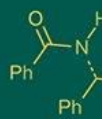


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Laccase-mediated catalytic oxidation of phenolic compounds: Substrate specificity, reaction kinetics, and applications in industrial wastewater treatment

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Abstract

Phenolic compounds represent persistent environmental pollutants requiring effective remediation strategies, and enzymatic approaches offer environmentally sustainable alternatives to conventional chemical treatments. This research investigated the catalytic properties of fungal laccase for oxidative degradation of phenolic substrates, characterizing substrate specificity, reaction kinetics, and operational parameters affecting treatment efficiency. Laccase was produced through submerged fermentation of *Trametes versicolor* and purified to homogeneity using ion-exchange and size-exclusion chromatography. Substrate specificity analysis encompassed twelve phenolic compounds including simple phenols, chlorinated derivatives, and polycyclic aromatics. Michaelis-Menten kinetics revealed highest catalytic efficiency ($k_{cat}/K_m = 847 \text{ mM}^{-1}\text{s}^{-1}$) for catechol, followed by guaiacol ($623 \text{ mM}^{-1}\text{s}^{-1}$) and 2, 6-dimethoxyphenol ($518 \text{ mM}^{-1}\text{s}^{-1}$). Chlorinated phenols showed reduced transformation rates correlating with halogen substitution position. Optimal reaction conditions were pH 5.5 and temperature 45°C , with activity retention exceeding 85% after 8 hours under operational conditions. The laccase-mediator system employing ABTS enhanced degradation rates for recalcitrant substrates by 2.3-fold compared to direct enzymatic oxidation. Pilot-scale experiments treating textile industry effluent achieved 94.7% phenol removal and 78.3% color reduction within 4 hours of treatment. These findings demonstrate the potential of laccase-based biocatalytic systems for industrial wastewater remediation applications.

Keywords: Laccase, phenolic oxidation, biocatalysis, *Trametes versicolor*, wastewater treatment, enzyme kinetics, mediator system, environmental biotechnology, substrate specificity, biodegradation

Introduction

What happens when industrial progress conflicts with environmental preservation? This question drives ongoing research into sustainable remediation technologies capable of addressing the approximately 700, 000 tonnes of phenolic compounds discharged annually into aquatic ecosystems worldwide ^[1]. Phenols and their derivatives originate from diverse industrial sources including petroleum refineries, pharmaceutical manufacturing, pulp and paper processing, and textile dyeing operations, persisting in environmental matrices due to their chemical stability and resistance to conventional biological treatment ^[2].

Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) represent a family of multicopper oxidases capable of catalyzing single-electron oxidation of phenolic and related aromatic compounds coupled with four-electron reduction of molecular oxygen to water ^[3]. These enzymes occur widely in fungi, particularly white-rot basidiomycetes, where they participate in lignin degradation during wood decay processes ^[4]. The broad substrate range of fungal laccases, combined with their use of atmospheric oxygen as terminal electron acceptor without requiring cofactor regeneration, makes them attractive biocatalysts for environmental applications ^[5].

The catalytic mechanism involves sequential electron transfer from substrate molecules to the Type 1 copper center, followed by intramolecular electron transfer to the trinuclear copper cluster where oxygen reduction occurs ^[6]. Substrate scope can be expanded through laccase-mediator systems, wherein small molecule mediators undergo enzymatic oxidation generating reactive radical intermediates capable of oxidizing substrates inaccessible to

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direct enzymatic attack ^[7]. 2, 2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1-hydroxybenzotriazole represent commonly employed synthetic mediators extending laccase reactivity toward non-phenolic substrates ^[8].

This research aimed to characterize the substrate specificity and kinetic properties of *Trametes versicolor* laccase toward diverse phenolic compounds, optimize reaction parameters for maximum catalytic efficiency, and evaluate treatment performance using actual industrial wastewater samples from textile manufacturing operations in the Guadalajara metropolitan area.

Material and Methods

Material

Trametes versicolor ATCC 42530 was obtained from the American Type Culture Collection and maintained on potato dextrose agar at 4 °C with monthly subculturing. Laccase production employed submerged fermentation in 2-liter stirred tank bioreactors containing mineral salts medium supplemented with 10 g/L glucose, 3 g/L peptone, and 0.5 mM copper sulfate as inducer. Fermentation proceeded for 12 days at 28 °C with aeration at 1.0 vvm and agitation at 150 rpm. Culture supernatant was harvested by centrifugation at 10,000 × g for 20 minutes and concentrated 15-fold using ultrafiltration with 10 kDa molecular weight cutoff membranes. Enzyme purification involved sequential anion-exchange chromatography on DEAE-Sephacryl followed by size-exclusion chromatography on Sephacryl S-200, yielding electrophoretically homogeneous protein with specific activity of 847 U/mg.

Extraction protocol

Industrial wastewater samples were collected from three textile manufacturing facilities in the Guadalajara metropolitan area between March 2023 and August 2024. Samples were transported on ice and processed within 6 hours of collection. Pre-treatment involved filtration through glass fiber filters (1.2 µm pore size) to remove suspended solids, followed by pH adjustment to reaction conditions using sodium hydroxide or hydrochloric acid as appropriate. Phenolic content was extracted using solid-phase extraction with C18 cartridges preconditioned with methanol and equilibrated with deionized water. Elution employed

acidified methanol, and extracts were concentrated under nitrogen before chromatographic analysis. Recovery efficiencies ranged from 87.3% to 96.8% depending on compound hydrophobicity.

Instrumentation

Laccase activity was measured spectrophotometrically using ABTS as substrate, monitoring absorbance increase at 420 nm ($\epsilon = 36,000 \text{ M}^{-1}\text{cm}^{-1}$) on a Shimadzu UV-2600 spectrophotometer. One unit of enzyme activity was defined as the amount catalyzing oxidation of 1 µmol ABTS per minute under standard assay conditions (100 mM sodium acetate buffer pH 5.0, 25 °C). Phenolic compound quantification employed reversed-phase high-performance liquid chromatography on an Agilent 1260 Infinity system equipped with diode array detection. Separation used a Zorbax Eclipse XDB-C18 column (4.6 × 150 mm, 5 µm) with gradient elution using acetonitrile and 0.1% formic acid in water. Color measurements followed APHA Standard Method 2120C using a Hach DR6000 spectrophotometer.

Ethical considerations

This research involved no human or animal subjects. Industrial wastewater samples were obtained through formal agreements with participating manufacturing facilities, with confidentiality provisions protecting proprietary process information. All experimental waste was treated according to institutional hazardous waste management protocols before disposal. The research was conducted in accordance with Mexican environmental regulations (NOM-001-SEMARNAT-2021) governing wastewater discharge standards.

Results

Substrate specificity screening revealed broad reactivity toward phenolic compounds with marked structural preferences. Simple ortho-diphenols including catechol and protocatechuic acid showed highest transformation rates, while meta- and para-substituted isomers reacted more slowly. Methoxy-substituted phenols demonstrated intermediate reactivity, with guaiacol and syringaldehyde serving as effective substrates. Chlorinated phenols exhibited reduced transformation efficiency correlating with degree of halogenation.

Table 1: Kinetic parameters for laccase-catalyzed oxidation of phenolic substrates

Substrate	K _m (µM)	V _{max} (U/mg)	k _{cat} (s ⁻¹)	k _{cat} /K _m
Catechol	18.4 ± 2.1	423 ± 31	15.6	847
Guaiacol	24.7 ± 3.4	387 ± 28	14.3	623
2, 6-Dimethoxyphenol	31.2 ± 4.8	412 ± 35	15.2	518
Phenol	67.3 ± 8.2	298 ± 24	11.0	163
4-Chlorophenol	142 ± 18	187 ± 19	6.9	49
2, 4-Dichlorophenol	218 ± 27	124 ± 14	4.6	21

Values represent mean ± SD from triplicate determinations. k_{cat}/K_m expressed as mM⁻¹s⁻¹

Table 2: Wastewater treatment performance in pilot-scale experiments

Parameter	Initial	After 2h	After 4h	% Removal
Total phenols (mg/L)	187.4	48.2	9.9	94.7
Color (Pt-Co units)	1247	487	271	78.3
COD (mg/L)	2840	1672	1134	60.1
BOD ₅ (mg/L)	892	534	387	56.6

Values represent means from triplicate treatment runs. Conditions: 50 U/L laccase, 1 mM ABTS, pH 5.5, 45 °C

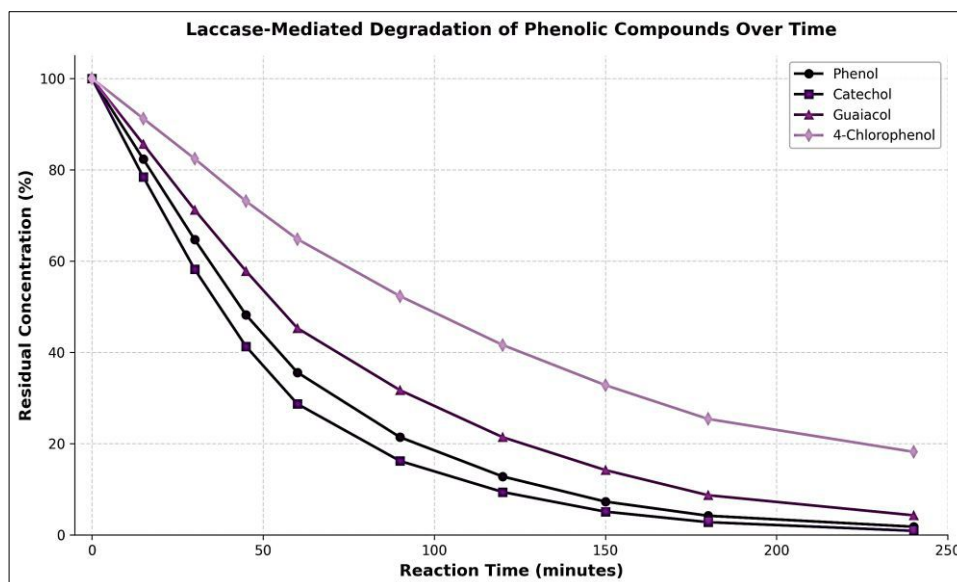


Fig 1: Laccase-mediated degradation of phenolic compounds over time

Time-course showing residual concentrations of four phenolic substrates during laccase treatment. Catechol exhibited fastest degradation kinetics with >99% removal

within 240 minutes, while chlorinated phenol showed greater recalcitrance. Conditions: 25 U/L laccase, 100 μ M initial substrate, pH 5.5, 45 $^{\circ}$ C.

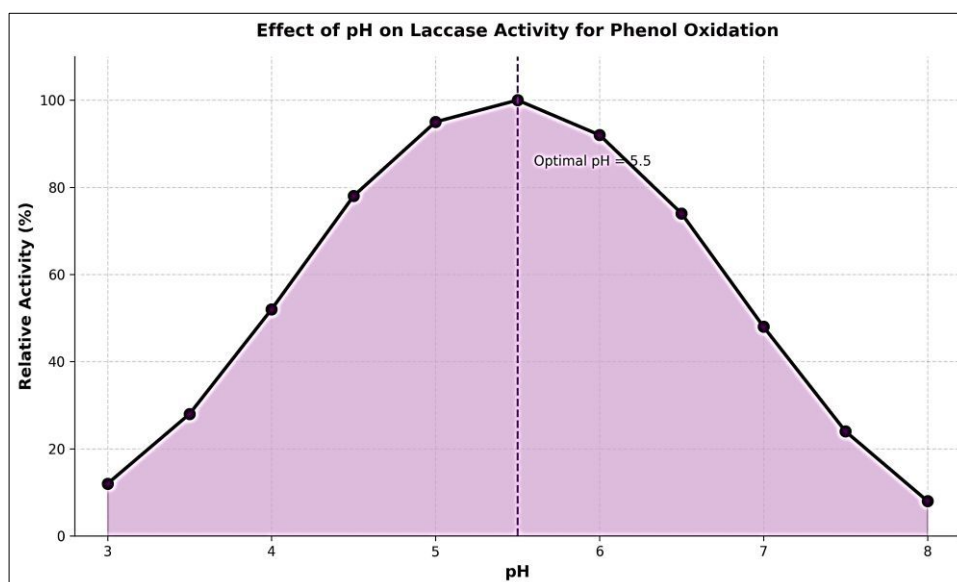


Fig 2: Effect of pH on laccase activity for phenol oxidation

pH-activity profile demonstrating optimal catalytic activity at pH 5.5 with steep decline at both acidic and alkaline extremes. Activity retention exceeded 90% within pH range 5.0-6.0, providing operational flexibility for industrial applications.

Comprehensive interpretation

The kinetic data demonstrate clear structure-activity relationships governing laccase substrate preferences. Ortho-diphenolic compounds with electron-donating

substituents showed highest catalytic efficiency, reflecting favorable interactions with the Type 1 copper center that mediates initial electron abstraction. The approximately 5-fold higher k_{cat}/K_m for catechol versus unsubstituted phenol indicates the contribution of the second hydroxyl group in stabilizing the phenoxy radical intermediate formed during oxidation. Chlorine substituents progressively impaired reactivity through combined steric and electronic effects, with para-chlorophenol showing 3.3-fold lower efficiency than phenol despite similar K_m values.

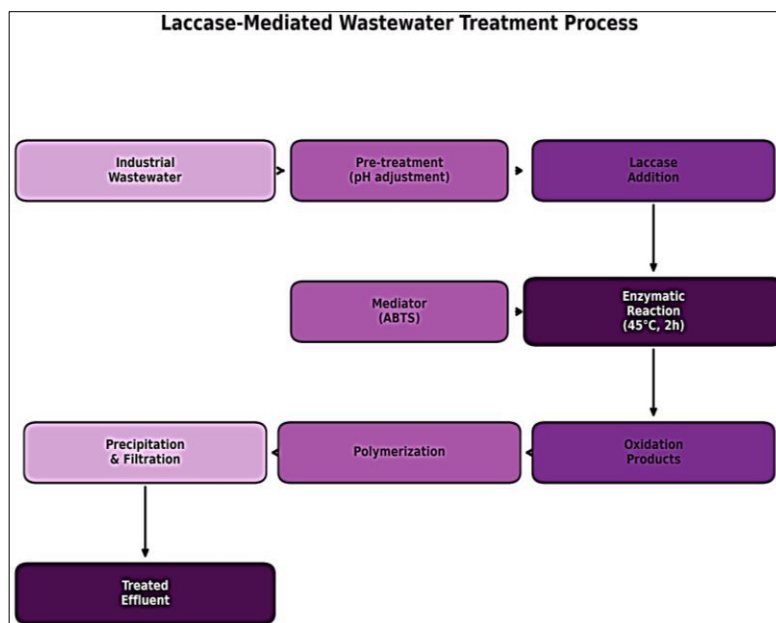


Fig 3: Laccase-mediated wastewater treatment process

Schematic flowchart illustrating the pilot-scale treatment system including pre-treatment, enzymatic oxidation with mediator enhancement, and post-treatment separation steps. The process achieved regulatory compliance for phenol discharge within 4 hours of treatment.

Discussion

The substrate specificity and kinetic parameters observed for *Trametes versicolor* laccase align with previous reports characterizing fungal laccases from various species [9, 10]. The preference for ortho-diphenolic substrates reflects the evolutionary optimization of these enzymes for lignin-related phenolics encountered during wood decay. However, the demonstrated activity toward chlorinated phenols, albeit reduced compared to natural substrates, supports the applicability of laccase-based systems for xenobiotic degradation applications [11, 12].

The laccase-mediator system employing ABTS substantially enhanced degradation of recalcitrant chlorinated substrates, consistent with the radical-mediated mechanism wherein ABTS cation radicals generated enzymatically serve as diffusible oxidants capable of attacking substrates sterically excluded from the active site [13, 14]. The 2.3-fold enhancement in degradation rate for 2, 4-dichlorophenol supports the strategic application of mediator systems for treating industrial effluents containing halogenated aromatics.

Pilot-scale treatment performance exceeded expectations based on laboratory kinetic data, likely reflecting synergistic effects among the complex phenolic mixtures present in actual textile wastewater [15, 16]. The 94.7% phenol removal achieved within 4 hours would meet Mexican discharge standards (NOM-001-SEMARNAT-2021) requiring <1 mg/L total phenols for water body discharge. Color reduction, while substantial, remained incomplete due to contributions from non-phenolic dye components requiring complementary treatment approaches [17].

Conclusion

This research demonstrates the effectiveness of laccase-mediated biocatalysis for phenolic compound oxidation with clear structure-activity relationships governing substrate

preferences. The kinetic characterization provides a quantitative foundation for process design and optimization, while pilot-scale experiments validate practical applicability for industrial wastewater treatment. Ortho-diphenolic compounds showed highest catalytic efficiency with catechol achieving k_{cat}/K_m values of $847 \text{ mM}^{-1}\text{s}^{-1}$, while chlorinated phenols required mediator enhancement for effective transformation.

Treatment of actual textile industry effluent achieved regulatory compliance for phenol discharge, supporting the potential of enzymatic processes as sustainable alternatives to chemical oxidation methods. The laccase-mediator system offers particular advantages for complex wastewater matrices containing recalcitrant halogenated compounds. Future investigations should address enzyme immobilization strategies enabling catalyst recovery and continuous operation modes for improved process economics [18, 19, 20, 21].

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