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Functional screening pipeline to uncover laccase-like multicopper oxidase enzymes that transform industrial lignins

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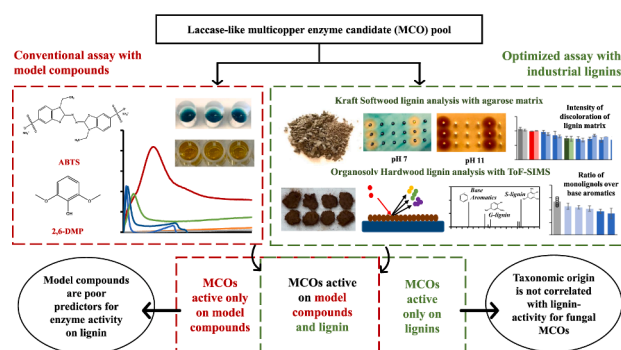
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HIGHLIGHTS

- Complex lignin substrates are critical for functional screening of industrial enzymes.
- ABTS is a poor predictor of multicopper-oxidase activity on industrial lignins.
- Twelve lignin-active multicopper-oxidases from diverse fungi were identified.
- Multicopper-oxidase activity on lignin did not correlate with taxonomic origin.

GRAPHICAL ABSTRACT



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ABSTRACT

Laccase-like multicopper oxidases are recognized for their potential to alter the reactivity of lignins for application in value-added products. Typically, model compounds are employed to discover such enzymes; however, they do not represent the complexity of industrial lignin substrates. In this work, a screening pipeline was developed to test enzymes simultaneously on model compounds and industrial lignins. A total of 12 lignin-active fungal multicopper oxidases were discovered, including 9 enzymes active under alkaline conditions (pH 11.0). Principal component analysis revealed the poor ability of model compounds to predict enzyme performance on industrial lignins. Additionally, sequence similarity analyses grouped these enzymes with Auxiliary Activity-1 sub-families with few previously characterized members, underscoring their taxonomic novelty. Correlation between the lignin-activity of these enzymes and their taxonomic origin, however, was not observed. These are critical insights to bridge the gap between enzyme discovery and application for industrial lignin valorization.

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1. Introduction

Lignin is the most abundant heteroaromatic polymer in nature. It is found in plant cell walls and formed by the polymerization of three main monolignols linked through C–C and C–O bonds (Fig. 1). The relative abundance of each monolignol and bonding network depends on the botanical source (Sethupathy et al., 2022). From an applied perspective, lignin reactivity is desirable and correlated to comparatively high C–O linkages (e.g., alkyl aryl ether (β -O-4) linkages). By contrast, condensed lignin structures characterized by C–C linkages (e.g., phenylcoumaran (β -5'), resinol (β - β) and biphenyl (5-5') bonds) reduce lignin reactivity (Suota et al., 2021) and limit access to C–O sites through steric hindrance effects. The industrial application of lignin requires the release of lignin from plant biomass. However, industrial processing, such as kraft pulping of wood, can increase the formation of aryl aliphatic units, quinone methides and stilbene-type structures containing unreactive C–C linkages (Fig. 1) (Crestini et al., 2017).

Enzymatic treatment of industrial lignins with oxidoreductases, including laccase and laccase-like multicopper oxidases (MCOs), are promising catalysis methods to overcome the structural inertness of industrial lignins under relatively environmentally benign conditions. Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) belong to the diverse group of MCO enzymes that are characterized by the presence of copper-binding domains where each domain typically consists of four spectroscopically distinct copper ions coordinated by three sites. Laccases catalyze O₂-dependent oxidation of polyphenols, methoxy-substituted phenols, aromatic diamines as well as certain electron-rich inorganic substrates (Maté and Alcalde, 2017). Laccase-like MCOs are found across all domains of life including plants, insects, fungi, and bacteria (Janusz et al., 2020). During a catalytic cycle the type I (T1) copper (Cu) site ("blue" copper, $\lambda_{\text{max}} = 600 \text{ nm}$) is the centre for substrate oxidation. Reduction of O₂ to water happens at the tri-nuclear centre containing interlinked type II (T2) and type III (T3) (paired) Cu

ions (Komori and Higuchi, 2015). Fungal laccases have a higher redox potential (E_0) compared to other laccases, potentially due to the amino acid responsible for axial coordination of the catalytic T1 Cu centre. This enables activity of fungal laccases on bulky, polymeric phenolic substrates. Based on the type of amino acid at this position, laccases are grouped into three classes of increasing E_0 : Lac 1 (Met) (400–500 mV), Lac 2 (Leu) and Lac 3 (Phe) (700–800 mV) (Cázares-García et al., 2013).

The oxidation of phenolic sites in lignin catalysed by laccases proceeds through a free radical mechanism where the radical generated at the oxidised phenolic site can migrate through the central aromatic ring as well as onto the methoxy groups or alkyl side-chains (Mai et al., 1999) (Fig. 1). These free radical reactions can be initiated by laccases alone (Perna et al., 2019) or in tandem with small compounds known as mediators that improve access to non-phenolic sites in lignin. As a result, other desirable structural modifications such as demethoxylation, demethylation, decarboxylation can also occur (Christopher et al., 2014). In addition to independent or mediator-assisted oxidation, laccase catalysed grafting of monomers containing alkyl-hydroxyl groups, such as acrylate, onto industrial lignin substrates, has been previously demonstrated (Arefmanesh et al., 2022).

Most reported screens of recombinantly produced laccases and laccase-like MCOs use model compounds including synthetic mediators such as 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), to assay enzyme activity (Curran et al., 2021). Phenolic substrates that can result from lignin degradation such as 2,6-dimethoxyphenol (DMP), guaiacol, syringic acid and ferulic acid (Sherif et al., 2013), or modified forms of these compounds such as syringaldazine, are also commonly used (Wang et al., 2018). Using defined compounds to uncover MCOs that act on industrial lignins, however, does not capture the chemical complexity of the intended substrate (Goacher et al., 2014). Besides model compounds, complex lignocellulosic substrates such as wood powders as well as water-insoluble (organosolv) lignins have been used to study laccase activity where the modification of lignin is then assessed

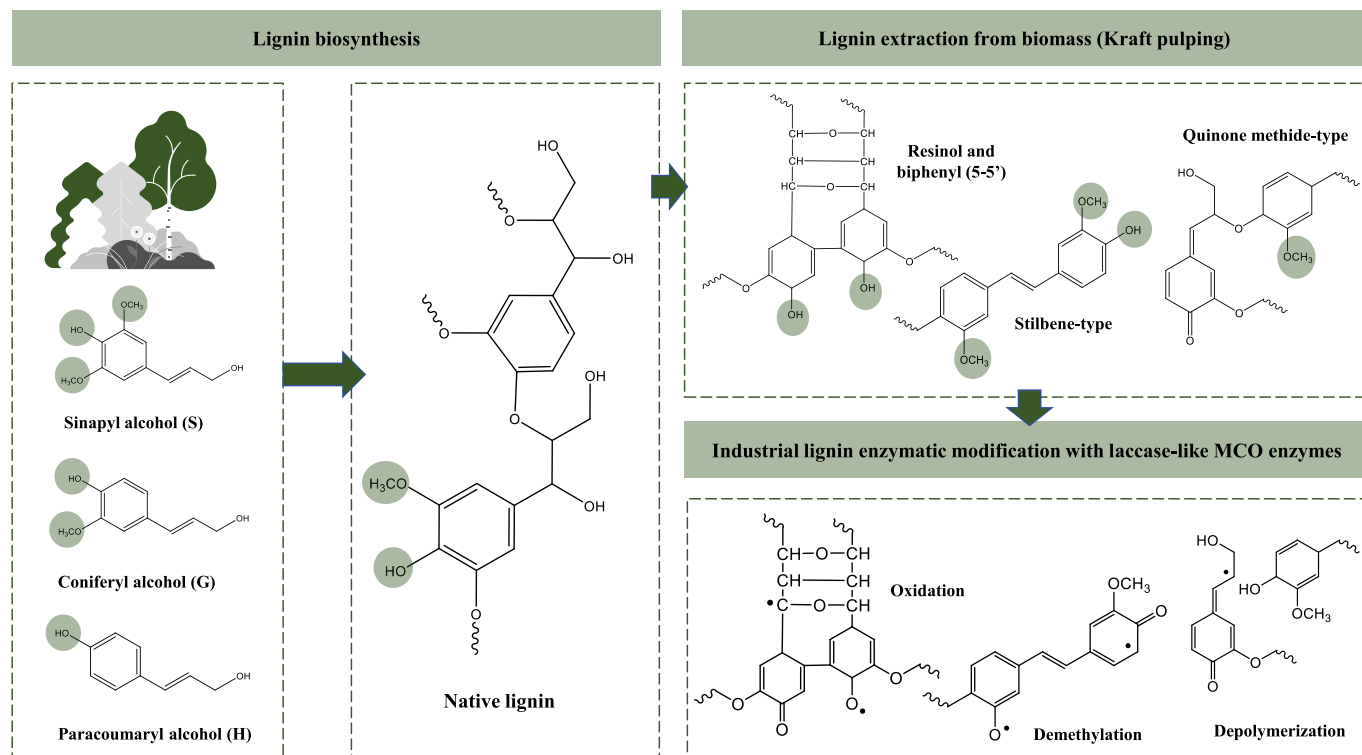


Fig. 1. Proposed effect of industrial extraction and enzymatic modification on lignin structure. Lignin is a natural hetero-polyphenol formed by the combination of three distinct phenylpropanoid alcohols. Following industrial processing such as kraft pulping, condensed substructures and unreactive linkages accumulate within lignin. Laccase and other multicopper oxidase enzymes (MCOs) target phenolic and phenyl-methoxy sites (circled in green) and generate reactive sites via a free-radical oxidation. This reactive lignin can then be used for high-value applications such as bio-based polymer production.

using mass spectrometry (Goacher et al., 2018). Considering industrial lignins, sulphonated lignin is most frequently used in enzymatic studies (Agustin et al., 2021). However, this lignin has higher water solubility and reactivity compared to substantially more abundant industrial lignins such as kraft lignin.

The aim of this study was to establish a functional screen for laccase and laccase-like MCOs that incorporate complex industrial substrates. In this way, enzymes that directly transform industrially relevant lignin substrates could be discovered. While this study focused on fungal enzymes produced in the industrially relevant host, *Aspergillus niger*, the same approach can be adapted to screen enzymes from other biological sources such as bacteria. In the developed screen, the selected MCOs were subject to activity tests on kraft softwood lignin (KSL) and organosolv hardwood lignin (OHL), in addition to defined mediator compounds such as ABTS and 2,6-DMP. A total of 12 fungal laccase-like MCOs displayed activity towards the industrial lignins, which could not be predicted by activity on the mediator compounds alone.

2. Materials and methods

2.1. Materials

ABTS (Ultra Pure, cat. no. AD0002) was purchased from Bio Basic Canada Inc. (Markham, ON, Canada) and 2,6-DMP (99 %, cat. no. D135550) was purchased from Sigma Aldrich (India). Stock solutions of ABTS (200 mM) and 2,6-DMP (100 mM) were prepared in milliQ water. Lignin substrates used in the study were obtained from kraft softwood pulping and hardwood organosolv treatment processes and sourced from industrial partners in North America. The physical properties and origin of the substrates have been previously detailed (Arefmanesh et al., 2020, Arefmanesh et al., 2022). The commercial multicopper oxidase (Novozym® 51003, *Aspergillus oryzae*) was obtained from Novozymes (Franklinton, NC, USA) and bovine serum albumin (BSA) (Quick Start Bovine Serum Albumin Standard #5000206) was purchased from Bio-Rad (Hercules, CA, USA). All other chemicals used in the study were of reagent grade, purchased from various vendors and used without any further purification.

2.2. Selection and analysis of multicopper oxidases

Fungal genomes available in the Joint Genome Institute (JGI) MycoCosm database (available at: <https://mycocosm.jgi.doe.gov/mycocosm/home>, accessed: November, 2022) were used to obtain putative Auxiliary Activity 1 (AA1) MCO gene sequences. After removing redundancies and following multiple sequence alignment, a maximum likelihood (ML) phylogenetic tree was constructed and overlaid with previously characterized MCOs. Fifty-seven gene sequences from under-characterized clades were selected for recombinant production in *Aspergillus niger*.

MCO sequences that were successfully produced in *A. niger* (section 2.3) were analysed using InterPro scan. Multiple-sequence alignment was done with default MUSCLE options in Geneious software (V. 8.1.9) and the alignment was manually refined to exclude large gap regions. The refined alignment was used for ML sequence similarity dendrogram construction using RAxML (V.7.2.8) with GAMMABLOSUM62 protein model and rapid bootstrapping ($n = 100$), also in Geneious (V. 8.1.9).

Additionally, SSNPipe (Viborg et al., 2019) was used to create a sequence similarity network (SSN) with the recombinantly produced and lignin-active MCOs, together with previously characterized AA1 enzymes reported in the Carbohydrate-Active enZYme (CAZy) database (available at: <http://www.cazy.org/>, accessed: November, 2021); an E-value threshold of 10^{-130} was used for clustering. The generated SSN was visualised and edited in Cytoscape (V.3.8.0).

2.3. Recombinant production of selected multicopper oxidases in *Aspergillus niger*

Gene targets (see supplementary material) were isolated from the source organism by growing each fungal strain on Emerson YpSs agar medium (HIMEDIA®, USA) at 45 °C. Exceptions were *Aureobasidium pullulans* and *Oculimacula yallundae*, which were grown at 22 °C, and *Trametes versicolor* which was grown at 30 °C. After three days of incubation, ten plugs from agar grown mycelia were harvested and transferred to 50 mL of liquid mycological broth (HIMEDIA®, Maharashtra, India). A volume of 25 mL of *Trametes* defined medium (Addleman and Archibald, 1993) supplemented with 2 % of a mix of alfalfa and barley was inoculated with 2.5 mL of the primary culture. Liquid cultures were incubated at respective optimum temperatures (30 °C, 22 °C or 45 °C) for 24 h with shaking at 220 rpm. Mycelia were harvested and ground to a powder in liquid nitrogen as described previously (Bellemare et al., 2018). Total RNA was extracted using the RNeasy® Plant Maxi Kit (Qiagen, Maryland, USA) and complementary DNA (cDNA) was synthesized using the Superscript™ III reverse transcriptase (Invitrogen, Massachusetts, USA) as per manufacturer instructions. Laccase genes were PCR amplified from cDNA using the Phusion® High-Fidelity DNA Polymerase (New England BioLabs Inc., Ipswich, MA, USA) and cloned into a variant of the pGBFIN expression vector (Tambor et al., 2012) using a ligation-independent cloning technique (Aslanidis and de Jong, 1990). CRISPR/Cas9 genome editing was used to replace the glucosylase gene (*glaA*, A2QHE1) of *A. niger* strain FGSC A1513 (Fungal Genetics Stock Center, USA) with each laccase gene by co-transformation of the expression plasmid and the CRISPR/Cas9 plasmid (Song et al., 2018). Guide RNA (5'-GCAGTGTGACTGT-CACCTCG-3') targeting the *glaA* gene was designed using Geneious (V.11.1.4) based on the method described by Doench et al. (2014).

CRISPR/Cas9 plasmid construction and *A. niger* transformation were done as described previously (Song et al., 2018). Selected transformants were inoculated in liquid modified minimal medium (MMJ) supplemented with 0.1 % arginine and screened for protein expression as described previously (Master et al., 2008). Transformants expressing the proteins of interest were cultured in liquid MMJ and supernatants were concentrated and desalted into 10 mM citrate buffer (pH 5.0). Following buffer exchange, produced laccases were confirmed by mass spectrometry (section 2.4). These concentrated supernatants were used for all activity assays without additional purification and are referred to henceforth as MCO enzymes.

2.4. Mass spectrometry based confirmation of enzyme identity

Two volumes of cold methanol were added to a 100 µL aliquot of cleared culture supernatant and kept on ice for 30 min. The sample was centrifuged for 30 min at 15,000g on a table-top centrifuge cooled to 4 °C. The supernatant was discarded, and the precipitated protein pellet was washed once with 300 µL of cold 60 % methanol in water. The pellet was suspended in 30 µL of 6 M urea, 100 mM ammonium bicarbonate solution (pH 8.0) and digested with trypsin as previously described (Budak et al., 2014). Aliquots of peptide digest were analyzed by liquid-chromatography-tandem mass spectrometry (LC-MS/MS) using an Agilent 1260 Infinity II chromatography system connected in-line with a Thermo-Finnigan 7 Tesla LTQ-FT MS system. Chromatography separation was done using a 150 x 0.5 mm Agilent Zorbax C18 column and gradient of water: 0.1 % formic acid for solvent A, and 99.9 % acetonitrile: 0.1 % formic acid for solvent B at a flow rate of 50 µL/min. The gradient started at 3 % B, increased to 40 % B over 45 min, then to 55 % B over 8 min, then to 90 % B over 2 min, and finally held at 90 % for 5 min. Column eluent was connected to a Thermo-Finnigan Ion Max electrospray source. Survey scans (300–1600 m/z) were acquired in positive mode with resolution $r = 50,000$ at 400 m/z . MS/MS data were acquired using data-dependent acquisition method for the selection of the top six most intense doubly, triply, and quadruply charged

precursors for collision induced dissociation scans in the linear ion trap mass analyzer. MS/MS data were processed for protein identification using the precursor ion quantitation workflow from Proteome Discoverer 2.4. MS/MS fragmentation data were queried against a database of 17,897 protein sequences comprising the *A. niger* NRRL3 protein models (available at: <https://mycocosm.jgi.doe.gov/mycocosm/home>, accessed: November, 2022) plus a collection of recombinant protein sequences, including the laccases of interest. Sequest HT search criteria used were one mis-cleavage allowed, carbamydomethylation of cysteine as a fixed modification and oxidation of methionine as a variable modification. Precursor ion tolerance used was 7 ppm and MS/MS fragment tolerance was 0.6 Da. A minimum of two peptides of high confidence (false discovery rate, FDR < 0.01) was selected as minimum requirement for protein identification. Relative protein quantitation was based on the reported protein area values calculated by Proteome Discoverer using the summed peak area abundances of extracted ion chromatograms of the top five identified peptides for each protein with pair-wise peptide matching across samples.

2.5. Activity assay with synthetic and natural mediators

MCO activity was measured using 1 mM ABTS ($\epsilon_{420} = 36 \text{ mM}^{-1} \text{ cm}^{-1}$) and 1 mM 2,6-DMP ($\epsilon_{468} = 14.8 \text{ mM}^{-1} \text{ cm}^{-1}$) (Sherif et al., 2013). Reactions (200 μL) were performed at room temperature in standard transparent 96 well plates (Falcon®) with 50 mM universal buffer (50

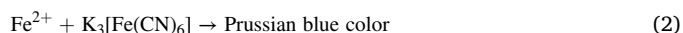
$$\text{Ratio of average intensity} = \left[\frac{\text{Mean pixel intensity}(a)}{\text{Mean pixel intensity}(b)} \text{(I)} + \frac{\text{Mean pixel intensity}(a)}{\text{Mean pixel intensity}(b)} \text{(II)} + \frac{\text{Mean pixel intensity}(a)}{\text{Mean pixel intensity}(b)} \text{(III)} \right] / 3, \quad (3)$$

mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid) adjusted to pH 4.5 and pH 8.0 for ABTS and 2,6-DMP, respectively. The reactions contained 0.04–3 μg of MCO enzymes, which ensured a linear relation between product formation and time. The reactions were initiated by adding the substrate. Substrate depletion was measured using an Infinite 200 plate reader (Tecan Trading AG, Switzerland) based on the absorption coefficients. Control reactions included reaction mixtures that 1) lacked enzyme, 2) replaced the MCO with Novozym® 51003 (positive control), 3) replaced the MCO with BSA (negative control), and 4) replaced the new MCO with non-transformed host strain extracellular protein concentrate (HSP) recovered from an *A. niger* cultivation. Reactions were run in triplicate and significant difference between reported values were determined with the highest significant difference (HSD) method using Tukey's test level at $p \leq 0.05$.

2.6. Agarose plate assay with kraft softwood lignin substrates

This assay was modified from the method developed by Choolaei et al. (2021). Briefly, 1.5 % agarose solutions containing 0.1 % w/v of solid kraft softwood lignin substrates (KSL 1–2) in 100 mM Tris buffer (pH 7.0 or pH 11.0) were prepared in $150 \times 150 \text{ mm}$ clear rectangular bioassay plates (Thermo Scientific™ Nunc™ Square BioAssay Dishes). Circular holes (5 mm diameter) were punched in the plate using a metallic puncher, spaced 2.5 cm apart. 5–20 μg of MCO enzymes were loaded in the wells with a total reaction volume of 50 μL and diluted as required with 100 mM Tris buffer adjusted to pH 7.0 or 11.0. Control reactions, number of samples, and statistical analyses were set up as described previously (section 2.5). The plates were incubated for 16 h at 37 °C.

Following incubation, the plates at pH 7.0 were developed using c.a. 20 mL of solution comprising 1 % (w/v) potassium ferricyanide ($\text{K}_3[\text{Fe}(\text{CN})_6]$) and 1 % ferric chloride (FeCl_3) (Peterson et al., 2009) (Eqs. (1)–(2)).



This staining method could not be implemented at pH 11.0 due to the instability of the stain. However, at both pH 7.0 and pH 11.0 enzyme action could be tracked through the formation of dark circular areas centered on wells containing active enzyme. Consequently, non-stained gels were used to estimate activity at both pH values. Visual indication of enzyme action was converted to a semi-quantitative estimate of enzyme activity using ImageJ software. The lignin-containing agarose gels was scanned using a Canon Lide 400 photo scanner and the corresponding image was imported into the software, focusing on areas around reaction wells. Color change due to enzyme action was quantified using intensity units that measure the brightness of a given pixel area in the image. Since enzyme action leads to reduction in brightness, this parameter is inversely correlated to enzyme activity. Using a fixed area of analysis for a given gel image, the intensity inside the enzyme area of activity is divided by the intensity outside the area, right on the edge of transition. The average value of this ratio over three technical replicates is then used as a proxy for enzyme activity and is calculated as follows (Eq. (3)):

$$\text{The ratio of intensity for selected area of analysis} = \frac{\text{Mean pixel intensity (a)}}{\text{Mean pixel intensity (b)}}$$

where area of a = area of b, and (I) - (III) are technical replicates for a given enzyme.

2.7. Time-of-flight secondary ion mass spectrometry-based assay with organosolv hardwood lignin

The recombinantly produced MCOs were tested for activity on solid organosolv hardwood lignin (OHL) using ToF-SIMS by tracking the change in ionic mass fragment peaks uniquely attributed to lignin and base aromatics (Saito et al., 2005). Briefly, 4–6 mg of powdered substrate was treated with 20 or 50 μg of MCO in 10 mM Tris buffer (pH 11.0). Reactions (200 μL) were set up in a 96-well plate equipped with a vacuum filter and 1.2 μm PVDF membrane attachment. Control reactions contained only the substrate in buffer. Control reactions, number of samples and statistical analyses were set up as described previously (section 2.5). Plates were shaken at 250 rpm in Eppendorf thermomixers for 16 h under ambient conditions. Reaction supernatant was recovered using a Tecan liquid handler equipped with vacuum filtration (Tecan Trading AG, Switzerland). The remaining solid was then washed 10 times with milliQ water to remove soluble impurities and left to air dry for 16 h. Air dried samples were fixed on a glass slide for surface analysis using ToF-SIMS (Goacher et al., 2018).

ToF-SIMS spectra were acquired and analysed as described previously by Goacher et al. (2018) and Vuong et al. (2021) with a few modifications. The modifications include acquiring 9 different spectra for each sample and maintaining total ion intensity counts for each sample below 1×10^6 . In addition to the Guaiacyl (G)-lignin (summation of intensity of methoxylated G-lignin peaks at m/z 137 and 151 over intensity of nonfunctionalized aromatic rings m/z values at 77 and 91) and Syringyl (S)-lignin modification metrics (summation of intensity of methoxylated S-lignin peaks at m/z 167 and 181 over intensity of non-functionalized aromatic rings) (Vuong et al., 2021), the S/G metric is

also reported as an estimation of demethylation potential of the enzyme.

2.8. Functional activity correlation analyses in R

Correlation analyses between the MCO activities on model compounds and industrial lignin substrates was done using principal component analysis (PCA) with the “prcomp” function. Prior to correlation analyses, the activity datasets for individual substrates were normalized and mean centered using custom functions. The loading plot depicting the PC1 and PC2 correlation scores was generated using ggplot options.

3. Results and discussion

3.1. Heterologous production of fungal multicopper oxidases in *A. niger*

Forty percent of the selected MCO sequences were successfully produced in *A. niger*, totalling 23 proteins for characterization. The percentage pairwise similarity of the produced sequences ranged from 15 to 85 % with an average value of 25 %. Based on the identity of the T1 Cu axial coordination residue, 7 sequences can be classified as Lac 1 (Met) and 13 sequences as Lac 2 (Leu) laccases. However, three sequences: MCO1A_CHAOL (Ile), MCO1A_THITE (Val), and MCO1A_RHIPU (Ile), did not have typical residues in this position therefore could not be placed into specific classes (Cázares-García et al., 2013) (see supplementary material). SDS-PAGE profiles of the produced enzymes are included in supplementary materials and the protein yields ranged from 3.4 to 200 mg/L.

The recombinantly produced proteins covered the breadth of taxonomy represented in the initial selections. Most of the sequences belong to phylum *Ascomycota* including seven sequences each from *Sordariomycetes* and *Eurotiomycetes*, three sequences from

Dothideomycetes and one sequence from *Leotiomycetes*. The rest of the sequences were from *Basidiomycota* including three sequences from *Trametes versicolor* and two sequences from *Mucoromycota* (*Rhizomucor pusillus*). Notably, 50 % of the recombinantly produced MCOs originated from thermophilic organisms including all candidates from *Eurotiomycetes* and two candidates from *Sordariomycetes* (*Thermothelomyces myriococcoides* and *Thielavia terrestris*) (see supplementary material).

Interpro domain analysis confirmed the presence of cupredoxin domains in all 23 heterologously produced MCO sequences. The cupredoxin domains in 21 of the sequences were annotated as laccase-type, similar to those found in *Melanocarpus albomyces*, *Aspergillus fumigatus* and *Trametes versicolor*. The two MCO sequences from *Rhizomucor pusillus* (MCO1A_RHIPU and MCO1B_RHIPU), however, were instead annotated as L-ascorbate oxidase type domains (PANTHER database: PTHR11709:SF136, PTHR11709:SF136) (see supplementary material). Among MCOs, L-ascorbate oxidases are most closely related to laccases (Wu et al., 2020). Indeed, MCO1A_RHIPU and MCO1B_RHIPU displayed activity towards L-ascorbic acid (see supplementary material).

3.2. Activity of recombinant fungal multicopper oxidases on common mediators

Initial measurements of MCO activity on 2,6-DMP (pH 8.0) and ABTS (pH 4.5) showed seven MCOs active on both ABTS and 2,6-DMP and three active only on 2,6-DMP (Fig. 2A-B). All new MCO targets displayed a preference for either 2,6-DMP or ABTS and the highest activity was observed for MCO1B_RHIPU on 2,6-DMP. The positive control Novozym® 51003 laccase displayed similar activities on both 2,6-DMP and ABTS. Notably, three MCOs demonstrated activity only on 2,6-DMP at pH 8.0 namely MCO1A_MYRTH from *Thermothelomyces myriococcoides*, MCO1A_OCUYA from *Oculimacula yallundae* and MCO1A_RASBY from *Rasamsonia byssochlamydoidea*. While the optimal pH to grow

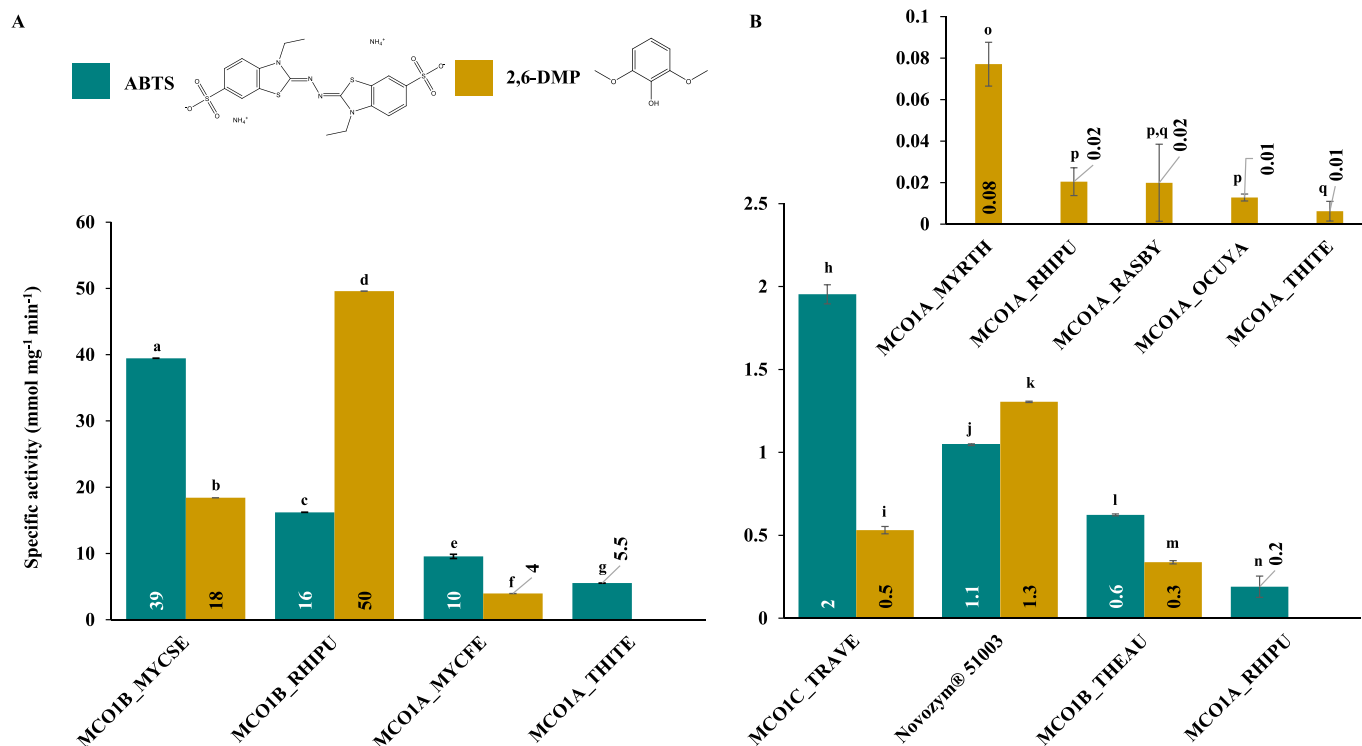


Fig. 2. Specific activity (mmol mg⁻¹ min⁻¹) of multicopper oxidase (MCO) enzymes on ABTS and 2,6-DMP. Activity was measured at pH 4.5 (ABTS) or at pH 8.0 (2,6-DMP) using 1 mM of substrate in 50 mM universal buffer and variable enzyme loading. Error bars represent standard deviation of 3 technical replicates (n = 3). Numerical values of average specific activities are reported as bar charts and are grouped based on MCO activity being (A) higher than or (B) lower than positive control (Novozym® 51003 laccase) (except MCO1C_TRAVE ABTS activity). (B inset) MCOs with activity on 2,6-DMP lower than 0.1 mmol mg⁻¹ min⁻¹. Average activity values followed by the same letter(s) are not significantly different (at p ≤ 0.05) according to Tukey’s HSD test.

Thermothelomyces myriococcoides is pH 5.0 and *Rasamsonia byssoclamydoides* is pH 3.0, *Oculimacula yallundae* grows well between pH 5.0–7.0 (see [supplementary material](#)). This difference between the pH range for growth of the source organism and pH optimum for laccase activity is also reported in previous studies that show the enzyme pH optimum can be impacted by the substrate ([Baldrian, 2006](#)), source organism and recombinant host ([Rodríguez-Escribano et al., 2017](#); [Camarero et al., 2012](#)).

The specific activities of MCOs from *Trametes versicolor* (MCO1C_TRAVE) and *Thielavia terrestris* (MCO1A_THITE) are lower than previously reported values for MCOs from the same organisms ([Chiadò et al., 2021](#); [Gutiérrez-Antón et al., 2023](#)), with the exception of MCO1A_THITE activity towards ABTS. This could be explained by the comparative purity of the previously characterized MCOs. For all other MCOs, there are no previous reports of MCOs characterized on either ABTS or 2,6-DMP from the respective source organisms.

3.3. Activity of recombinant fungal multicopper oxidases on kraft softwood lignin (KSL)

All 23 MCOs were tested for activity on kraft softwood lignin (KSL-1 and KSL-2) using a modified version of the agarose plate assay ([Choolaei et al., 2021](#)) ([Fig. 3A](#)). The activity of the enzyme was extrapolated from a visual parameter quantifying the color change of the lignin-embedded agarose matrix caused by the oxidation of phenolic moieties in lignins by MCOs. Briefly, the color formation indicates the production of oxidized species such as aromatic and aliphatic quinones and localized condensed 5-5' and 4-O-5' linked dehydropolymers ([Matsumura et al., 1986](#); [Agustin et al., 2021](#)).

At pH 7.0, 11 MCOs were active on KSL-1, 13 MCOs were active on KSL-2, and 10 MCOs were not active on either lignin source ([Fig. 3B](#)). Notably, all MCOs with activity on both ABTS (pH 4.5) and 2,6-DMP (pH 8.0) were also active on both KSL substrates at pH 7.0. Physical properties of the lignins including molecular weight, polydispersity index (PDI) and ash content were previously demonstrated to be inversely correlated with enzyme activity due to steric hindrance and inhibitory effects ([Wang et al., 2021](#); [Kalyani et al., 2015](#)). The average molecular weights (M_w) of KSL-1 and KSL-2 are 9700 Da and 6600 Da, polydispersity indices are 3.3 and 7.1 and ash contents are <3 % and c.a. 0.3 % (dry weight basis), respectively ([Arefmanesh et al., 2020](#); [Arefmanesh et al., 2022](#)). This supports the slightly higher number of MCOs having activity on KSL-2 compared to KSL-1. Although the PDI of KSL-2 is higher than KSL-1, the enzyme action seems more impacted by M_w . Lignin purity, as determined by carbohydrate content, can also potentially impact oxidative enzyme action on lignin. A higher carbohydrate content can indicate higher number of lignin-carbohydrate linkages that can serve as sites of laccase action ([Du et al., 2013](#)). The carbohydrate content is highly variable (c.a. 2–50 mg/g) for KSL substrates ([Liu et al., 2022](#)) and is ~4 % (xylan + glucan) in KSL-2 ([Arefmanesh et al., 2022](#)).

Three MCOs with activity on KSL substrates at pH 7.0 did not act on ABTS or 2,6-DMP. These included MCO1A_THEAU from *Thermoascus crustaceus* that transformed both KSL substrates, and MCO1A_MALCI and MCO1B_MALCI from *Malbranchea cinnamomea* that transformed KSL-2. Notably, MCO1B_THEAU also from *Thermoascus crustaceus* was active on both mediators and KSL substrates. These results underscore the potential to miss lignin-active MCOs when screening using mediator compounds alone.

At pH 11.0, 10 MCOs were active on KSL-1, 8 MCOs were active on

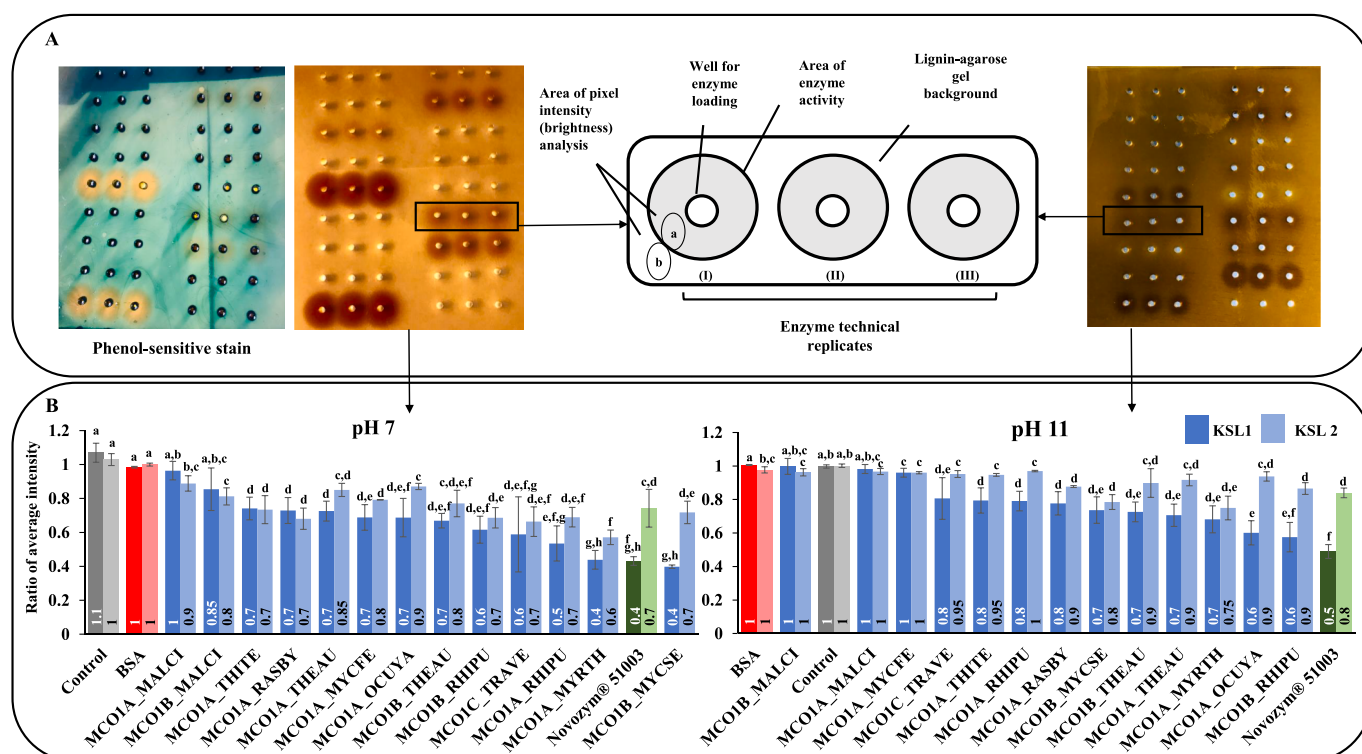


Fig. 3. Activity of multicopper oxidases (MCOs) on kraft softwood lignin (KSL) substrates. (A) 5–20 μ g of MCOs were added to wells in agarose plates containing 0.1 % of lignin in 100 mM Tris-buffer (pH 7.0 or 11.0) and incubated at 37 °C for 16 h. Plates at pH 7.0 were optionally stained with a phenol sensitive stain. The clearing zones indicate areas where MCOs oxidized phenolic sites in lignin. Non-stained plates were used for estimations of enzyme activity, where activity is calculated by taking the ratio of color intensity at the enzyme position in the agarose gel (a) and gel background (b), and then reported as average of three technical replicates (I–III). (B) MCO activity reported as inversely correlated to average intensity ratio and compared to following controls: (1) lacking enzyme (control) (2) BSA (negative control) and (3) Novozym® 51003 laccase (positive control). Error bars represent standard deviation of 3 technical replicates ($n = 3$) with average ratios reported as bar charts with numerical values inside the columns. Ratio values followed by the same letter(s) are not significantly different (at $p \leq 0.05$) according to Tukey's HSD test.

KSL-2, and 13 MCOs were not active on either lignin source (Fig. 3B). All MCOs that were active on a KSL substrate at pH 11.0 were also active on a KSL substrate at pH 7.0. Conversely, five MCOs that were active on a KSL substrate at pH 7.0 lost activity at pH 11.0, including MCO1A_MYCFE, MCO1A_MALCI and MCO1B_MALCI that lost activity on both KSL-1 and KSL-2. Interestingly, MCO1C_TRAVE and MCO1A_RHIPU only lost activity on KSL-2 but retained activity on KSL-1 at pH 11.0, suggesting the impact of pH cannot be simply explained by enzyme instability in alkaline solutions (Renfeldt et al., 2023). Still, qualitative assessment of MCO activity using the agarose plate assay showed that for all lignin-active enzymes, MCO activity was lower at pH 11.0 than pH 7.0. The only exception to this trend was MCO1A_OCUYA from *Oculimacula yallundae*, which performed better on KSL-1 at pH 11.0 compared to pH 7.0.

MCO1A_OCUYA showed activity only on 2,6-DMP under alkaline conditions (pH 8.0) and no activity on ABTS at pH 4.5 (Fig. 2B). Thus, MCO1A_OCUYA may be particularly suited for kraft lignin valorization given the higher solubility and accessibility of lignin at alkaline pH. To the best of the author's knowledge, there have not been any laccase or MCO enzymes characterized from *Oculimacula yallundae* to date. This organism is a well-known pathogen with high resistance to abiotic stress caused by fungicides (Leroux et al., 2013). However, it is not clear how these characteristics might influence the evolution of an alkaline tolerant MCO. Notably, there are no studies to date that describe a specific tolerance of this organism to alkaline conditions. Unfortunately, due to low viability of *A. niger* transformants expressing MCO1A_OCUYA, this MCO could not be stably produced in the recombinant host and was omitted from further analyses.

3.4. Activity of recombinant fungal multicopper oxidase on organosolv hardwood lignin

Fungal laccases have been previously applied for the oxidative structural modification of organosolv lignins (Perna et al., 2019). However, the specific evaluation of fungal MCO activity by quantifying changes in S and G subunits of OHL using ToF-SIMS is unprecedented. ToF-SIMS is a sensitive surface compositional analysis technique and has been used to evaluate enzyme activity directly on complex lignocellulosic substrates (Goacher et al., 2014; Vuong et al., 2021). Herein, ToF-SIMS was used to investigate MCO activity on organosolv hardwood lignin, focusing on the 12 MCOs that were stably produced in *A. niger* and active on KSL-2 at pH 7.0 (Fig. 3B). OHL is the preferred lignin substrate for ToF-SIMS investigation since it contains both methoxylated G and S monomers due to its origin from hardwood (Suota et al., 2021) and existing m/z peak assignments can be used (Saito et al., 2005).

Eleven of the 12 MCOs transformed both G and S lignin monomers. The average value of S/Ar of the OHL active MCOs is ~40 % lower than the control sample compared to G/Ar average which is ~33 % lower (Fig. 4 A-B). Moreover, the S/G ratio values are also lower than the value of the control sample (~1.5) for most enzymes (Fig. 4C). Together these observations point to the preferential activity of MCOs on S moieties compared to G moieties. This preference for S moieties in OHL is supported by previous similar observations for bacterial laccases and peroxidases by Vuong et al. (2021).

3.5. Correlating multicopper oxidase activity on lignin to activity on model compounds and protein sequence

Twelve MCOs were tested using model mediator compounds, two KSLs, and OHL, which permitted a correlation analysis to reveal how well the selected model compounds predict MCO activity on lignin.

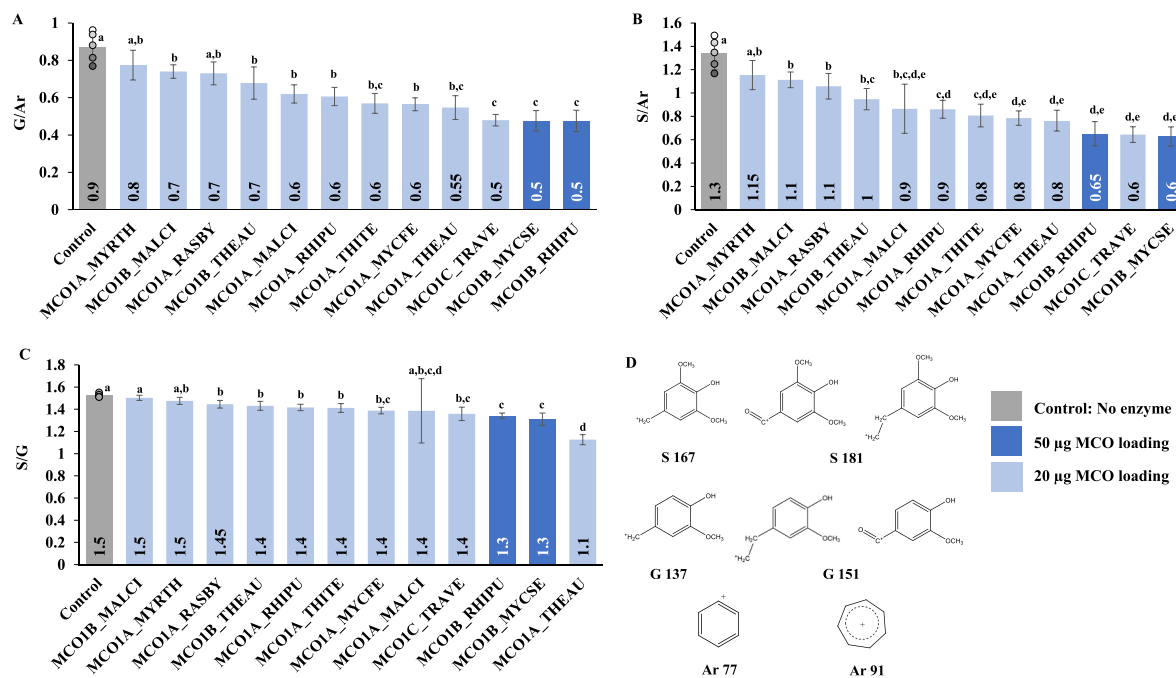


Fig. 4. Activity of multicopper oxidase (MCO) enzymes on organosolv hardwood lignin (OHL) substrate measured by ion intensity of specific lignin aromatic moieties (A) Guaiacyl (G) and (B) Syringyl (S) normalized by ion intensity of base aromatics (Ar) measured using time-of-flight secondary ion spectrophotometry (ToF-SIMS). 20–50 µg of MCOs were mixed with 4 mg of solid OHL using 200 µL of 10 mM Tris buffer at pH 11.0 and incubated shaking at 250 rpm for 16 h at room temperature. Error bars represent combined standard deviation over 9 ToF-SIMS spectra collected for each MCO ($n = 9$) with averages reported as bar charts with numerical values inside the columns. For control samples (no enzyme) from different ToF-SIMS runs, the dots represent the average value from each run ($n = 9$), and the average over different runs is presented inside columns ($n = 5$). (C) Ratio of S and G moieties as a measure of demethylation potential of enzymes. (D) Chemical structures of all aromatic ions used for activity measurement. Ratio values followed by the same letter(s) are not significantly different (at $p \leq 0.05$) according to Tukey's HSD test.

The model compounds used in this study include ABTS and 2,6-DMP. ABTS remains the most widely used substrate to measure laccase and laccase-like enzyme activities. However, it is a synthetic compound and does not share structural similarity with lignin. Conversely, 2,6-DMP is of natural origin and representative of syringyl (S) moieties in lignin. Additionally, it offers the advantage of enzyme activity measurement under alkaline (pH 8.0) and low redox potential (E_0) conditions (Pavitt et al., 2017). Consequently, both these compounds are representative of the spectrum of model compounds typically used to assay laccase-like enzymes and good candidates for conducting correlation analyses between enzyme activity on typical model substrates versus activity on industrial lignins.

When comparing MCO activity on mediator compounds and lignin substrates through correlation analyses, it was observed that activity on ABTS was a poor predictor of activity on lignin substrates with a high PC1 differentiation of ~75 %. Activity on 2,6-DMP was a better predictor of activity on lignin (PC1 and PC2 differentiation of ~25 %) (Fig. 5). It is unclear, however, if this is the result of 2,6-DMP being structurally similar to methoxylated phenolic moieties in lignin or because activity measurements using 2,6-DMP were performed under alkaline conditions, similar to activity measurements with lignin

substrates. Rodríguez-Escribano et al. (2017) previously compared the activity of a laccase clone library on ABTS and lignosulfonate. The authors similarly found no correlation between activities on these substrates.

MCO activity on OHL was correlated to activity on KSL substrates with a low PC1 differentiation (c.a. 3%). However, the potential impacts of substrate processing (e.g., kraft versus organosolv) and botanical source (e.g., softwood versus hardwood) was revealed through PC2 (3–5 %) which differentiated MCO activity on OHL versus KSL. PC2 (2 %) also differentiated MCOs based on preferential activity on KSL substrates at either pH 7.0 or 11.0. Clearly, the impacts of heterogeneous industrial lignin structures on the complex oxidative reaction mechanisms of MCOs (Perna et al., 2019) require further investigation and will ultimately inform the successful application of the alkaline-tolerant enzymes discovered herein for the valorization of industrial lignins.

Besides PCA, sequence similarity network (SSN) analysis was performed to investigate the potential correlation between MCO activity and sequence. The analysis included previously characterized AA1 enzymes from the CAZy database together with the 12 lignin-active MCOs characterized in this study. The resulting SSN largely grouped the sequences based on their taxonomic origin and most MCOs characterized

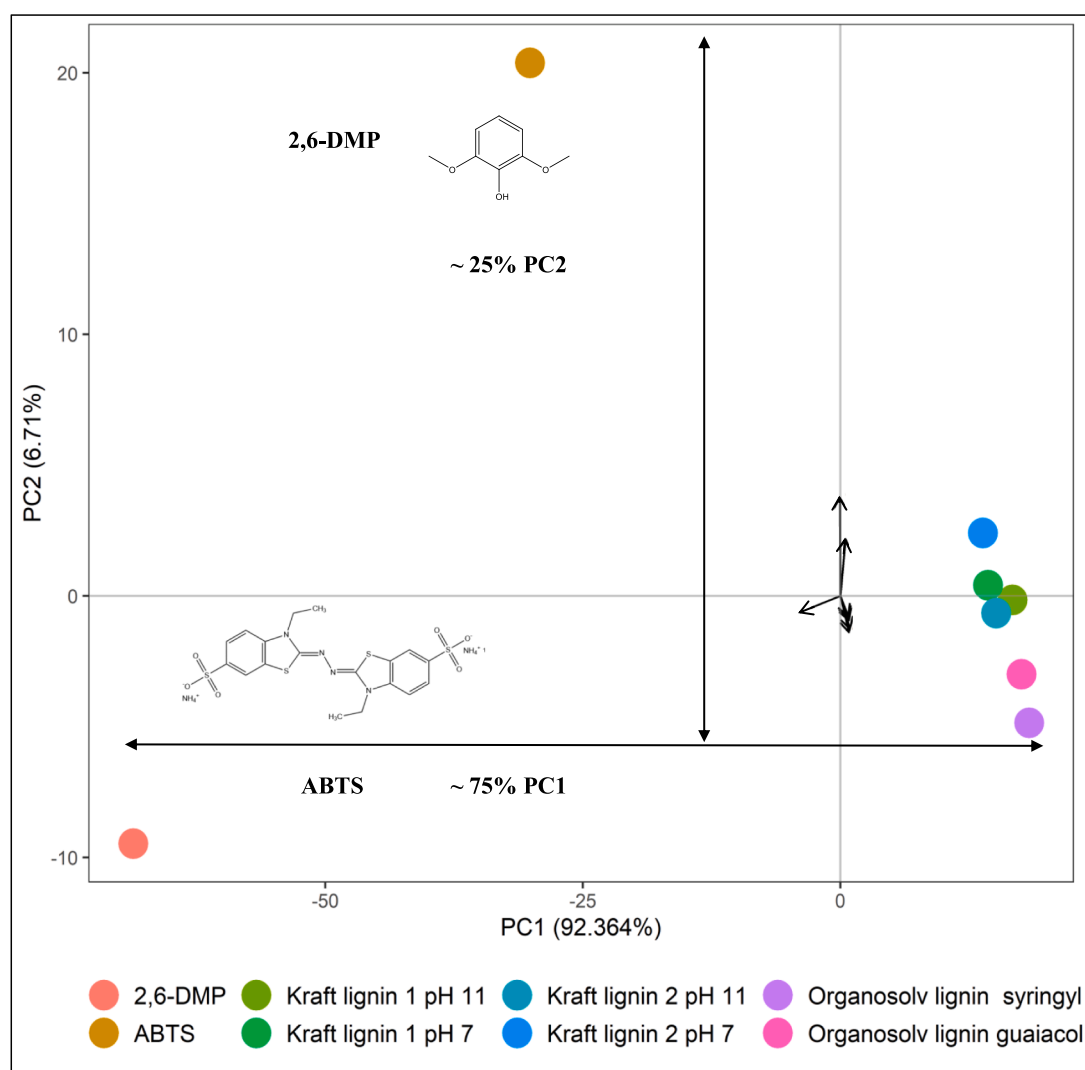


Fig. 5. Principal component analysis (PCA) biplot of combined activity data of 12 lignin-active multicopper oxidase (MCO) enzymes. Each dot represents the type of enzyme activity: ABTS and 2,6-DMP, specific activities as measured by monitoring absorbance of oxidised products; kraft lignin substrate activity measured by visual quantification of phenolic oxidation potential and organosolv lignin activity measured by modification of syringyl and guaiacyl moieties using ToF-SIMS. PCA loading values are depicted by vector length of arrows at origin ranging from a maximum of ~10 % (PC1) – ~5% (PC2) which indicates that there is minimal impact of individual enzyme performance on the analysis. (inset) Chemical structures of ABTS and 2,6-DMP.

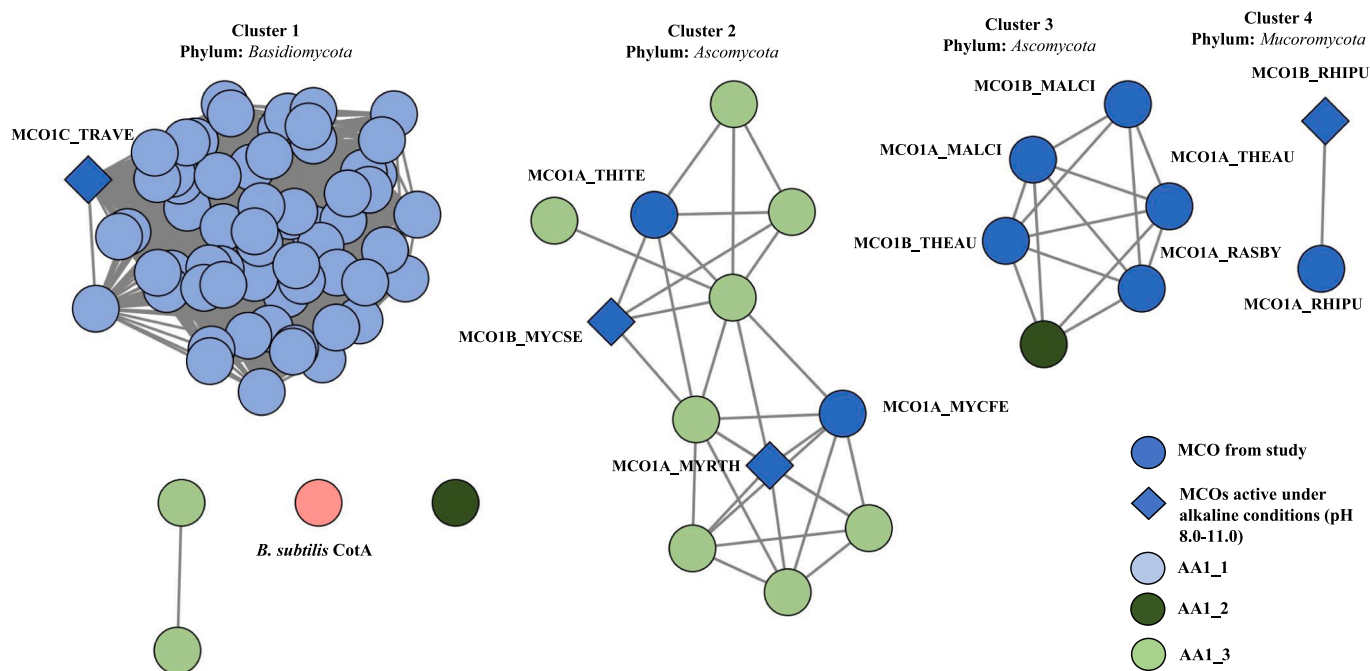


Fig. 6. Sequence similarity network (SSN) of 12 lignin-active multicopper oxidase (MCO) enzymes from this study, together with previously biochemically characterized Auxiliary Activity 1 (AA1) candidates from the carbohydrate-active enzyme (CAZy) database. Nodes represent the amino acid sequences connected by edges below E -value threshold of 10^{-130} for sequence similarity and are colored by AA1 subfamily as characterized on CAZy. Taxonomic classifiers for each cluster at the phylum level are indicated with CotA spore coat protein laccase from *B. subtilis* as outgroup. Diamond nodes indicate MCOs retaining high activities under alkaline pH conditions.

herein grouped with AA1 sub-families with few previously characterized members. The MCOs active in alkaline conditions are indicated by diamond markers and no significant clustering of the corresponding sequences was observed (Fig. 6). Consequently, no significant correlation was observed between taxonomic origin and enzyme activity on industrial lignins. This could be explained by the non-natural origin of the substrates used in this study as well as the typically broad substrate specificity of laccases, as discussed previously. Notably, MCOs grouped in cluster 3 represent those that generally showed lowest measured activities. Given that all MCOs in this cluster originate from thermophilic organisms, their lower measured performance could be explained by having run the assays in ambient conditions.

4. Conclusion

Simple model compounds are conventionally employed in enzyme screens for conversion of complex biomass substrates such as lignin. In a departure from this approach, the current study reports screening options that evaluate MCOs on three different industrial lignins. Twelve kraft lignin-active MCOs were discovered, including 9 MCOs active under alkaline conditions. Eleven out of the 12 MCOs were also active on organosolv lignin. Critically, the observed lack of correlation between activity of enzymes on mediator compounds and industrial lignins was quantitatively demonstrated, underscoring the importance of incorporating complex substrates early on in screens for industrially relevant enzymes.

CRedit authorship contribution statement

Anupama A. Sharan: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – Review and Editing, Visualization. **Annie Bellemare:** Methodology, Investigation, Writing – original draft, Writing – Review and Editing.

Marcos DiFalco: Methodology, Investigation, Writing – original draft. **Adrian Tsang:** Conceptualization, Writing – Review and Editing, Resources, Supervision, Project Administration, Funding acquisition. **Thu V. Vuong:** Writing – Review and Editing, Project Administration. **Elizabeth A. Edwards:** Writing – Review and Editing, Supervision, Project Administration, Funding acquisition. **Emma R. Master:** Conceptualization, Writing – Review and Editing, Resources, Supervision, Project Administration, Funding acquisition.

Declaration of competing interest

The authors have no competing interests to declare.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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