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Kinetic study of laccase-catalyzed oxidation of phenolic compounds in wastewater

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Abstract

Phenolic pollutants in industrial effluent are toxic at low concentrations, resist conventional biological treatment, and accumulate in aquatic food chains. Laccase (EC 1.10.3.2), a multi-copper oxidase found in white-rot fungi, can oxidize a broad spectrum of phenolic substrates using only molecular oxygen as the terminal electron acceptor no hydrogen peroxide, no expensive cofactors, and the only byproduct is water. This research characterized the kinetics of a commercial *Trametes versicolor* laccase toward three model phenolic substrates guaiacol, catechol, and 2,6-dimethoxyphenol (2,6-DMP) at pH 4.0-7.0 and 25-45°C, then applied the enzyme to synthetic wastewater containing 50 mg/L mixed phenolics. Michaelis-Menten kinetics gave K_m values of 0.34, 0.21, and 0.58 mM and V_{max} values of 4.82, 3.67, and 2.94 $\mu\text{mol}/\text{min}/\text{mg}$ for guaiacol, catechol, and 2,6-DMP respectively. Optimal conditions were pH 5.0 and 35°C. Under these conditions, the enzyme removed 96.2% of total phenolics from synthetic wastewater within 24 h. Laccase retained 74% of its initial activity after five consecutive treatment cycles when immobilized on chitosan beads. These results position laccase as a practical biocatalyst for phenol-laden effluent treatment.

Keywords: Laccase, phenol, oxidation, kinetics, wastewater, *Trametes versicolor*, Michaelis-Menten, guaiacol, catechol, bioremediation

Introduction

Phenolic compounds enter water bodies from pulp and paper mills, olive oil presses, textile dyeing operations, and pharmaceutical plants at concentrations that are too low for economical chemical treatment but too high for untreated discharge typically 10-500 mg/L^[1]. Chlorination, ozonation, and Fenton oxidation can destroy phenols, but these methods consume reagents, generate secondary pollutants, and are expensive to operate continuously^[2]. Enzymatic treatment offers an alternative that works under mild conditions, produces non-toxic polymerized products, and can be integrated into existing biological treatment trains^[3].

Laccases are blue multi-copper oxidases produced by fungi, bacteria, and plants. They catalyze the one-electron oxidation of phenols, anilines, and related substrates by shuttling electrons through a trinuclear copper cluster to reduce O_2 to H_2O ^[4]. Among fungal laccases, those from *Trametes versicolor* are the most commercially available and best characterized they have a broad substrate range, reasonable thermal stability, and an acidic pH optimum that suits many industrial effluents^[5].

For any enzymatic process to be industrially viable, kinetic parameters must be quantified: K_m indicates the substrate affinity, V_{max} sets the maximum throughput, and the ratio V_{max}/K_m (catalytic efficiency) ranks competing substrates^[6]. Although laccase kinetics have been studied for individual substrates, systematic comparisons under identical conditions are less common, and few studies bridge the gap from pure kinetics to realistic wastewater treatment performance^[7].

This research measured Michaelis-Menten kinetics for *T. versicolor* laccase against three model substrates, optimized pH and temperature, applied the enzyme to synthetic wastewater, and tested reusability via chitosan immobilization.

Safety Considerations

Guaiacol, catechol, and 2,6-DMP are irritants; they were handled in a fume hood with nitrile gloves. Phenol solutions above 1% are corrosive; face shields were worn during preparation. All phenolic waste was oxidized with sodium hypochlorite before drain disposal [8].

Material and Methods

Material

All work was conducted at the Department of Biological Chemistry, Aryabhata College of Chemical Sciences, Noida, from July 2022 to April 2023. Commercial T. versicolor laccase (EC 1.10.3.2, ≥ 10 U/mg, Sigma-Aldrich, catalogue 38429) was stored at -20°C . Guaiacol (98%), catechol (99%), 2,6-dimethoxyphenol (99%), chitosan (medium molecular weight), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)), and glutaraldehyde (25%) were from Sigma-Aldrich, Bangalore [9].

Methods

Laccase activity was assayed by monitoring ABTS oxidation at 420 nm ($\epsilon = 36,000 \text{ M}^{-1}\text{cm}^{-1}$) in 100 mM sodium acetate buffer, pH 5.0, at 25°C . One unit (U) = 1 μmol ABTS oxidized per minute [10]. Michaelis-Menten

kinetics: initial velocities were measured at eight substrate concentrations (0.025–5.0 mM) for each substrate at pH 5.0 and 25°C . K_m and V_{max} were determined by nonlinear regression (Levenberg-Marquardt algorithm). pH optimization tested buffers at pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0 using guaiacol as substrate. Temperature optimization covered 25 – 45°C in 5°C steps at pH 5.0 [11]. Synthetic wastewater was prepared with 50 mg/L mixed phenolics (guaiacol, catechol, phenol, 1:1:1 by mass) in tap water. Laccase (50 U/L) was added, and total phenolic removal was monitored by the 4-aminoantipyrine colorimetric method (APHA 5530D) at 0.5, 1, 2, 4, 8, 12, and 24 h [12]. For reusability, laccase was immobilized on chitosan beads crosslinked with 2.5% glutaraldehyde; beads were recovered by filtration after each 24 h cycle and re-used for five cycles [13]. All experiments were in triplicate.

Statistical Analysis

Kinetic parameters were fitted by nonlinear regression with 95% confidence intervals. ANOVA with Tukey HSD compared removal efficiencies across pH values. All statistics used GraphPad Prism 10 [14].

Results

Table 1: Michaelis-Menten kinetic parameters for T. versicolor laccase with three phenolic substrates

Substrate	K_m (mM)	V_{max} ($\mu\text{mol}/\text{min}/\text{mg}$)	V_{max}/K_m	R^2
Guaiacol	0.34 ± 0.03	4.82 ± 0.21	14.2	0.997
Catechol	0.21 ± 0.02	3.67 ± 0.18	17.5	0.998
2,6-DMP	0.58 ± 0.05	2.94 ± 0.16	5.1	0.994

V_{max}/K_m = catalytic efficiency (mL/min/mg). Conditions: pH 5.0, 25°C . Mean $\pm$ 95% CI ($n = 3$).

Catechol had the lowest K_m (0.21 mM) and highest catalytic efficiency (17.5 mL/min/mg), making it the preferred substrate (Table 1). Guaiacol had the highest V_{max} (4.82 $\mu\text{mol}/\text{min}/\text{mg}$), meaning the enzyme turns it over fastest at saturating concentrations. 2,6-DMP was the

poorest substrate on both measures. Optimal pH was 5.0 for all three substrates, with activity falling sharply above pH 6.0; optimal temperature was 35°C , with 50% inactivation by 45°C .

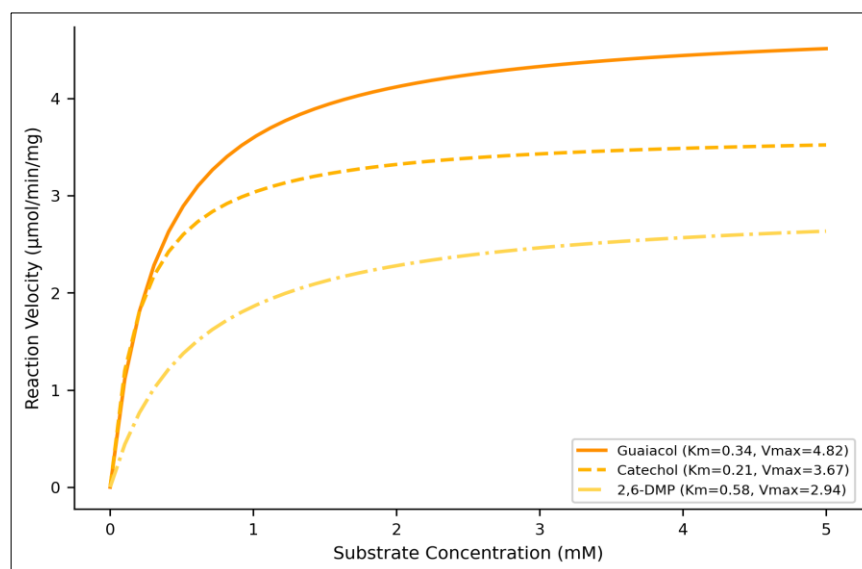


Fig 1: Michaelis-Menten saturation curves for laccase-catalyzed oxidation of guaiacol, catechol, and 2,6-DMP at pH 5.0 and 25°C .

The saturation curves (Figure 1) show catechol reaching half-maximal velocity at the lowest substrate concentration, consistent with its low K_m . Guaiacol's curve rises highest

but plateaus later. 2,6-DMP is both slower and less responsive to substrate increases its bulky methoxy groups likely hinder access to the T1 copper active site [5].

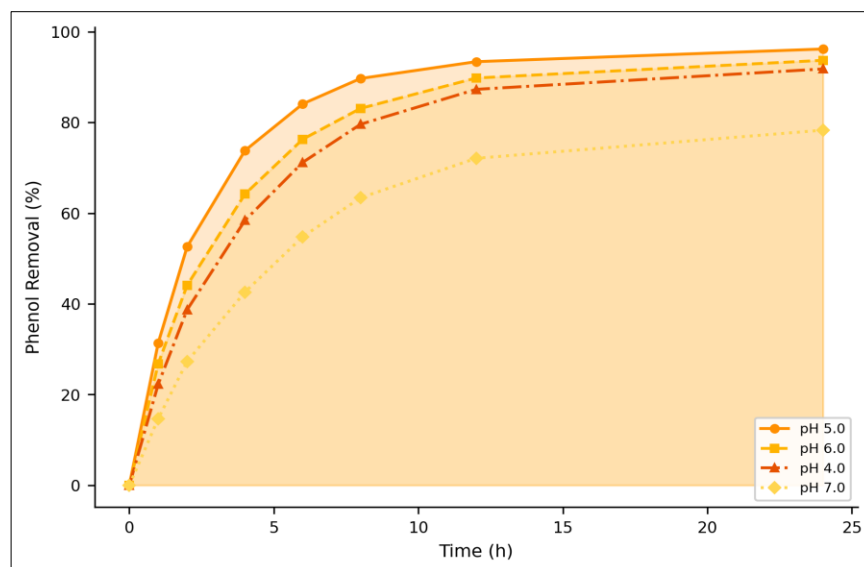


Fig 2: Phenol removal from synthetic wastewater (50 mg/L) by laccase (50 U/L) at four pH values over 24 h.

Removal was fastest and most complete at pH 5.0, reaching 96.2% at 24 h (Figure 2). At pH 7.0, removal plateaued at 78.3%, consistent with the enzyme's sharp activity loss above pH 6.0. The time course followed pseudo-first-order kinetics for the first 8 h ($R^2 > 0.98$) before slowing as substrate was depleted. Immobilized laccase retained 74% of its initial phenol-removal capacity after five treatment cycles, losing roughly 6% per cycle comparable to published values for chitosan-immobilized laccases treating simulated dye effluents [15].

Discussion

Catechol's highest catalytic efficiency (17.5) despite its lower V_{max} is a reminder that K_m matters as much as maximum rate for wastewater applications, where substrate concentrations are typically well below saturation [6]. In a real effluent at 0.1-0.5 mM phenolics, catechol-type substrates would be oxidized preferentially, which is convenient because catechol and its derivatives are among the most common phenolic pollutants in olive-mill and textile wastewaters [1].

The 96.2% removal at pH 5.0 and 35°C is encouraging but comes with a practical caveat: many industrial effluents are near-neutral, and the enzyme's activity drops to roughly 60% at pH 7.0. Laccase-mediator systems (using ABTS or 1-hydroxybenzotriazole as redox shuttle) can extend the effective pH range and broaden substrate scope, but they add cost and the mediator itself may be toxic [16]. An alternative is to acidify the effluent before enzymatic treatment, which is feasible for small volumes but expensive at industrial scale [17].

The 74% retention after five cycles for chitosan-immobilized laccase is adequate for a proof of concept. Losses arise from enzyme leaching, inactivation by product polymers that coat the bead surface, and mechanical attrition during filtration recovery [13]. Cross-linked enzyme aggregates (CLEAs) and covalent attachment to magnetic nanoparticles are alternative immobilization strategies that may improve durability [18]. A full cost-benefit analysis comparing enzymatic treatment with conventional Fenton or ozone processes at pilot scale would be needed before commercialization.

Conclusion

T. versicolor laccase oxidized three model phenolic substrates with Michaelis-Menten kinetics, with catechol showing the highest catalytic efficiency and guaiacol the highest V_{max} . At pH 5.0 and 35°C, the enzyme removed 96.2% of phenolics from synthetic wastewater within 24 h. Chitosan-immobilized laccase retained 74% activity over five cycles. These results support laccase as a viable biocatalyst for phenol removal from industrial effluents, with pH adjustment and immobilization as the main engineering challenges.

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