic resonance (EPR) spectroscopy has been shown to be a label-free method to directly measure the kinetics of radical formation in lignin by laccases (Munk et al., 2017). The kinetics of lignin (organosolv) conversion by fungal laccases *T. versicolor*, *M. thermophila*, and *Ganoderma lucidum* has been measured using this technique. The apparent kinetic parameters based on the extent of radical formation were determined to be $V_{max} = 0.30 \pm 0.04\ \mu M\ min^{-1}$ and $K_M = 73.0 \pm 5.9$ mM lignin for *T versicolor* laccase (Perna et al., 2019). Different apparent kinetic parameters were obtained for the different laccases when compared after normalization for their syringaldazine oxidation activities. These relative kinetic parameters showed that *T. versicolor* laccase had a five to tenfold higher specific activity than *M. thermophila* and *G. lucidum* laccase. We find that the specific activity for lignin conversion by *T. versicolor* laccase is circa threefold higher than for *M. thermophila* laccase after normalization for ABTS oxidation activity (Table 3). Without normalization *M. thermophila* laccase has a circa 80-fold higher specific activity than *T. versicolor* laccase, although this represents a much lower extent of lignin conversion.

The modeling of the enzyme kinetics assuming (global) Michaelis–Menten is a simplification and more advanced kinetic modeling will be necessary to fully understand the process (Cajnko et al., 2021). Lignin degradation products have been shown to act as a competitive inhibitor for laccase catalyzed ABTS oxidation, and therefore are likely to influence the lignin oxidation kinetics as well (Pamidipati & Ahmed, 2020).

Here we present the conversion of kraft lignin (with low sulfonate content) by three different laccases, which resulted in an apparent molar enthalpy of −263 to −2063 kJ mol^{-1} depending on the type of enzyme. On the basis of the molar enthalpy values that have been previously reported for laccase oxidation of phenolic substrates (−50 to −100 kJ mol^{-1} per phenolic group), this may correspond to the conversion of 3–5 to 21–41 phenolic groups per 10 kDa unit of lignin. The large difference in the extent of conversion of the lignin substrate by the different laccases can be partially explained by the differences in their T1 Cu reduction potentials. Another explanation could be the accessibility of the substrate or substrate recognition. The performance of the enzymes at their optimal pH and temperature may be different, but here we chose to compare the enzymes under identical conditions.

The slower uncatalyzed polymerization or depolymerization reactions brought about by the phenoxy radicals are more difficult to analyze using ITC. In principle, one can distinguish the two processes, enzyme-catalyzed oxidation, and uncatalyzed radical reactions, by performing either relative short measurements (minutes) and very long measurements (hours/days). This has been observed for the oxidation of syringic acid by laccase using ITC (Volkova et al., 2012). The effect of the uncatalyzed radical reactions on the enthalpy is difficult to predict. As the polymerization and depolymerization reactions involve different reaction types, each of which can be either exothermic or endothermic (also depending on conditions), the observed thermogram will be the net result of all these contributions.

Most studies describing lignin degradation using laccase involve so-called laccase-mediator systems (Christopher et al., 2014). Mediators are small molecules that undergo one-electron oxidation to form cation radicals with some stability. The radical forms of the mediators subsequently chemically react with the different linkages in lignin. Also, small molecule lignin degradation products can act as a natural mediator. It has been suggested that the phenolic units of lignin are initially oxidized, followed by the more sluggish transformation of the non-phenolic part. We may assume that, in the absence of added mediators, laccase will only be able to oxidize phenolic lignin units within the timeframe of the measurements. Degradation of Kraft lignin using different isoforms of *T. versicolor* laccase with ABTS as mediator has been reported (Bourbonnais et al., 1995). Lignin conversion with laccase without added mediator increased the average molecular weight of the product, while it decreased after the addition of ABTS. These reactions were performed up to 6 days, so they do reflect a different timescale than the reactions reported here.

Although this study focused on laccase as a lignin converting enzyme, the outcomes have implications for both other lignin converting enzymes and enzymatic conversions of other complex polymeric substrates. ITC is a powerful tool to gain independent information on the extent and rate of conversion of complex substrates such as lignin. In principle, this method can be adapted for reporting on enzymes that catalyzes similar reactions, for example, similar enzymes from different sources or genetically engineered enzyme variants. A number of lignin-converting enzymes have been identified, including lignin peroxidase, versatile peroxidase, dye decolorizing peroxidase, and Mn peroxidase, β-etherase, C-C hydrolase, O-demethylase, and other oxidative enzymes in lignin degradation pathways (Pollegioni et al., 2015). We envision that by combining the conversion and rate information obtained by ITC with advanced analytical approaches, such as the aforementioned MS and EPR approaches as well as NMR and HPLC to establish the precise nature of the observed transformation, a very powerful approach for the biotransformation of lignin compounds and materials can be established.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Jie Zhang and Peter-Leon Hagedoorn contributed to the conception and design of the study. Shams T. A. Islam, Jie Zhang, Fabio Tonin, Renske Hinderks, Yanthi N. Deurloo performed the experiments. Shams T. A. Islam, Jie Zhang, and Peter-Leon Hagedoorn analyzed the data. Shams T. A. Islam, Jie Zhang, Vlada B. Urlacher, and Peter-Leon Hagedoorn wrote the manuscript

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available upon request from the corresponding author.

ORCID

Shams T. A. Islam https://orcid.org/0000-0001-9419-8596
Vlada B. Urlacher http://orcid.org/0000-0003-1312-4574
Peter-Leon Hagedoorn http://orcid.org/0000-0001-6342-2022

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