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**Environmental Significance Statement**

Chlorophenols are toxic, thus are regulated for discharge into surface waters or municipal treatment systems. Existing methods are inefficient, energy intensive, and/or generate large quantities of sludge. Here, a manganese peroxidase treats selected chlorophenols efficiently under mild conditions, effecting removal by an oligomerization-precipitation mechanism. Moreover, the enzyme used, a heterologous expression product from corn, is amenable to cost-effective scaleup.significance



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# Manganese Peroxidase-Catalyzed Treatment of Aqueous Chlorophenols

by

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## ABSTRACT

Chlorophenols (CPs) are recalcitrant pollutants commonly detected in industrial effluents due to their widespread use in agrochemical, pharmaceutical, and materials manufacturing. Their persistence, toxicity, and resistance to conventional biological treatment necessitate the development of sustainable remediation strategies. This study evaluates the feasibility of manganese peroxidase (MnP)-catalyzed treatment for the removal of aqueous 2-chlorophenol (2-CP), 3-chlorophenol (3-CP), 4-chlorophenol (4-CP), and 2,4-dichlorophenol (2,4-DCP) individually in synthetic wastewater under ambient conditions. Batch reactions were conducted at pH 4.5 in the presence of $Mn^{+2}$ and hydrogen peroxide, with systematic optimization of enzyme activity and oxidant addition strategy. Stepwise addition of $H_2O_2$ was found to preserve catalytic activity and significantly enhance substrate removal compared to single-aliquot addition. Under optimized conditions, removal efficiencies ≥90% were achieved for 2-CP, 4-CP, and 2,4-DCP within 60 minutes, whereas 3-CP exhibited markedly lower efficiency. First-order kinetic analysis confirmed compound-dependent initial conversion rates. Liquid chromatography-high resolution mass spectrometry revealed the formation of higher-order oligomers (up to decamers), supporting a radical-mediated oligomerization–precipitation pathway accompanied by partial dechlorination. These findings demonstrate that MnP, a product of heterologous expression in corn, can effectively transform chlorophenols into readily separated insoluble polymeric products, offering an environmentally compatible and potentially cost-effective alternative to conventional physicochemical treatment methods for chlorophenol-contaminated wastewater.

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**Keywords:** enzymatic, chlorophenol, hazardous, treatment, wastewater

## 1- INTRODUCTION

Chlorophenols (CPs) are a diverse group of aromatic compounds characterized by the presence of one or more chlorine atoms attached to a phenolic ring. They are widely used in various industrial and agricultural applications, including the manufacture of pesticides, herbicides, fungicides, pharmaceuticals, dyes, and wood preservatives, as well as in the production of plastics and textiles, and thus can occur in the effluents of these processes [1-3]. Their extensive use and persistence have led to their ubiquitous presence in the environment, where they are recognized as highly toxic, carcinogenic, and mutagenic substances that pose significant risks to both human health and ecosystems [1-4]. CPs are notably resistant to natural degradation processes, resulting in their accumulation in water, soil, sediments, and in the tissues of living organisms, including fish and humans, where they can induce acute and chronic toxic effects such as oxidative stress, immune disruption, endocrine interference, and carcinogenesis [1, 3-5]. The hazardous properties of CPs, including offensive odor, poor biodegradability, and potential to form even more toxic by-products during degradation or incineration, have led to their classification as toxic and/or priority pollutants by major regulatory agencies [6]. To give an idea of the size of the problem, US EPA Toxics Release Inventory [7] data for 2024 for chlorophenols (N084) gives total emissions (air and water ) of 350,000 lb and total chlorophenolic waste managed of 1.3 million lb. Thus, the United States Environmental Protection Agency (US EPA) and the World Health Organization (WHO) have established stringent regulatory limits for CPs in drinking water and industrial effluents, typically not exceeding 0.5 µg/L and 100 µg/L, respectively [1, 8]. The European Union Drinking Water Directive sets a maximum admissible concentration for total phenols of 0.5 µg $L^{-1}$ and 0.1 µg $L^{-1}$ for any individual phenolic compound in drinking water, reflecting their high toxicity and low organoleptic thresholds [9-11]. The WHO drinking-water guidelines for 2,4-DCP and 2-CP are 0.04 and 0.01 µg $L^{-1}$, respectively [11]. For pentachlorophenol (PCP), US EPA National Primary Drinking Water Regulations specify a maximum contaminant level (MCL) of 1 µg $L^{-1}$, with WHO and other agencies recommending allowable concentrations in the 0.01–0.1 µg $L^{-1}$ range [12]. Furthermore, PCP has been listed as a persistent organic pollutant under the Stockholm Convention, mandating international control and phase-out [1, 13]. Under the EU Water Framework Directive, pentachlorophenol is a priority substance with a water-based annual average EQS of 0.4 µg $L^{-1}$ and a maximum admissible concentration of 1 µg $L^{-1}$ in inland and other surface waters [14]. Advanced monitoring and remediation technologies, such as molecularly imprinted polymer-based sensors and photocatalytic degradation, are being developed to detect and mitigate CP pollution, reflecting the urgent need for effective management of these regulated industrial pollutants [1-4]. CPs exemplify the challenges posed by industrial chemicals that are both essential for modern manufacturing and agriculture yet require strict regulation due to their profound and lasting environmental and health hazards.

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Conventional wastewater treatment methods, particularly those based on standard biological processes such as activated sludge, are widely recognized as inadequate for the effective removal of regulated CPs from industrial and municipal effluents. The primary reason for this inefficiency lies in the chemical structure and properties of CPs: they are recalcitrant and often present at low concentrations, making them resistant to biodegradation and difficult to eliminate using traditional treatment approaches [15-18]. Biological treatment systems, which rely on microbial metabolism to break down organic pollutants, are especially challenged by CPs because these compounds inhibit microbial activity and are not readily metabolized, leading to poor removal rates and accumulation in the environment [15, 17, 18]. Physical treatment methods, such as sedimentation and filtration, are also largely ineffective for CPs, as these processes do not alter the chemical structure of pollutants and cannot address their solubility and persistence [19]. Chlorophenols as contaminants and their removal from wastewater by adsorption has also been comprehensively reviewed [20]. Chemical methods, such as chlorination or coagulation, do not fully degrade chlorophenols and can even lead to the formation of more toxic by-products, such as dioxins, [17, 19]. The limitations of these conventional approaches have driven the development and adoption of advanced treatment technologies, including advanced oxidation processes (AOPs), electrocatalytic filtration, and specialized adsorption or extraction techniques, which are specifically designed to target the recalcitrant and hazardous nature of chlorophenols [15-17, 21]. However, these too have drawbacks such as large energy consumption, sludge generation and/or oxidant consumption [22-24].

Recent advances in enzymatic treatment have positioned this approach as a highly promising and sustainable solution for CPs' removal [24-27]. Enzymatic treatment leverages the catalytic power of oxidoreductases—such as laccases, peroxidases, and tyrosinases—to transform chlorophenols into less toxic or insoluble products through oxidative polymerization and dechlorination, due to the following advantages. Enzymatic systems can achieve high removal efficiencies rapidly under mild conditions, avoiding the need for harsh chemicals or extreme temperatures typical of advanced oxidation or adsorption processes. In many instances, once feasibility has been demonstrated, enzymes are immobilized [28]. Importantly, enzymatic treatment pathways often result in the formation of oligomers that are less soluble and less toxic, and the release of chloride ions indicates partial dechlorination, a contribution to detoxifying CPs [29]. Recent studies also show that enzymatic treatment can be effective across a wide range of CP congeners, with some enzymes displaying broad substrate specificity and adaptability to a wide temperature range, making them suitable for diverse environmental and industrial applications [28]. A recent report on sulfate radical-induced coupling of chlorophenols [30] another on laccase-catalyzed coupling of chlorophenols [27] and a review of chemical oxidative coupling of phenols in water treatment [31] validate the oligomerization-precipitation [27] pathway, which is the central hypothesis of the current study. A recent perspective article further buttresses the concept [32].

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The current study is based on manganese peroxidase (MnP), a relatively understudied, ligninolytic heme protein produced by various white-rot fungi and some bacteria, which has demonstrated a remarkable ability to oxidize a wide range of phenolic and non-phenolic substrates, including chlorophenols, by catalyzing the oxidation of $Mn^{2+}$ to $Mn^{3+}$, which then acts as a diffusible oxidizer [33]. The equations operating in this work are (1) to (6), below. The peroxidase cycle consists of 2-electron oxidation of the heme by hydrogen peroxide to form reactive enzyme intermediate Compound I (equation (1)). Compound I reacts with $Mn^{2+}$ to form $Mn^{3+}$ and Compound II (equation 2)); the latter reacts with a second $Mn^{2+}$ to produce another $Mn^{3+}$ (equation 4) while the enzyme is returned to its resting state [34, 35]. Compound I can also react directly with phenols (ArOH) to generate a phenoxy radical (ArO•) (equation (3)) and anilines, but Compound II cannot; thus, $Mn^{2+}$ mediation is necessary to complete the MnP cycle via Compound II [36-38]. In a non-enzymatic, mediator mechanism, $Mn^{3+}$ converts phenols to phenoxy radicals (equation 5), which couple non-enzymatically to form dimers; the dimers can undergo subsequent Compound I or $Mn^{3+}$ reactions to form higher oligomers (equation 6), which precipitate [39, 40]. Thus, MnP offers the prospect of being substrate-agnostic, compared with other heme peroxidases such as soybean peroxidase (SBP) and horseradish peroxidase (HRP). Moreover, the MnP used here (ultrahigh-resolution crystal structure) [37, 41] is a heterologous expression product from corn and, thus, has the potential for low-cost production at scale [42, 43]. This report provides a comprehensive assessment of the mechanisms, operational parameters, and products in MnP-mediated enzymatic treatment to validate the oligomerization-precipitation pathway and establish its viability as an effective and environmentally friendly approach for the remediation of chlorophenol-contaminated wastewater. The underlying strategy of the current approach is to develop a wild-type enzyme that is cheap enough for single use, thereby avoiding the constraints of immobilization (such as need for relatively purified enzyme, fouling of the solid phase with precipitate). With these baseline operating conditions establishing feasibility, future work could target real industrial wastewater samples and an appropriate reactor design, recognizing that each such sample would be industry-specific in terms of toxicity and matrix complexity, including mixtures of substrates.

$$MnP + H_2O_2 \rightarrow Cpd\ I + H_2O \quad (1)$$

$$Cpd\ I + Mn^{2+} \rightarrow Cpd\ II + Mn^{3+} \quad (2)$$

$$Cpd\ I + ArOH \rightarrow Cpd\ II + ArO\cdot \quad (3)$$

$$Cpd\ II + Mn^{2+} \rightarrow MnP + Mn^{3+} \quad (4)$$

$$Mn^{3+} + ArOH \rightarrow Mn^{2+} + ArO\cdot \quad (5)$$

$$ArO\cdot + ArO\cdot \rightarrow dimers/oligomers \quad (6)$$

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## 2- MATERIALS AND METHODS

### *Materials*

The peroxidase derived from *Arthromyces ramosus* (ARP) was provided by Novozymes Inc., Franklinton, NC, USA. GreenLab Inc. (Jonesboro, AR, USA) supplied a crude liquid preparation of MnP (E.C. 1.11.1.13) produced from the MnP1 gene of *Phanerochaete chrysosporium*, which was heterologously expressed in corn [43]. The following reagents, each with a minimum purity of 99%, were purchased from Sigma Aldrich (Oakville, ON, Canada): 2-chlorophenol (2-CP), 3-chlorophenol (3-CP), 4-chlorophenol (4-CP), 2,4-dichlorophenol (2,4-DCP), sulfuric acid, tartaric acid, sodium hydroxide (NaOH), manganese sulfate, sodium L-tartrate dibasic dihydrate, 2,6-dimethoxyphenol, formic acid, and bovine liver catalase. BDH Inc. (Toronto, ON, Canada) supplied 4-aminoantipyrine (4-AAP). A 30% (w/v) hydrogen peroxide solution, stored at 4 °C, was obtained from ACP Chemicals Inc. (Montreal, QC, Canada). From Fisher Scientific (Ottawa, ON, Canada), high-performance liquid chromatography-(HPLC)-grade water and acetonitrile (ACN) were acquired. Sarstedt (Montreal, QC, Canada) supplied polyethersulfone syringe filters with a pore size of 0.22 µm and a diameter of 26 mm. Waters Ltd. (Mississauga, ON, Canada) provided Symmetry C18 columns.

### *Buffer preparation*

For pH 4.5, a 0.5 M tartrate buffer was prepared by titrating either sodium L-tartrate dibasic dihydrate with sulfuric acid or tartaric acid with NaOH.

### *MnP activity assay*

Activity of MnP was quantified and expressed in standard catalytic units per milliliter (U/mL). One unit (1.0 U) corresponds to the quantity of enzyme necessary to consume 1.0 µmol of $H_2O_2$ per minute. Oxidative dimerization of 2,6-dimethoxyphenol (2,6-DMP) in the presence of manganese (II) sulfate ($MnSO_4$), $H_2O_2$ and MnP produces a pink chromophore, $\lambda_{max}$ 469 nm ($\varepsilon$ = 53,200 $M^{-1}*cm^{-1}$). MnP activity was determined from the initial reaction rate, which was quantified using the built-in kinetic rate calculation function of the UV–Vis spectrometer. To prepare the reagent, 5.0 mL of 10 mM 2,6-DMP, 50 µL of 100 mM $H_2O_2$, 5.0 mL of 5 mM $MnSO_4$, and 4.0 mL of 0.5 M tartrate buffer were combined, and the final volume was adjusted to 47.5 mL with water [44]. The enzyme solution obtained from Greenlab Inc. was diluted 50-fold, and 50 µL of this diluted preparation was transferred into a quartz cuvette. Subsequently, 950 µL freshly prepared reagent was rapidly introduced. In kinetic mode, absorbance at 469 nm was recorded every 5 s over a 30 s period. The spectrometer software subsequently determined the reaction rate ($\Delta A_{496}*min^{-1}$) and calculated the enzyme activity in U/mL.

### *Analytical Equipment*

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The $\lambda_{max}$ of each compound, as well as reaction progress and enzyme activity, were determined using an Agilent diode-array UV–Vis spectrophotometer (model 8453) operated via a Hewlett Packard Vectra ES/12 computer. The instrument, supplied by Agilent (Mississauga, ON, Canada), covered a wavelength range of 190–1100 nm with a resolution of 1 nm. The HPLC detector was set at the $\lambda_{max}$ determined for each compound. UV-Vis measurements were conducted using 1 cm path length quartz cuvettes (type 104-QS) purchased from Hellma (Concord, ON, Canada). Substrate concentrations were determined by HPLC using a Waters Ltd (Mississauga, ON, Canada) system fitted with a model 2489 dual-wavelength absorbance detector, a model 2707 autosampler, and model 1525 binary pumps. The system was equipped with a Symmetry C18 column (100 Å, 5 µm, 4.6 mm × 150 mm) supplied by Waters. Breeze 2.0 software was used to operate the HPLC system. Table 1 presents the selected mobile phase compositions and ratios, along with the corresponding detection wavelengths and flow rates applied to each substrate [44]. An injection volume of 10 µL was used for all samples.

*Table 1 HPLC conditions for substrates run under isocratic elution*

| Substrate | Mobile phase ratio | | Flow mL/min | λmax nm |
|---|---|---|---|---|
| | Pump A | Pump B | | |
| 2-Chlorophenol | 50% formic acid (0.1%) | 50% ACN + formic acid (0.1%) | 1.0 | 280 |
| 3-Chlorophenol | 50% formic acid (0.1%) | 50% ACN | 1.0 | 275 |
| 4-Chlorophenol | 50% formic acid (0.1%) | 50% ACN + formic acid (0.1%) | 1.0 | 280 |
| 2,4-Dichlorophenol | 50% formic acid (0.1%) | 50% ACN + formic acid (0.1%) | 1.0 | 285 |

At Brock University (St. Catharines, ON, Canada), high-resolution mass spectrometric analysis was performed using an Agilent 1290 Infinity II liquid chromatography system with a quaternary pump, interfaced to an Agilent 6546 QTOFMS.

A Thermo Scientific (Ottawa, ON, Canada) Orion pH probe (9110DJWP, refillable DJ semi-micro glass; ±0.02 pH accuracy) was coupled to an Oakton (Vernon Hills, IL, USA) pH 700 benchtop meter, which provided a measurement range of 0.00–14.00 with a resolution of 0.01 pH units. ACP Chemicals Inc. provided the calibration buffer solutions at pH 4.00, 7.00, and 10.00. A Corning (New York, NY, USA) LSE™ compact centrifuge, equipped with 6 × 50 mL and 6 × 15 mL tube capacities and operating at a maximum speed of 6000 rpm, was used to perform the centrifugation. The Micro V magnetic stirrers (model 4805-00, 0–1100 rpm) and the VWR Magstirrers (model 82026-764, 100–1500 rpm) were supplied by VWR International Inc. (Mississauga, ON, Canada). The magnetic stir bars were from Cole-Parmer Canada Inc. (Montreal,

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QC). Model K-550-G vortex mixer (50/60Hz, 0.5 Amps, 120 volts) was from Scientific Industries, Inc (Bohemia, NY, USA).

***Batch reactions using MnP***

Reactions were carried out in 30-mL open glass batch reactors at ambient conditions (approximately 19-25 °C) without active temperature control. For the enzymatic treatment experiments, synthetic wastewater samples (20 mL) were formulated in triplicate to achieve 95% removal of each target substrate. In the 40 mM tartrate buffer system, the reaction mixture consisted of 1.0 mM of an individual substrate, supplemented with 0.20 mM manganese sulfate and varying MnP activity levels and hydrogen peroxide concentrations. Hydrogen peroxide was introduced last, either as a single aliquot or divided into seven separate aliquots, each providing 1.0 mM at 10-minute intervals. The reaction was terminated after 180 minutes of stirring by adding catalase (100 µL of a 10 mg/mL stock solution), and the samples were subsequently filtered as described above before HPLC analysis. For all substrates, the effects of enzyme activity, hydrogen peroxide concentration, and reaction time were systematically investigated as key reaction parameters. The reactions were conducted at pH 4.5, the optimal pH for MnP [43]. Each batch reactor set included two control reactors that were prepared using the same formulation as the corresponding samples. In one setup, hydrogen peroxide was omitted to evaluate the enzyme's effect on the substrate independently, while in the other, the enzyme was excluded to assess the influence of hydrogen peroxide alone on the substrate. Reactions were terminated by the addition of catalase, followed by microfiltration before HPLC analysis. Hydrogen peroxide optimization was conducted after completing the enzyme optimization process. The identical control experiments were also conducted. At optimal pH, enzyme activity, and hydrogen peroxide concentration, the reaction time course was evaluated over 180 min. At designated time intervals, 5.0 mL aliquots were withdrawn and immediately quenched with catalase, followed by vortex mixing to terminate the reaction. The supernatants were microfiltered and subjected to HPLC analysis to determine the remaining substrate concentration. Statistically, the triplicate results are presented as average ± standard deviations represented by error bars on the plots.

***Identification of products***

High-resolution mass spectrometry (HRMS) was used to examine the reaction mixtures for the identification of polymerization products. Under optimized conditions (pH, enzyme activity, and hydrogen peroxide concentration) batch reactors were set up in 10 mM tartrate buffer (to minimize interference from buffer ions during MS analysis). The catalase-treated reaction mixture was centrifuged at 4000 rcf for 20 min. An aliquot of this supernatant was microfiltered for MS analysis; a second 1.0 mL aliquot of the reaction mixture was mixed with 4.0 mL of

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acetonitrile (ACN), vortexed several times over one h, and then microfiltered. These two samples for each batch reactor were analyzed by MS under the same instrumental conditions.

## 3- RESULTS AND DISCUSSION

The focus here is on developing a treatment for industrial, not municipal, wastewater, where the matrix will be industry-specific, likely less complex than typical municipal wastewater, at substantially higher concentrations (10s to 100s of ppm; for example, a petroleum refinery wastewater was reported to have 28-32 mg/L of 2,4-DCP [23]). Ideally, the treatment would be within a plant, where CPs are at their highest concentration, not after mixing in the general waste circulation.

In the catalytic mechanism of MnP, pH is especially important because it influences both the redox potential of the manganese couple [45-47] and the stability of the enzyme as well as its reaction intermediates. MnP achieves its maximum catalytic efficiency at pH 4.5 in the presence of a Mn-chelating agent, tartrate (the buffer) [38], under which the enzyme's active site conformation, substrate ionization, and electron transfer kinetics are all favorably aligned [43]. The $pK_a$s for the chlorophenols studied are in the range 8.3-9.4 for the monochloro isomers and 7.5-8.1 for 2,4-DCP [20]; thus, at pH 4.5, all compounds are in the uncharged hydroxyl form. $Mn(OH)_2$ is a sparingly soluble salt, $K_{sp}$ 2 x $10^{-13}$ [48], its solubility decreasing with increasing pH. However, at the $Mn^{+2}$ concentration used here, 0.2 mM, it can be calculated that the onset of precipitation would be at pH 9.65, well above the operating pH of 4.5. Furthermore, the presence of a chelating buffer would forestall precipitation of $Mn^{+2}$ to even higher pH [49]. In this study, the pH was maintained at 4.5 rather than being optimized for the target compounds, as their conversion occurs through a non-enzymatic process.

### MnP optimization

Enzyme optimization tests were performed to identify the minimum activity necessary to achieve 95% removal of the target organic contaminants. All experiments were carried out for 3 h under consistent conditions, in 40 mM tartrate buffer at pH 4.5, supplemented with 0.20 mM manganese sulfate and hydrogen peroxide as specified below. For this preliminary investigation, the $Mn^{+2}$ concentration was arbitrarily set at 20% of the substrate molar concentration (thus requiring cycling of the Mn-couple to achieve high CP conversion); however, this level aligns with previously reported millimolar ranges in the literature [50, 51]. Future studies will systematically examine how varying $Mn^{2+}$ concentrations influence enzymatic activity, as well as evaluate the reaction kinetics between $Mn^{3+}$ and phenolic compounds during the chemical coupling process. Hydrogen peroxide was introduced in 1.0 mM increments every 10 minutes, reaching a cumulative concentration of 7.0 mM during the first 60 minutes to achieve the conversions. By applying the stepwise addition approach [44], a controlled oxidative condition was preserved,

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minimizing the risk of enzyme deactivation caused by excessive hydrogen peroxide. This approach maintained catalytic performance during the reaction and facilitated nearly complete conversion of the contaminant. Unless otherwise specified, any reference in the text or figure captions to 7 mM hydrogen peroxide or its stepwise addition denotes the use of the incremental protocol involving 1.0 mM aliquots introduced at 10-minute intervals. When samples were collected to determine residual substrate during a time-course experiment, they were withdrawn immediately before the addition of the subsequent peroxide aliquot. Figure 1 shows that, at a phenolic compound concentration of 1.0 mM, maximum removal efficiencies were obtained at the following MnP activities (U/mL): 0.90 for 2-CP (93%), 2.5 for 3-CP (41%; this only increases to 49% with 6 U/mL of MnP, data not shown), 0.70 for 4-CP (95%), and 0.60 for 2,4-DCP (96%). These conversion levels indicate that the sub-stoichiometric Mn redox couple was undergoing continuous cycling. The MnP activities identified as optimal for each compound were used in the following experiments. For wider comparison, previous work with phenol (Ph) and the *o*-, *m*-, and *p*-cresols under the same conditions, the highest removal efficiencies were achieved at MnP activities (U/mL) as follows: phenol (Ph) 0.73 (93%), *o*-cresol (*o*-C) 0.60 (100%), *m*-cresol (*m*-C) 0.80 (96%), and *p*-cresol (*p*-C) 0.70 (97%). Aside from 3-CP, there is not much variation in these efficiencies, perhaps not surprising in view of the fact that radical generation and coupling is not enzymatic. Future work will examine these MnP-independent features. That the two *meta*-substituted phenols are quite different in their efficiencies, suggests that it is not steric influence on their radical generation/coupling, but an electronic substituent effect.

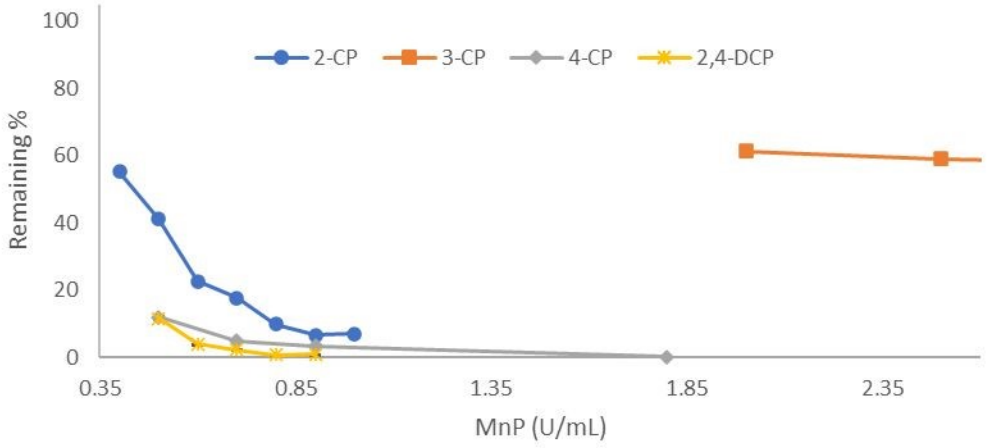


*Figure 1. MnP optimization. Conditions: 1.0 mM 2-CP, 3-CP, 4-CP, 2,4-DCP, 7 mM hydrogen peroxide, 40 mM buffer, pH 4.5, 0.2 mM manganese sulfate, incremental addition of hydrogen peroxide, 3-hour reaction, room temperature, triplicate experiments (average±SD, error bars, if not visible are smaller than the symbols), analysis by HPLC.*

**Hydrogen peroxide optimization**

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In the peroxidase cycle, one mole of $H_2O_2$ functions as the oxidant, for the conversion of two moles of $Mn^{+2}$ into $Mn^{+3}$; the generated $Mn^{+3}$ then reacts with the phenolic compound. Accordingly, this mechanism implies a predicted peroxide:phenol stoichiometric ratio of 0.5. A stoichiometric value of 1.0 would be anticipated under an oligomerization–precipitation mechanism [52].

At pH 4.5, corresponding to the respective optimal MnP activities determined above, the dependence of the target compounds on $H_2O_2$ was assessed, as illustrated in Figure 2, with the $H_2O_2$ concentration added in a single initial aliquot. The results are universally poor (note the expanded y-scale), showing that not only did increasing the initial $H_2O_2$ concentration not enhance removal efficiency, but it also actually made it much worse in three of the four cases. This suggests that a gradual or incremental addition of peroxide is necessary to promote more effective substrate conversion.

Beyond the previously mentioned oligomerization, a plausible explanation may be attributed to interactions occurring between the enzyme and hydrogen peroxide during the oxidation reaction. Certain substrates exhibit comparatively low reaction rates with MnP (more precisely, they act as slow-reacting partners in their chemical interaction with $Mn^{3+}$). Introducing a high concentration of $H_2O_2$ in a single aliquot can trigger two inhibitory mechanisms. First, the sudden formation of excessive $Mn^{3+}$ may cause oxidative deactivation of the enzyme before meaningful substrate conversion occurs. Second, excessive hydrogen peroxide can directly inhibit enzyme activity by promoting over-oxidation to Compound III, a reversibly inactive form of the enzyme [36, 53]. Despite the greater availability of oxidant, MnP catalytic activity would be diminished through both pathways, ultimately restricting substrate removal. Furthermore, any residual peroxide from the initial aliquots could be consumed by the endogenous catalase activity associated with MnP [53]. These findings underscore that, in MnP-catalyzed systems, especially for substrates exhibiting slower oxidation kinetics, a controlled, stepwise $H_2O_2$ addition strategy is critical for effective treatment.

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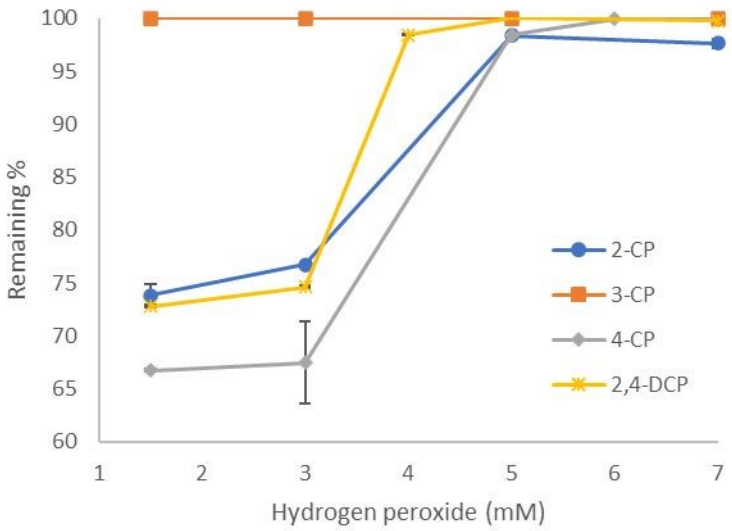


*Figure 2. Hydrogen peroxide optimization. Conditions: 1.0 mM 2-CP, 3-CP, 4-CP, 2,4-DCP, optimal U/mL MnP, 40 mM buffer, pH 4.5, 0.2 mM manganese sulfate, hydrogen peroxide added as a single aliquot at the start, 3-h reaction, room temperature, triplicate experiments (average±SD, error bars, if not visible are smaller than the symbols), analysis by HPLC.*

Figure 3 presents a stepwise approach to hydrogen peroxide addition for substrate removal under conditions optimized for MnP activity. Before each subsequent 1.0 mM $H_2O_2$ aliquot was added, samples were collected at 10-minute intervals. Qualitatively, Figure 3 shows reactivity in the order 2,4-DCP>4-CP>2-CP>3-CP. The relative order for the first three compounds is consistent with steric and electronic influences on radical coupling. Qualitative results from previous work with phenol and the cresols indicated that the reactivity followed the sequence *p*-C > *o*-C > *m*-C, Ph. Among the cresol isomers, this trend reflects similar steric effect of steric hindrance on radical coupling [54].

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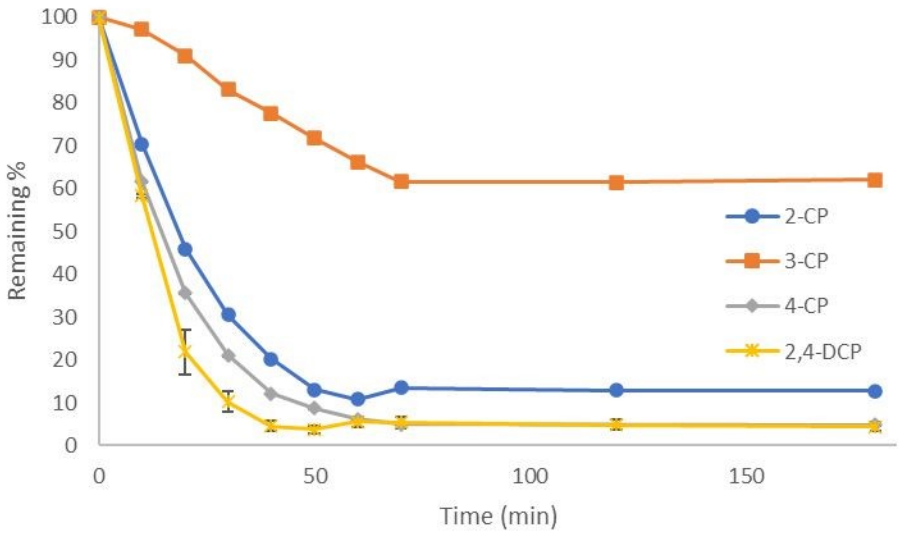


*Figure 3. Hydrogen peroxide optimization. Conditions: 1.0 mM 2-CP, 3-CP, 4-CP, 2,4-DCP, optimal U/mL MnP , 7 mM hydrogen peroxide; 40 mM buffer, pH 4.5, 0.2 mM manganese sulfate, incremental addition of hydrogen peroxide, 3-h reaction, room temperature, triplicate experiments (average±SD, error bars, if not visible are smaller than the symbols), analysis by HPLC.*

Figure 3 indicates that for 2-CP, increasing the cumulative $H_2O_2$ concentration above 5 mM—reached at approximately 50 min—did not produce any further improvement in the removal efficiency. The entire 7 mM of $H_2O_2$ was required to achieve approximately 40% removal of 3-CP, with this level of conversion reached after about 70 min of treatment. A 94% removal of 4-CP was achieved, at a cumulative concentration of 6 mM by the 60-min mark. Results indicate that 4 mM $H_2O_2$ was sufficient to achieve 95% removal of 2,4-DCP, which occurred within 40 min. The wider comparison with phenol and the cresols from previous work [53] showed complete removal of *p*-C (100%) in 20 minutes using only 1.5 mM $H_2O_2$. In contrast, the full 7.0 mM $H_2O_2$ was required to obtain 95% removal of *m*-C and Ph within 70 minutes, whereas for *o*-C, 4.0 mM $H_2O_2$ was sufficient to achieve a 99% reduction within 40 minutes.

Although the peroxide requirement has been empirically optimized here, it is acknowledged that for all compounds, it is well in excess of the requirement for an oligomerization–precipitation mechanism, thus opening the paths for peroxide-induced MnP inactivation. A more detailed investigation into how these enzymatic and non-enzymatic reactions interact will be undertaken in future studies, with the aim of increasing MnP efficiency.

## Summary of optimum conditions for substrate treatment

Table 2 presents the optimal conditions for the room-temperature removal of the aqueous chlorophenols using MnP. Both single- and stepwise-additions of hydrogen peroxide are included; the stepwise approach led to greater removal.

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*Table 2. Optimized conditions – MnP-catalyzed removal with single or stepwise additions of hydrogen peroxide*

| Compound (mM) | MnP (U/mL) | Single $H_2O_2$ (mM) | Remaining % | Time (min) | $H_2O_2$ stepwise (mM) | Remaining % | Time (min) |
|---|---|---|---|---|---|---|---|
| 2-Chlorophenol (1.0) | 0.90 | 5 | 98 | 180 | 5 | 7 | 50 |
| 3-Chlorophenol (1.0) | 2.50 | 7 | 100 | 180 | 7 | 59 | 180 |
| 4-Chlorophenol (1.0) | 0.73 | 6 | 100 | 180 | 6 | 6 | 60 |
| 2,4-Dichlorophenol (1.0) | 0.60 | 4 | 98 | 180 | 4 | 5 | 40 |

To normalize the treatment efficiencies reported in Table 2, the optimum enzyme activity was divided by the respective substrate concentration. The normalized efficiency range indicates that the MnP reaction is relatively independent of substrate (aside from 3-CP), which is consistent with the involvement of the Mn redox couple as an intermediate. A comparison of the normalized efficiencies with data reported for 35 compounds treated with soybean peroxidase [54, 55] (Table S1), indicates that both enzymes have comparable efficiencies with the chlorophenols, aside from 3-CP with MnP, and both enzymes with the chlorophenols (3-CP excepted) are categorized within the high-efficiency range (<1 U/mL/mM) relative to the compounds listed. In prior studies, the enzyme was also classified in the high-efficiency category for the treatment of phenol and cresols [54]. Two enzymes, two *meta*-substituted substrates undergoing oxidative coupling: the exceptional behavior of 3-CP with MnP suggests that the difference lies in an electronic, not steric, substituent effect on the non-enzymatic $Mn^{3+}$ reaction with 3-CP, not on the subsequent coupling (the product analysis below shows that coupling occurs as usual). One hypothesis is that the $Mn^{3+}$-3-CP reaction is slower than the corresponding reactions with the other CPs, and that the $Mn^{3+}$ generated in a burst by the enzymatic reaction undergoes competing reactions, including enzyme inactivation.

Most of the enzymatic work by others on CPs was conducted about 25-30 years ago, for example, definitive work [56] reported ≥93% conversion of the CPs used in the current study by HRP (activities unspecified); 3-CP was much less reactive than the other CPs. Immobilized HRP removed 95% of 2,4-DCP and 100% of p-CP [23]; Laccase, in 10 h, removed 94, 69, and 75% of 2,4-DCP, 4-CP, and 2-CP, respectively [24]; potato peroxidase catalyzed removal of 2,4-DCP (1-3 mM), mesquite peroxidase removed 90-92% of CP (at 25°C, 75% at 80°C; but HRP only achieved 40-60% at 25°C), and an immobilized laccase removed 65% of 2-CP (in 90 min for 1mM, lower efficiency at higher CP concentration [25]). In other enzymatic work [57] SBP removed up to 96% of 2,4-DCP in 24 h and concomitantly reduced toxicity [57].

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With conventional and advanced non-enzymatic methods, a recent mini-review [17] and a full review [58] provide context, pointing out benefits and drawbacks. Biological methods are impeded by the toxicity of CPs to microbes; nevertheless, effective mixed aerobic-anaerobic systems have been reported [58]. Adsorption methods [20], reviewed recently, are effective (no specific efficiencies given) and have a large capacity but the adsorbents are expensive, incurring additional costs for disposal or regeneration. Advanced oxidation of CPs, primarily UV/$O_3$ (for example, removed 99% of 2-, 4-, and 2,4-DCP at pH ≥ 6) and UV/$H_2O_2$ (for example, removed 99% of 4-CP at pH 3-7, poor at pH 10), has been reviewed in detail [59]. A hybrid enzymatic membrane bioreactor, combining ultrafiltration with immobilized laccase to reduce effluent toxicity, achieved up to 88% removal of 2-CP and 74% of 4-CP at pH 5 and 30°C, with the added benefits of significant reusability and storage stability [28].

**Time course of reaction**

First-order treatment of the initial data in Figure 3 was used to calculate half-lives for the substrates, as shown in Figure 4. The exponential fits confirm pseudo-first-order behavior and allow for the precise determination of the initial rate constants.

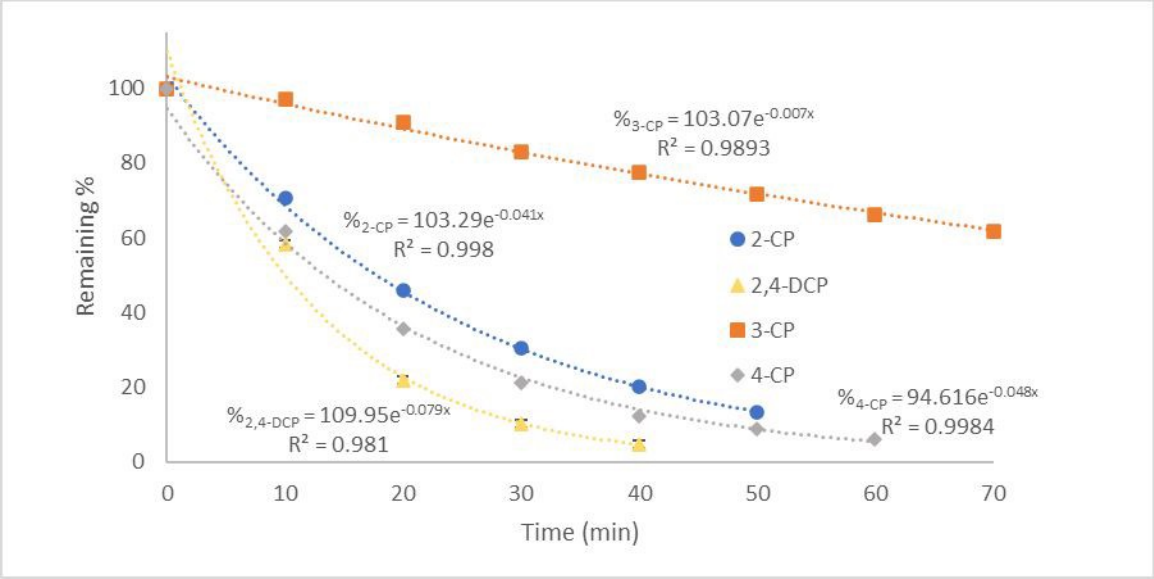


*Figure 4. First-order conversion of substrates – presented in Figure 3. The lines drawn are exponential fits to the data.*

**Summary of enzyme initial kinetic efficiencies**

Kinetic efficiencies, based on the half-lives calculated from the preceding time courses, are summarized in Table 3 (normalized rate constants calculated by dividing the fitted rate constants by the respective enzyme activities). Again, 3-CP's kinetic efficiency is more than an order of magnitude lower than the rest, and it mirrors its distinctly low treatment efficiency discussed above. Here, the qualitative order of initial reactivities noted with Figure 3 is quantified. In

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contrast, other work with phenol and the cresols showed a burst (fast phase), roughly equivalent to one turnover of $Mn^{3+}$, followed by a slow phase, akin to the decay curves in Figure 4; slow-phase data for phenol and the cresols showed normalized half-lives: Ph 11 min, *o*-C 3.5 min, *m*-C 11 min, and *p*-C 0.041 min [54]. Thus, 3-CP is about 20-fold slower than *m*-C, whereas *p*-C is 8-fold faster than 4-CP. Wider comparison of the kinetic efficiencies given here may be made with those tabulated by [55] for soybean peroxidase with 29 compounds, supplementary Table S2: except 2,4-DCP, the normalized half-lives in Table 3 for the chlorophenols with MnP fall into the slow range (>10 min.U/mL). Future work will dissect the kinetic complexity of this system, as noted above and below.

*Table 3. Half-lives and normalized half-lives of various MnP substrates*

| Compound (mM) | MnP (U/mL) | Rate constant ($min^{-1}$) | Normalized rate constant ($min^{-1}$) | Half-life (min) | Normalized half-life (min.U/mL) | Remaining % |
|---|---|---|---|---|---|---|
| 2-Chlorophenol | 0.90 | 0.041 | 0.045 | 17 | 15 | 7 |
| 3-Chlorophenol | 2.50 | 0.0072 | 0.0029 | 96 | 240 | 59 |
| 4-Chlorophenol | 0.73 | 0.048 | 0.065 | 15 | 11 | 6 |
| 2,4-Dichlorophenol | 0.60 | 0.079 | 0.13 | 8.8 | 5.3 | 5 |

**Polymerization products**

Through radical-mediated coupling reactions, MnP can oxidatively convert phenolic compounds into a diverse array of oligomeric products. C–C or C–O bonds are formed through *ortho*- or *para*-oriented coupling reactions of the phenoxyl radicals. The substituents present on the parent molecules, along with the applied oxidation conditions, determine the structure and degree of complexity of the resulting oligomers. The peroxidase-mediator cycle continues until the oligomers formed reach their solubility threshold [60].

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Filtered reaction mixtures obtained after enzymatic treatment were analyzed using liquid chromatography–high resolution mass spectrometry (LC-HRMS) to identify and characterize the oligomerization products (up to pentadecamers). Nevertheless, the solid products formed during the reaction could not be directly examined by the MS method. To verify oligomer precipitation, an aliquot of each unfiltered reaction suspension was combined with acetonitrile to achieve a final composition of 80% acetonitrile and 20% aqueous in an extract (Aq-ACN). It was hypothesized that dissolving the reaction solids in aqueous acetonitrile would enable partial or complete solubilization of any precipitates, thereby permitting the detection of higher oligomers formed during the MnP reaction.

By allowing the separation and accurate mass determination of both soluble oxidative oligomers and their precipitated counterparts, this method would provide valuable insight into the coupling pathways and resulting transformation products. The MS analysis results of the filtered samples, indicating the highest oligomers identified, are presented in Table 4, which qualitatively confirms the oligomerization-precipitation pathway.

*Table 4. Mass Spectrometry Analysis of Filtered Samples*

| Sample | | Aqueous [a] | Aq-ACN [a,b] |
|---|---|---|---|
| 2-Chlorophenol | 2-CP | trimer | heptamer |
| 3-Chlorophenol | 3-CP | octamer | decamer |
| 4-Chlorophenol | 4-CP | tetramer | octamer |
| 2,4-Dichlorophenol | 2,4-DCP | NF[c] | pentamer |

[a] the highest-order oligomers are reported; the relative LC–HRMS peak area ratios for the dimer, trimer, and other species were calculated and are presented in Table S3.
[b] the combined total area ratios Aq-ACN:aqueous were: 2-CP 63, 3-CP 11, 4-CP 161, 2,4-DCP NA (2,4-DCP solvent area comparable to areas of the other solvent extracts).
[c] none found

For a quantitative understanding, the relative peak area ratios (as % of total) were calculated for the oligomer distributions in the aqueous samples and in the solvent extracts. For example, a plot of the distributions for 3-CP is given in Figure 5; the data for all four compounds and plots for the remaining three are given in Supplementary Table S3 and Figures S1-S3. In addition, when the combined oligomer peak areas were corrected for dilution and compared, (Aq-ACN:aqueous) sample (ratios given in Table 4, footnote [b]), more material, by 1-2 orders of magnitude, was present in the respective extracts. This comparison could not be performed for 2,4-DCP (because

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no oligomers were detected in the aqueous sample), but the total area of its oligomers in the extract was comparable to the corresponding total areas in the extracts of the other three.

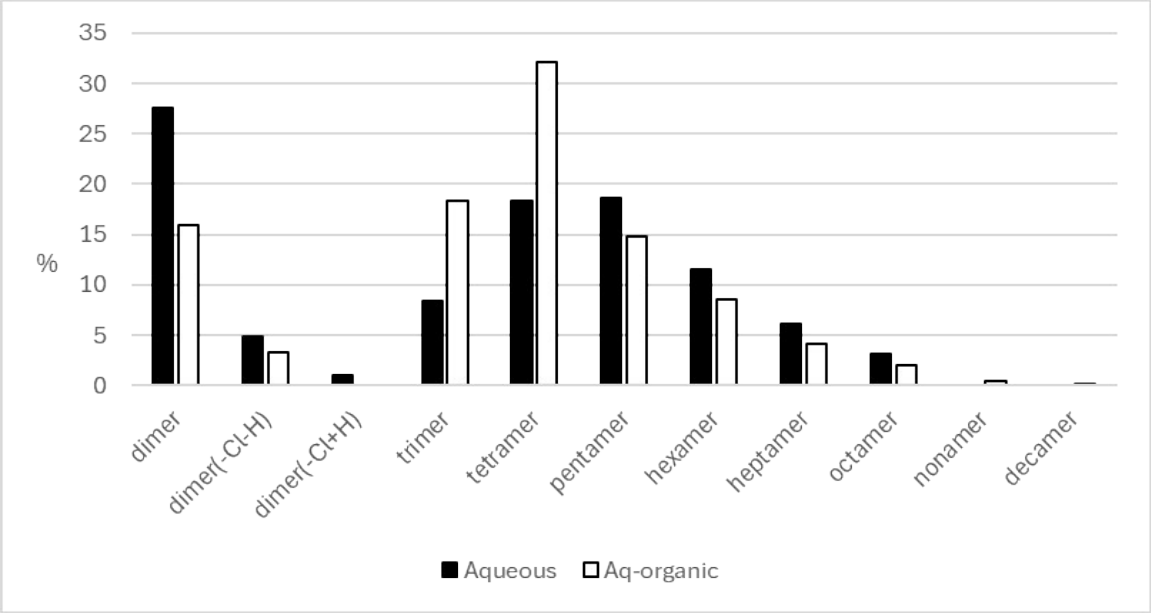


*Figure 5. Oligomer distribution for 3-CP in aqueous reaction mixture and in Aq-ACN extract. The enzymatic reaction mixture was filtered directly for the aqueous sample; for the extract, the reaction mixture was diluted 4-fold with acetonitrile, vortexed for an hour, then filtered. MS peak areas (all isomers) are expressed as a % of the total set of areas for that solvent (Table S3).*

Figure 5 (and Figures S1-S3) shows that partial dechlorination was evident at the dimer stage, with compounds showing loss of HCl and/or replacement of Cl by H. Since dechlorination products stem from the dimer coupling, the combined dimer components in Figure 5 are 34% for the aqueous sample and 19% for the aqueous-organic one. Partial loss of chlorine during enzymatic oxidative oligomerization was first reported by [56] (significantly less relative dechlorination for 3-CP than its isomers and 2,4-DCP) for HRP-catalyzed reactions of chlorophenols, and it has been more recently reviewed [61, 62]. The process stems from the reactive intermediates of radical coupling as they form stable dimers by re-aromatization (schematic provided [56]). Thus, it is not surprising that dechlorination should also occur in the enzyme-independent oligomerization stage of the MnP mediator system studied here. It is noted that the product distribution for 3-CP is analogous to those of the other CPs, despite its low relative treatment and kinetic efficiencies identified in the preceding sections. It is anticipated that future studies to tease apart the enzymatic and non-enzymatic contributions to this mediator system will find conditions to improve the treatment of 3-CP.

While toxicity studies have not been conducted in the work, it is noted that previous studies, for example by [57] and [27], have demonstrated that oligomerization in and of itself reduces toxicity. Furthermore, there is substantial physical removal of oligomeric products by precipitation.

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Toxicity studies would be conducted in future, in conjunction with individual real wastewater samples [28-32].

**Mechanism and novelty of the process**

Substantial evidence has been cited for the MnP kinetic mechanism operating through radical chemistry on phenols and anilines; furthermore, there is extensive literature on the radical-coupling oligomerization-precipitation mechanism for other peroxidases and laccases [28-32]. As noted in the Introduction, MnP Compound I can be Mn-independent with simple phenols and anilines, but Compound II cannot; thus, mediation is necessary to complete the MnP cycle. The Mn concentration used here is sub-stoichiometric; thus, there has to Mn cycling to account for the conversions reported. A huge amount of material was recovered in the Aq-ACN extracts, confirming that the oligomers detected by HRMS had indeed precipitated.

The underlying hypothesis of this work is that MnP, a relatively understudied peroxidase, effects removal by oligomerization-precipitation as argued above, and as is well established for SBP, HRP and laccases. The novelty here resides in the MnP studied, a heterologous expression product from a byproduct of corn processing, the germ, and its application to chlorophenols. It also has the potential for being largely substrate-agnostic (provisionally, 3-CP is an outlier). Thus, it has the economic potential of wild-type SBP (from the seed coat) and recombinant laccases. In contrast, HRP is derived from a niche agricultural product, and its extraction involves production of toxic residual biomass of no particular use; HRP will not be economical for wastewater treatment unless it can be cost-effectively produced heterologously [63].

**CONCLUSIONS**

The chlorophenols studied, except 3-chlorophenol, can be efficiently removed via an oligomerization-precipitation mechanism from aqueous solution by MnP in the presence of hydrogen peroxide and sub-stoichiometric $Mn^{2+}$; thus, future work will delve into the interplay of peroxide consumption by the enzyme, $Mn^{3+}$ reactivity with the phenols, and $Mn^{2+}/Mn^{3+}$ cycling, followed by extension to real wastewater samples. The promise of enzymatic treatment for chlorophenol removal is rooted in its high efficiency, operational flexibility, environmental compatibility, and capacity to reduce both pollutant concentration and toxicity, thereby offering a green and effective alternative to conventional, chemical-intensive methods for the remediation of chlorophenol-contaminated water and wastewater. Use of MnP derived from a byproduct of an agricultural commodity poises the system for cost-effectiveness (see cost estimate of [42]).

**Author contributions**

Samira Narimannejad, Keith Taylor, Elizabeth Hood and Nihar Biswas contributed to the study conception, design and analysis. Material preparation and data collection were performed by

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Samira Narimannejad. The first draft of the manuscript was written by Samira Narimannejad. Samira Narimannejad, Keith Taylor, Elizabeth Hood and Nihar Biswas commented on and contributed to subsequent versions of the manuscript. Samira Narimannejad, Keith Taylor, Elizabeth Hood and Nihar Biswas read and approved the final manuscript.

**Conflicts of Interest**

Nihar Biswas and Keith E. Taylor declare no relevant financial or non-financial competing interests. Samira Narimannejad and Elizabeth E. Hood are co-applicants on World/US Patent application PCT/US2024/056724, entitled "Compositions and methods for degradation of pollutants by plant-produced manganese peroxidase". Elizabeth E. Hood is a shareholder in GreenLab, Inc.

**Data availability**

All the data supporting the findings of this study are available within the paper or its Supplementary material.

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**Manganese Peroxidase-Catalyzed Treatment of Aqueous Chlorophenols**

by

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All the data pertaining to the manuscript are included in the manuscript file or in the *Supplementary information* file.

References 60-74 are cited in the Supplementary information (SI), aside from reference 54 and 55 which were already cited in the main manuscript.

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