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Optimization of Laccase Production, and Characterization of Lignin Degradation Products by *Fusarium oxysporum* JUMAD-053

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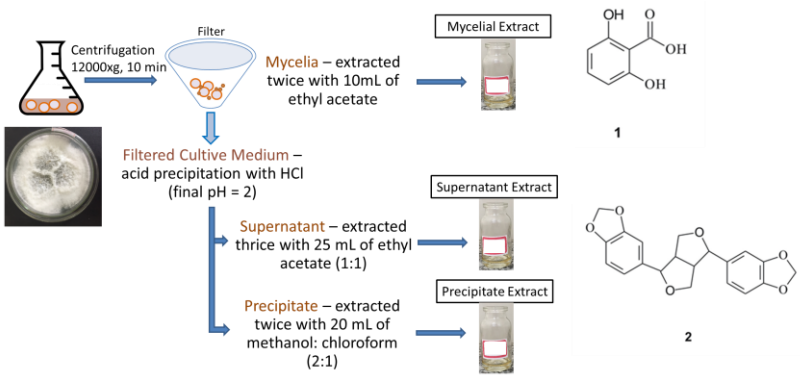
The objective of this work was to optimize the culture conditions for laccase production in the presence of Kraft lignin by *Fusarium oxysporum* JUMAD-053, and to evaluate the biodegradation products of lignin. The fungal isolate that presented highest laccase activity had its production optimized by a statistical factorial design 3^3 in 15 experimental runs. *F. oxysporum* presented the highest constitutive laccase titer (5.37 U/mL). Statistical factorial design demonstrated a maximum laccase titer of 9.8 U/mL when assayed against ABTS under the conditions optimized: 1.125% (w/v) yeast extract, 0.5% (w/v) Kraft lignin and 10 days of cultivation. The maximum laccase titer when assayed on DMP was 8.4 U/mL, following the conditions optimized: 1.125% yeast extract, 0.25% Kraft lignin and 7 days of cultivation. The analysis of cultures led to identification of metabolites; two being aromatic: 2,6-dimethoxy benzoic acid and sesamin; also, fumonisin and long-chain fatty acids. As a result of the study, the maximum laccase activities of 9.8 and 8.4 U/mL measured from ABTS and DMP substrates, respectively. The search shows new sources of fungal laccase for obtaining new metabolites of biodegradation from Kraft lignin in culture medium.

Graphical abstract



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1. Introduction

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The world dependence on non-renewable fossil resources has increased significantly during the last century, raising a concern with the environment and interest in a sustainable economy. Thus, the use of raw materials and technologies based on carbon, but free of fossil materials, has received special attention in research and industrial application. This is the case of the biorefinery process in which enzymatic conversion and fermentation of lignocellulosic materials - consisting of lignin, cellulose and hemicellulose - into biofuels and value-added chemical products takes place [1, 2].

Lignin is a natural aromatic biopolymer, where it represents about 30% of all organic carbon, in addition to being the most abundant aromatic compound in vascular plants [3–5]. Because it is an important natural source of carbon and aromatic compounds (lignin is used as a dietary fiber in some foods), lignin has been involved in renewable and sustainable production research, since its biodegradation aromatic products have important industrial applications [6].

Despite its potential from the 70 million tons of lignin produced annually, as a by-product of the paper production process [3]. Most of the lignin is discarded or burned for low-cost bioenergy production in the paper and pulp industries, which does not fit sustainable development, and may contribute to the generation of greenhouse gases and the loss of valuable chemical compounds present in wood [7–9].

Among the microorganisms capable of promoting lignin biodegradation, the white-rot fungi stand out. They produce and use enzymes such as laccases, peroxidases such as lignin peroxidase (LiP), manganese peroxidase (MnP), secreted by hyphae, and degrade lignin by oxidative depolymerization [10]. Laccase is capable of catalyze the mono-electronic oxidation of organic substrates, as lignin, while concomitantly reducing molecular oxygen to water [11]. White-rot fungi have received more attention in studies of lignin degradation, as they have a very efficient enzyme system degrading lignin [12]. The ascomyceteous fungi are becoming increasing recognized as playing an important role in the decomposition of lignocellulosic materials [13].

Laccases (diphenol:dioxigen oxidoreductases, EC 1.10.3.2) are polyphenol oxidases containing copper atoms in the catalytic centre [11,14,15]. It is a very versatile enzyme, of much biotechnological interest, for example, in organic synthesis [11], in the fabrication of biosensors [16], in the degradation of aromatic compounds, and as a bioremediation agent [17–19].

The objective of the work reported herein was to optimize the yeast extract concentration and incubation time in culture conditions, for optimal laccase production in the presence of Kraft lignin by *Fusarium oxysporum* JUMAD-053. Degradation metabolites of lignin were also examined and identified by UHPLC-HRMS/MS.

2. Material and Methods

2.1 Isolation of fungi

The forty-seven (47) fungi used in this work (**Table S1, Supplemental Material**) were isolated from decaying tree trunks. The collections were from different parks in Londrina and Maringá, Paraná, which constitutes part of the Atlantic Forest biome of Brazil. The fungal specimens were removed with the aid of a knife and placed in plastic bags. After external asepsis with 70% alcohol and 2.5% sodium hypochlorite, they were aseptically placed on solid medium comprizing potato-dextrose-agar (PDA), and colonies isolated, and purified by successive culturing. The isolates were stored on PDA slants in tubes, and also in sterile distilled water by the Castellani method [20].

2.2 Screening for constitutive laccases

The 47 fungal isolates were evaluated for the production of constitutive laccases. The fungal isolates were cultured in Petri dishes on solid medium containing PDA for 7 days at 28 °C. Subsequently, four disks (agar plugs) of 5 mm in diameter, completely covered by fungal mycelium, were transferred to 125-ml Erlenmeyer flasks containing 25 ml of basal medium (Vogel minimum salts medium [21]), 1% (w/v) glucose and 1% (w/v) yeast extract]. The cultures were kept under agitation (180 rpm) for 5 days at 28 °C.

The fungal cultures were harvested by centrifugation (1200 x *g*/10 min) and the supernatants (cell-free extracts, ELCs) used to assay for laccase activity. The microorganism with the highest laccase activity by the fungal isolates screened was JUMAD-053 (**Fig. S1, Supplemental Material**), and was studied further as described below. This isolate was collected from the trunk of a tree on the campus of Universidade Estadual de Maringá, Maringá, Paraná, (geographic coordinates: 23° 24' 28.5" S and 51° 56' 8.7" W).

2.3 Laccase assays

The cell-free extracts were used to assay for laccase activity against two substrates: putative ABTS (0.05M) and the natural substrate 2,6-DMP (10 mM) in 0.25 M citrate-phosphate buffer (pH 3.0), and were incubated at 50 °C for 5 min, and the absorbance measured at 420 nm (ξ = 36,000 mol^{-1} cm^{-1}) and 468 nm (ξ = 10,000 mol^{-1} cm^{-1}), for the two respective substrates. Laccase activity is expressed in units (U) as the number of µmols of oxidized ABTS or DMP formed per min under the conditions of the enzyme assays. The optimal pH was previously determined as 3.0 for both substrates.

2.4 DNA sequencing and phylogenetic analyses

2.4.1 Isolation of genomic DNA

To obtain genomic DNA, approximately 500 mg of mycelium was harvested and ground in liquid nitrogen. The mycelial powder was transferred to a microtube and 800 µL of lysis buffer (100 mM Tris–HCl (pH 8.0), 25 mM EDTA, 1% SDS, 25 mM NaCl) were added. The sample was then heated in a water bath at 65 °C for 20 min. Deproteinization was carried out by adding a volume of phenol, followed by an equal volume of chloroform: isoamyl alcohol (24:1). DNA was precipitated by addition of two volumes of ice-cold ethanol 10% and 3 M NaCl. The DNA precipitate was then resuspended in 25 µl of sterile deionized distilled water. Samples were quantified using a spectrophotometer, and the purity was estimated by measuring the absorbance at 260 nm/280 nm (validated when between 1.8 and 2.1). The integrity of the DNA was demonstrated in 1.2% agarose gel electrophoresis stained with ethidium bromide in TAE buffer (working solution: 40 mM Tris, 1 mM EDTA, glacial acetic acid 1.2 µL/mL, pH 8.0) at 100 V. DNA quantification was performed by spectrophotometry. A working DNA concentration of 20 ng/µL was used for PCR reactions.

2.4.2 Amplification of internal transcribed spacer (ITS) region

and sequencing

Primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) were used to amplify the ITS1-5.8S-ITS2 genomic region (Invitrogen, Life technologies, Carlsbad, California, USA) [22]. Each PCR reaction was carried out in 20 µl solution containing 2.0 mM $MgCl_2$, 0.2 mM of each primer, 0.2 mM of each dNTP, 0.1 mg of bovine serum albumin (BSA), and 0.01 U/µl of Taq DNA polymerase. An aliquot of 25 ng of DNA was used in each PCR reaction. The amplification protocol consisted of an initial denaturation step of 5 min at 95°C, followed by 35 cycles of amplification as follows: 1 min at 95 °C, 1 min at 55°C, and 1 min at 72 °C. A final extension of 10 min was performed at 72 °C. After amplification, an aliquot of 2 µl was analyzed by electrophoresis on 1.5 % agarose gel. The PCR products were sent for sequencing to ACTGene-Análises Moleculares Ltda, Alvorada, Rio Grande do Sul, Brazil. The generated gene sequences were compared with sequences available in GenBank by using the BLASTn program (Basic Local Alignment Search Tools) (**http://www.ncbi.nih.gov**).

2.5 Optimization of laccase production by statistical factorial design

Fungal isolate JUMAD-053 was selected, and was grown on solid medium comprizing VMSM, 2% (w/v) agar and 1% (w/v) glucose for 10 days at 28 °C. Subsequently, four agar plugs (diam. 5 mm) colonized with fungal mycelium were transferred to 125-mL Erlenmeyer flasks containing 25 mL of VMSM, 1% (w/v) glucose, and varying concentrations of yeast extract (0.5 to 3.0%, w/v) and Kraft lignin (0 to 0.5% (w/v) dissolved in 2% (v/v) DMSO). The time of cultivation varied from 4 to 10 days. The cultures were incubated at 28 °C under agitation (180 rpm), with a medium initial pH between 6.5-7.0.

The factors and levels described were evaluated in a Box-Behnken design [23] using three variables at three levels (3^3) in 15 experimental runs, of which three were repetitions at the central point. Cultivations for each condition were conducted in triplicate. Statistical analysis, including analysis of variance (ANOVA), the Pareto graph and the response surface plots were conducted using Statistica 10.0 software, StatSoft. The response surface design enabled the elaboration of an adjusted second order model, containing linear and quadratic effects, in addition to binary interactions.

After cultivation, the fungal cultures were harvested (filtration) and the mycelia and cell-free filtrates recovered. The latter in turn, were assayed for laccase activity against the substrate ABTS as described on section 2.3.

2.6 Biodegradation of Kraft lignin

The fungal isolate JUMAD-053 was grown in 25 mL of VMSM medium containing 1% glucose (w/v), according to the conditions optimized in the respective factorial plans and assayed against the substrates ABTS: 1.125% (w/v) of extract yeast, 0.5% (w/v) of Kraft lignin dissolved in DMSO (2%, v/v) and 10 days of culture.

Thereafter, the cultures were centrifuged (1200 x *g*/10 min) and filtered on Whatmann No. 5 paper. The mycelia recovered were treated with 10 mL of ethyl acetate (mycelial extract). The cell-free fermentation broths (supernatants) were subjected to acid precipitation to avoid emulsification by adding 37% hydrochloric acid to pH 2.0 and left stationary for 12 h at 7 °C [24], and thereafter were filtered giving rise to 2 fractions; a precipitate and filtrate. The filtrate was extracted thrice with ethyl acetate (1:1) (labeled supernatant extract). The precipitate recovered was extracted with 20 mL of methanol:chloroform (2:1) (precipitate extract). The protocol of the extraction steps is shown in the flowsheet (**Fig. S2, Supplemental Material**).

Samples of controls included: biotic (fungus and culture medium), abiotic with lignin (culture medium and Kraft lignin) and abiotic without lignin (only culture medium), and they were incubated in parallel with the growing fungal cultures. All extracts were analyzed for metabolites by LC-MS (see below).

2.7 Ultra High-Pressure Liquid Chromatography (UHPLC) and High-Resolution Mass Spectrometry (HRMS/MS)

Dried samples from organic extracts (mycelial, labeled supernatant, precipitate and controls) were diluted in an aqueous solution of 10% methanol to a concentration of 10 mg/ml. Chromatographic analysis was performed on an UHPLC Dionex Ultimate 3000 (Thermo Fisher Scientific, city, state, USA) with a C_{18} column (2.1 x 50 mm, 1.7 µm particle size, Syncronis, ThermoFisher). A Q Exactive™ Orbitrap (ThermoFisher Scientific, Bremen, GE), controlled by Xcalibur™ software was used for mass spectrometry (MS) data collection from the fungal extracts in quintuplicates. For metabolite separation, the mobile phase (A) consisted of 0.1% formic acid in ultra-pure water containing 5 mM ammonium formate, and mobile phase (B) consisted of 0.1% formic acid in methanol. The elution method followed the gradient: 10% B at 0 min; 10% B at 1 min; 100% B at 9 min; 100% B at 12 min and re-equilibration with 10% B from 12.5 to 15 min. The flow rate was 0.4 mL min^{-1} and the column was maintained at 40 °C. The injection volume of each sample was 5 µL. The mass data were acquired in the positive electrospray ion mode (ESI) with +3.0 kV. The sheath gas was set to 60 L/h, the capillary temperature was set to 325 °C, and the S-lens RF level 80. The desolvation gas was high-purity nitrogen and was set to 20 L/h at a temperature of 325 °C. The scan range selected was set from 67 to 1,000 *m/z*, as profile mode, with resolution of 70,000 on Full MS. The AGC (automatic gain control) was $1x10^6$ and the maximum IT (ion trap) was 100 ms. The MS^2 were acquired as data dependent (DDms2) with 17,500 resolution, AGC $1x10^5$ and maximum IT for 50 ms. The normalized collision energy (NCE) was alternated between 10/15/30 eV. Calibration with caffeine, MRFA, Ultramark 1,621, $[M+H]^+$ 195 - 1,522 *m/z*, and SDS, sodium taurocholate, Ultramark 1,621 $[M+H]^+$ 265 - 1,680 *m/z* was performed before the analyses.

2.7.1 UHPLC-HRMS/MS data processing and metabolite identification

After acquisition, the raw data were submitted to the identification of compounds using software Compound Discoverer 2.1 (Thermo Fisher Scientific, NY, USA), using the workflow to untargeted mode and a blank analysis as background. The identifications were based on ChemSpider, mzCloud, BioCyc pathways, KEEG, Human Metabolome Database and LipidMAPS databases. Some compounds could be identified only by their elemental composition, and others were completely unknown.

3. Results and Discussion

3.1 Selection of fungal isolates producing constitutive laccase

A total of 47 fungal isolates were screened and evaluated for the production of constitutive laccases by submerged fermentation on basal medium (**Table S2, Supplemental Material**). The isolate JUMAD-053 showed the highest laccase activity against ABTS as substrate (5.37 U/mL) when compared to the other fungal isolates tested. All isolates showed low laccase activity against DMP as substrate varying between 0.0 and 0.16 U/mL; that of JUMAD-053 was 0.01 U/mL. These results suggest the production of laccase isoforms. This isolate was molecularly characterized as *Fusarium oxysporum* (see below). The production of laccases by the fungus *F. oxysporum* (isolated from hard wood) was previously reported for the strain HUIB02 [25].

The production of laccase isoforms is commonly reported in the literature for fungi. For example, the ligninolytic basidiomycete *Pleurotus nebrodensis* produced three isoforms of laccase [26]. Another example is the basidiomycete *Trametes hirsuta*, which produced four laccase isoforms induced during the degradation of textile dyes [27]. The production and secretion of these isoenzymes are influenced by the conditions to which growing fungi are subjected [28]. The availability of different nutrients in the culture medium as a source of carbon (glucose), nitrogen, aromatic compounds, xenobiotic compounds, and under static or agitation conditions of the cultures, are examples of these conditions [29].

3.2 Molecular phylogenetic analyses and identification of the fungal isolates

As isolate JUMAD053 was the best laccase titer producer, it was selected for further study, and was consequently chosen for molecular identification. BLAST search in the NCBI database revealed high homology to fungal *Fusarium oxysporum* (ID: GenBank: MG356946.1). Phylogenetic reconstruction based on nucleotide sequences and similar sequences deposited in GenBank demonstrated that isolate JUMAD-053 was closer (bootstrap support 100%) to other *F. oxysporum* clusters. Reference strains of *F. oxysporum* in a well-supported clade in the Maximum Parsimony phylogenetic tree, and they were named accordingly (**Fig. 1**).

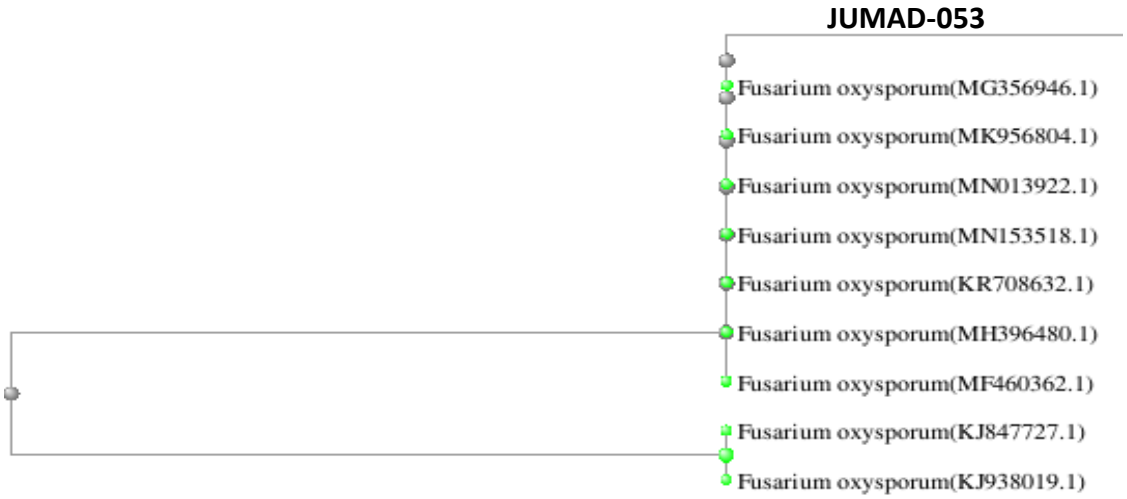

Fig. 1. Phylogenetic tree reconstructed for sequences of JUMAD-053 and its homologs of different *Fusarium oxysporum* strains.

3.3 Optimization of laccase production in the presence of lignin - factorial design

The production of laccase by isolate JUMAD-053 was optimized using the response surface method and considered the factors: concentration of yeast extract (X_1) and Kraft lignin (X_2), in addition to the cultivation time (X_3) and enzyme activity was assayed against the substrates ABTS and 2,6-DMP.

Table 1 shows the results of the optimization measured against the substrates ABTS and DMP. In factorial design, Pareto chart (**Fig. S3, Supplemental Material**) is an important tool to demonstrate the magnitude of significance for the selected independent variables [30].

The bars that intersect the dividing line (p = 0.05) on the Pareto charts represent the factors that resulted in significant effects on laccase production, with (L) the linear and (Q) the quadratic effects.

These results demonstrated that for the laccase activity determined against ABTS, the effect of time was the most significant factor in enzyme production by SmF, followed by the concentration of Kraft lignin and yeast extract. The Pareto chart also showed that the interactions of yeast extract versus Kraft lignin and time were not significant at the 95% confidence level.

The regression coefficients obtained were used in Equation 1, which represents laccase production against the substrate ABTS. All values represented are significant (p <0.05), and the terms that did not show statistical significance were omitted from the second order equation, represented here in its reduced form:

$$Y = 7.3 - 1.11250X_1 + 1.12875X_2 + 1.51625X_3 - 1.10625{X_2}^2 + 1.10250X_2X_3 \quad (1)$$

The response curves obtained in the optimization of the production of laccase, as determined against the substrate ABTS, and evaluated for the different levels of the stipulated factors are shown in **Fig. 2**, and **Fig. S4 - Supplemental Material**. **Fig. 2** shows the response surface plots (3D and contours) obtained for laccase activity determined against the substrate ABTS. The maximum laccase activity for the statistical model of 9.8 U/mL was obtained under the following conditions: 1.125% (w/v) yeast extract, 0.5% (w/v) Kraft lignin and cultivation time of 10 days.

Table 1. Central composite factorial (3^3) design to optimize laccase production assayed against the substrates ABTS and 2,6-DMP.

Experimental run	X_1	X_2	X_3	Responses (laccase activity, U/mL) ABTS $Y_{méd} \pm SD$	2,6-DMP
1	-1	-1	0	7.45 ± 0.39	6.61 ±0.67
2	+1	-1	0	3.90 ±0.40	3.25 ±0.07
3	-1	+1	0	8.26 ±0.39	5.74 ±0.94
4	+1	+1	0	6.61 ±0.81	3.94 ±0.35
5	-1	0	-1	6.64 ±0.30	6.19 ±0.13
6	+1	0	-1	4.71 ±0.28	3.38 ±0.05
7	-1	0	+1	9.49 ±0.42	4.05 ±0.10
8	+1	0	+1	7.72 ±0.71	2.57 ±0.57
9	0	-1	-1	3.83 ±0.77	4.38 ±0.72
10	0	+1	-1	4.38 ±0.37	4.45 ±0.29
11	0	-1	+1	4.76 ±0.71	2.15 ±0.71
12	0	+1	+1	9.72 ±0.31	2.26 ±0.17
13	0	0	0	7.30 ±0.32	8.09 ±1.87
14	0	0	0	7.16 ±0.62	8.05 ±0.44
15	0	0	0	7.44 ±0.18	8.14 ±3.40

Factors	Real Values -1	0	+1
X_1 – Yeast Extract (%)	0.5	1.75	3.0
X_2 – Kraft Lignin (%)	0	0.25	0.5
X_3 – Incubation time (days)	4	7	10

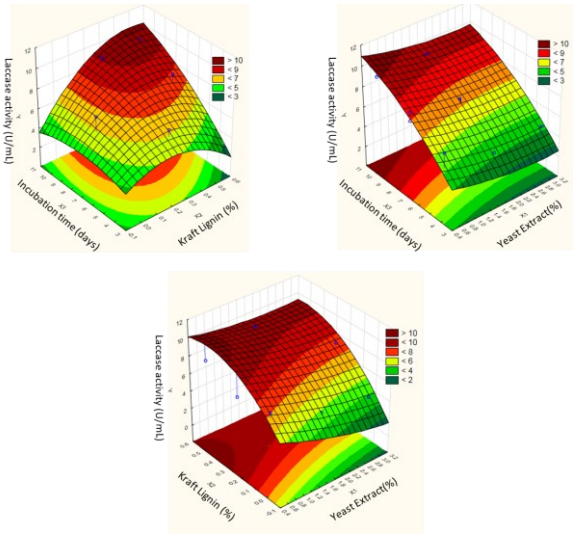

Fig. 2. Response surface plots for laccase production assayed against ABTS as substrate, and fixed at: a) X_1 at 1.125%, b) X_2 at 0.5% and c) X_3 at 10 days; yeast extract (X_1) and Kraft lignin (X_2) and fermentation time (X_3).

The value of R^2 in the optimized condition using ABTS as substrate was 0.9836, indicating that the values predicted by the model are well-aligned with the values obtained experimentally. To confirm the possible applicability of the model obtained, validation experiments of three replicates were performed under the optimum conditions. The average laccase activity obtained experimentally was 10.1 ± (0.33)U/mL, which showed no statistically significant difference in relation to the predicted value of 9.8 U/mL, thus demonstrating the adequacy of the proposed model.

Table 1 presents the optimization results measured against the substrate 2,6-DMP for laccase assay. The enzyme activity values for each replicated replicate ($Y`_1$, $Y`_2$ and $Y`_3$) are presented, as well as the average value (Y'_{med}), with their respective standard deviations.

The Pareto chart (**Fig. S5, Supplemental Material**) highlighted the statistically important factors for the expression of the laccase titres of *F. oxysporum* (JUMAD-053) against the substrate DMP. The effect of incubation time was the most significant factor, followed by the concentration of Kraft lignin and yeast extract. For laccase assayed against DMP as substrate, the statistical effects remained essentially in the same sequence of importance, but they differed from the results obtained with the ABTS substrate, where the interaction of Kraft lignin versus cultivation time was not significant for the production of laccase. The results indicate the possibility of laccase isoenzymes being produced by the studied fungal isolate, as isoenzymes have different affinities for the substrates ABTS and DMP. Ramírez-Cavazos and collaborators [31] reported the production of two laccase isoforms from *Pycnoporus sanguineus* CS43; Lac I showed higher affinity for ABTS and DMP, while Lac II showed higher affinity for guaiacol.

From the regression coefficients obtained against the substrate DMP, Equation 2 was obtained. All values represented are significant ($p < 0.05$), and terms that did not show statistical significance were omitted from the second order equation, represented here in its reduced form:

$$Y' = 8{,}09333 - 1{,}18125X_1 - 0{,}92125X_3 - 1{,}23542{X_1}^2 - 1{,}97292{X_2}^2 - 2{,}81042{X_3}^2 + 0{,}39X_1X_2 \quad (2)$$

The response surface curves (Fig. 3, **Fig. S6**) obtained for the optimization of laccase production determining the activity against the substrate DMP, evaluating the different levels of the stipulated factors. The curves showed that the optimal condition was within the range of the levels studied. Fig. 3 shows the response surface plots obtained when the laccase activity was determined against the substrate DMP. Maximum laccase activity of 8.4 U/mL was predicted under the following conditions: 1.125% yeast extract, 0.25% Kraft lignin and 7 days of culture. The value of R^2 in the optimized condition using the DMP substrate was 0.9939 demonstrating that the values obtained experimentally were adequate for the proposed model.

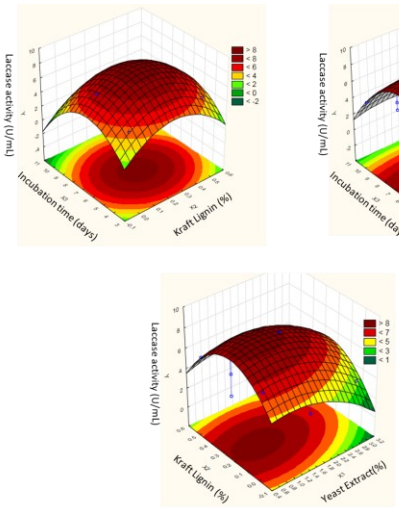

Fig. 3. Response surface plots of laccase production assayed against the substrate DMP, and fixed at: a) X_1 at 1.125%, b) X_2 at 0.25% and c) X_3 at 7 days, yeast extract (X_1) and Kraft lignin (X_2) and fermentation time (X_3).

According to the optimization of laccase reported by Hernández-Monjaraz and collaborators [32], a strain of *Fusarium oxysporum* f. sp. *lycopersici* positively benefited from the addition of an iron chelator (bathophenanthrolinedisulfonic acid) resulting in an increase of up to 114.32% in the laccase titer. The study developed by Medeiros and collaborators [33] optimized laccase production for a strain DM-1513 of the basidiomycete *Pleurotus ostreatus*, in which the influence of initial pH, yeast extract concentration and the action of some inducers were evaluated. Their results showed that the initial pH between 6.0 and 6.5 and yeast extract concentrations of 0 ± 0.25 % were statistically significant in the highest levels of production of laccase activity (489 ± 540 U/l) by this fungal strain. In this work, the highest enzymatic results were under the 1.125% yeast extract concentration. In addition, the work developed by Vasconcelos and collaborators [34] optimized the production of laccase against ABTS by the ascomycete *Botryosphaeria* sp. MAMB-05 at 5.6 U/mL, demonstrating that the concentration of veratryl alcohol (laccase inducer) and cultivation time were the 2 significant variables. By contrast, yeast extract had no influence on laccase production by this fungal isolate. The results obtained in our work suggest that yeast extract was a major factor in production of laccases by *Fusarium oxysporum* JUMAD-053. The ascomycete fungal *Nectriella pironii* [35] and *Aspergillus* sp. HB_RZ4 [36] revealed laccase activity using 3.0 and 2.5 g/l of yeast extract, respectively. Since for the *Aspergillus* sp. HB_RZ4 the optimization by statistical design studies shown optimum laccase yield with this concentration of yeast extract [36].

3.4 Identification of lignin degradation products by UHPLC and HRMS/MS in tandem mode

Table 2 shows the degradation metabolites produced by *F. oxysporum* JUMAD-053 when cultivated on media containing Kraft lignin under optimizated conditions for laccase production as assayed against ABTS as substrate, and that which appeared in the mycelial extracts (metabolites 1–7), and which were absent in the controls. Under DMP as substrate conditions there were no phenolic metabolites identified, only alpha-linolenic acid. The phenolic compounds identified as lignin metabolites were 2,6-dimethoxy benzoic acid and sesamin (**Fig. 4**). The 2,6-dimethoxy benzoic acid is lignin monomeric products from depolymerization [37]. Benzoic acid has been reported as aromatic products arising from lignin breakdown, and can be present as metabolites in soil, and produced by the ligninolytic fungus, *Phanerochaete chrysosporium*, respectively [38]. The production of this compound by *F. oxysporum* JUMAD-053 is evidence of lignin degradation, as these phenolic units are considered to be basic moieties that build the natural lignin polymer. In addition, these aromatic compounds from the biodegradation of lignin act as inducers of lignin in culture medium, such as veratryl alcohol [34] and ferulic acid [38].

Table 2. Characterization of named ions and MS/MS of metabolites of *Fusarium oxysporum* cultivated under conditions of SmF on media containing Kraft lignin.

Metabolites	Molecular formula	Found $[M+H]^+$	Precursor ion (m/z)	Product ions (m/z)
1. 2,6 -Dimethoxy benzoic acid	$C_9H_{10}O_4$	183.07707	183.07655	165.06696[a] (M-H_2O) 137.07095 (M-H_2CO_2)
2. not identified	$C_{15}H_{18}O_4$	263.12846	263.12756	245.11682 (M-H_2O) 231.10020 (M-H_2CO_2) 137.07095 (100%)
3. 13-Hydroxyoctadeca-6,9,11-trienoic acid	$C_{18}H_{30}O_3$	294.21886	277.21600	259.20636 (M-H_2O) 179.14319 (M-98) 161.13223 (M-98-H_2O)
4. not identified	$C_{17}H_{16}O_6$	317.10272	317.21115	299.19995 (M-H_2O) 271.20554 (M-H_2O -H_2CO_2) 243.21136 (M-H_2O H_2CO_2 - CO)
5. Hydrolyzed fumonisin B1	$C_{22}H_{47}NO_5$	406.35214	334.31058 (M – 72)[b,c]	317.28369 (M - $^+NH_3$) 137.13243, 123.11692* 109.10140*, 95.08588*
6. Sesamin	$C_{20}H_{18}O_6$	355.15464	355.15411	151.07526[d,e] 137.05969[d,e]
7. alpha-linolenic acid	$C_{18}H_{30}O_2$	279.23190	279.23196	261.22086 (M-H_2O) 223.16948[f] (M – C_4H_8) 123.11710, 109.10151[f,*] 95.16949[f,*], 81.07043* 67.05490[f,*]

[a][47]; [b][48]; [c][49]; * loss CH_2 units – straight alkane; [d][50]; [e][51]; [f][52].

Sesamin (furofuran, a class of the lignan family of natural products) is a pinoresinol derivative of two fused tetrahydrofuran rings linked by *β/β'* bonds [39,40] was also detected (Table 2). This natural metabolite is a lignan isolated from the bark of plants, and the fungi use the same kinds of enzymes (peroxidases and laccases) to initiate lignin biodegradation that plants use to make lignin [41]. The pinoresinol component (*d,l*-syringaresinol) from lignin was a biotransformation breakdown product by *Fusarium solani* via oxidation of the benzylic position. Additionally this fungus degraded a synthetic lignin (a dehydrogenation polymer of coniferyl alcohol, DHP), dilignols and *d,l*-pinoresinol [42].

Moreover, *Fusarium proliferatum* produced limited mineralizations and residual products of lignins [43]. In addition, α-linolenic acid and 13-hydroxyoctadeca-6,9,11-trienoic acid were detected in mycelial extract (**Table 2**). The long chain acids such as octadecanoic acid had been reported in ethyl acetate extract from *Bacillus* sp. degrading kraft lignin [44].

Fuminosin B1 was also found in mycelial extract. Fumonisins constitute a class of mycotoxins that are produced by certain *Fusarium* species. Their chemical structures are made up of two tricarballylic acid groups esterified to a 20-carbon backbone. Under certain conditions, or through fungal metabolism, the aminopentol backbone, also known as hydrolyzed fumonisin B1 (HFB1) can be formed and is itself toxic [45].

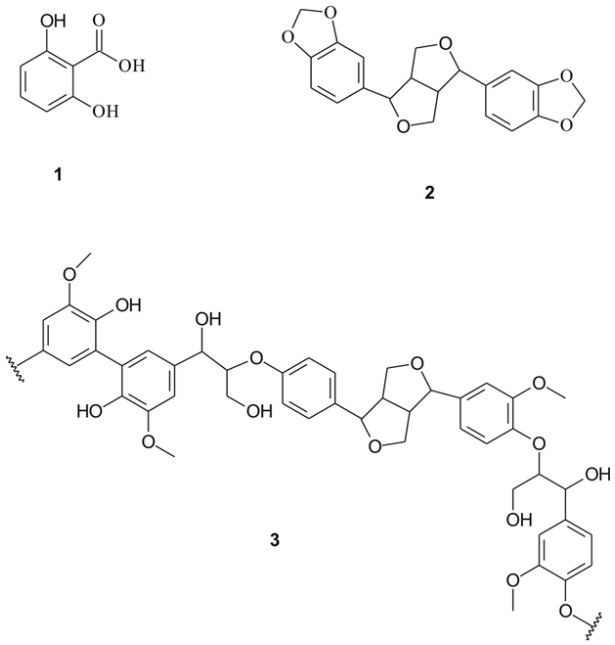

Fig. 4. Phenolic compounds: 2,6 -dimethoxy benzoic acid (1) and sesamin (2) identified as lignin metabolites of *Fusarium oxysporum*, appearing in mycelial extract extracted with ethyl acetate. Representation of a minor part of softwood lignin structure featuring the structure (3), [46].

4. Conclusions

Fusarium oxysporum isolate JUMAD-053 was selected based upon its production of constitutive laccases (5.37 U/mL). The optimal conditions for laccase production by this isolate were determined based on a 3^3 factorial design, taking into account the concentrations of yeast extract and Kraft lignin, and the time of cultivation, and assaying laccase activity against the substrates ABTS and DMP. The results predicted maximum laccase activities of 9.8 and 8.4 U/mL measured from ABTS and DMP substrates, respectively. The main metabolites produced from lignin through biodegradation by *F. oxysporum* JUMAD-053 were identified as 2,6 -dimethoxy benzoic acid and sesamin. The results obtained indicate that isolate JUMAD-053 can be used in the biodegradation of lignin, and to obtain aromatic metabolites.

Supporting Information

Tables S1-S2 and figures S1-S6.

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Author Contributions

Igor S. Shiraishi (master), Beatriz R. Ribeiro (graduate), Renato P. Dorte (master), Gabriela Pardinho (graduate): Investigation - Fungal cultivation, metabolites extraction and experimental assays. Dr. Robert F.H. Dekker: Assisted in writing and revising the paper. Dr. Luciana Furnaleto-Maia: Investigation - DNA sequencing and phylogenetic analyses. Dr. Aneli M. Barbosa-Dekker, Dr. Dionísio Borsato: Investigation - Analysed and discussed the statistical and assays results. Factorial design discussion. Dr. Joseph A.M. Evaristo, Dr. Geisa P.C. Evaristo: Investigation - Mass spectrometry analyses. Dr. Julliana I. Simionato: Factorial design discussion. Dr. Juliana F.S. Daniel: Project administration. Wrote the paper, supervision of experiments and was involved in discussion of the results. All authors read and approved the final manuscript.

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