Boosting Enzymes with Mediator Molecules for Water Purification Applications

Final report

ÅForsk Project **24-536**

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Project Summary

Contaminants of emerging concern (CEC) in aquatic environments remain a major challenge for conventional water treatment technologies, particularly due to their persistence and the formation of toxic transformation products. Enzyme-based oxidation has emerged as a promising green alternative, offering selective degradation of recalcitrant pollutants under mild conditions. However, the catalytic efficiency of oxidoreductases such as laccases and peroxidases is often limited by their redox potential and operational stability[1]. The use of mediator molecules can significantly enhance enzyme performance, yet practical implementation of these systems are very limited due to complications such as, mediator leaching, enzyme deactivation, and risks of secondary pollution[2]. This project addressed these limitations by exploring co-immobilized enzyme–mediator systems designed for improved activity, stability, and reusability. Both synthetic mediators and lignin-derived natural phenolic compounds were investigated to balance catalytic efficiency with environmental sustainability. Particular emphasis was placed on immobilization strategies that enable mediator retention while preserving enzyme activity. The performance of the resulting biocatalysts was evaluated through activity assays, stability tests, and degradation of representative organic pollutants.

Project aims:

(i) To screen and assess lignin-derived natural phenolic compounds as environmentally benign mediators for laccase and peroxidase catalysis.

(ii) To develop and evaluate co-immobilized enzyme-synthetic mediator systems for efficient and reusable degradation of organic pollutants in water.

Results

1. Screening of natural mediators

The mediator screening was performed in a homogeneous aqueous system using Rhodamine B (RhB) as a model organic contaminant, allowing direct evaluation of mediator effects on enzymatic degradation without immobilization related influences. In these experiments, RhB solution (1 mL) was mixed with the mediator solution (100 µL) and the corresponding enzyme solution (100 µL), and degradation was monitored spectrophotometrically.

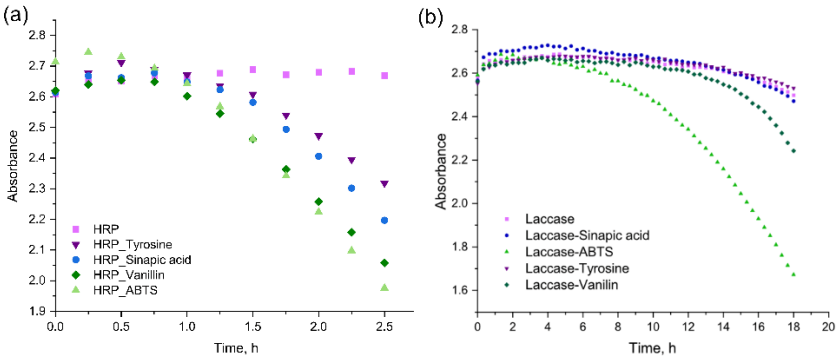

Figure 1. Degradation of Rhodamine B by (a) HRP and (b) Laccase in the presence of different mediator molecules.

The results revealed a consistent trend for both enzymes, with ABTS exhibiting the highest enhancement of catalytic activity, followed by vanillin, while sinapic acid showed only a limited

effect under the investigated conditions (ABTS > vanillin > sinapic acid). For HRP, the presence of tyrosine resulted in a slight increase in activity compared to the enzyme alone, indicating a modest mediator or co-substrate effect. In contrast, for laccase, the addition of tyrosine led to a decrease in activity relative to laccase without mediator, suggesting an inhibitory interaction rather than catalytic enhancement.

The superior performance of ABTS is most plausibly explained by favorable mediator kinetics rather than redox potential alone [3]. In laccase-mediator systems, ABTS is known to be rapidly oxidized by laccase to a relatively persistent radical species, enabling efficient electron shuttling and competitive oxidation of substrates, whereas several phenolic mediators can be oxidized more slowly and/or undergo side reactions (e.g., radical coupling or quenching) that reduce their effective mediator role[4]. Consistent with mechanistic studies on phenolic lignin model compounds, mediators with slow oxidation kinetics can exhibit negligible contribution even when their redox potentials are higher, because the substrate may be consumed through direct enzymatic pathways before sufficient mediator radical is generated. Vanillin showed intermediate performance for both enzymes, suggesting more favorable reactivity under the applied conditions than the other tested natural phenolics. Overall, these results demonstrate that mediator effectiveness is strongly enzyme- and condition-dependent, and that not all naturally occurring phenolic compounds act as beneficial mediators in oxidoreductase-based systems.

2. Degradation of phenolic dyes by enzyme-mediator systems

Following the initial mediator screening, the performance of selected mediators was further evaluated for the degradation of five structurally different dyes: **MB** (methylene blue), **MG** (methyl green), **CR** (Congo red), **PR** (phenol red), and **AF** (acid fuchsin). Laccase was selected for this extended evaluation, as the earlier screening showed comparable mediator performance trends for both laccase and HRP. In addition, laccase is particularly attractive for water treatment applications because it operates using molecular oxygen as the terminal electron acceptor and does not require external oxidants, allowing for simpler reaction conditions and more straightforward interpretation of mediator effects[5,6].

All experiments were conducted in solution using free enzyme and free mediators. In a typical assay, 5 mL of dye solution was mixed with laccase (500 µL, 1 U/mL) and the corresponding mediator solution (500 µL, 500 µM), and degradation was monitored spectrophotometrically by following changes in the characteristic maximum absorption wavelength of each dye over time.

The degradation behavior strongly depended on both dye structure and mediator presence. For **MB** (**Figure** 2a), no measurable degradation was observed for laccase alone or for any natural mediator systems, whereas laccase-ABTS achieved approximately 46% degradation within 20 h. This indicates that MB oxidation requires a mediator that can be rapidly oxidized by laccase to form a sufficiently reactive and persistent radical species, capable of oxidizing substrates that are otherwise recalcitrant to direct enzymatic attack. In contrast, **MG** (**Figure** 2b) was readily degraded by laccase alone, and all mediator-containing systems showed high degradation efficiencies. The final degradation followed the order laccase-vanillin > laccase > laccase-sinapic acid > laccase-tyrosine > laccase-ABTS, with laccase-vanillin reaching up to 98% degradation, while laccase-ABTS reached approximately 83%.

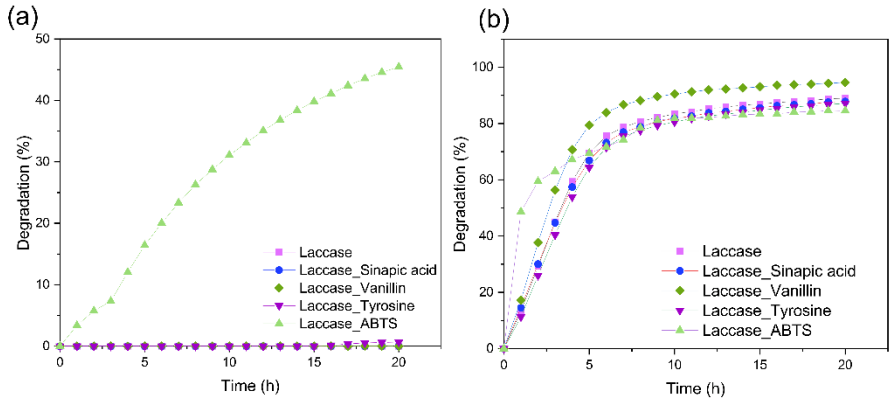

Figure 2. Degradation of MB (a) and MG (b) by Laccase and Laccase_mediator systems.

For **AF**, significant degradation was observed only in the presence of ABTS, with laccase-ABTS achieving approximately 68% removal (**Figure** 3a), while all other systems showed negligible activity. A similar mediator-dependent trend was observed for **CR**, where all systems exhibited some degree of degradation, but laccase-ABTS clearly outperformed the others (~49%), whereas laccase, laccase-tyrosine, laccase-vanillin, and laccase-sinapic acid showed comparable and lower degradation levels (approximately 23-27%) (**Figure** 3b). For **PR**, laccase-ABTS again resulted in the highest degradation (~40%), while the remaining systems, including laccase alone and natural mediators, showed similar removal efficiencies of approximately 30% (**Figure** 3c).

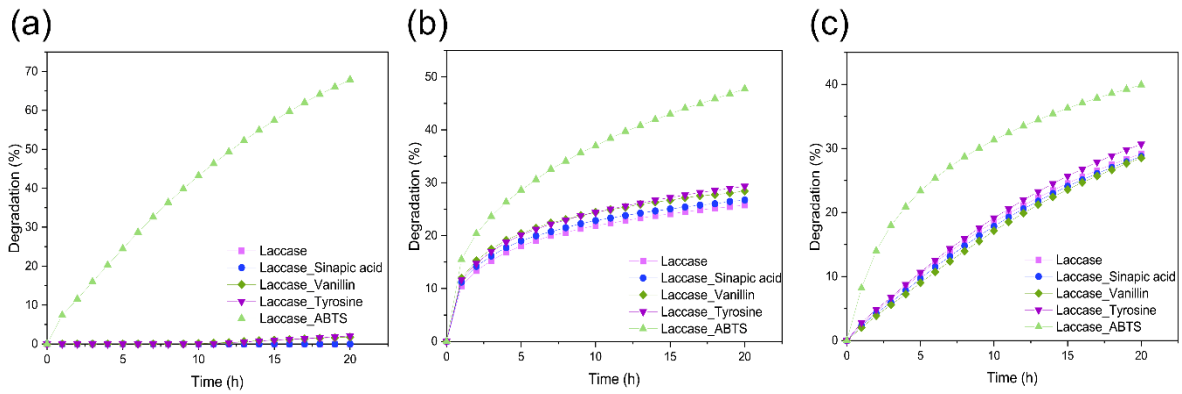

Figure 3. Degradation of AF (a), CR (b) and PR (c) by Laccase and Laccase_mediator systems.

Overall, these results demonstrate that mediator effects in laccase-catalyzed systems are strongly substrate-dependent. While certain dyes are readily degraded by laccase alone, others require the presence of a high-redox-potential mediator with fast kinetics, such as ABTS, to enable efficient oxidation, whereas natural phenolic mediators exhibit more moderate and dye-specific effects.

3. Co-immobilization of enzymes and mediators

Based on the mediator screening results, co-immobilization strategies were explored to enable mediator retention, enzyme reuse, and direct comparison between natural and synthetic mediators. An initial approach focused on covalent immobilization on silica-based supports. While this strategy is well suited for enzymes and phenolic mediators such as vanillin, it is not applicable to ABTS, which lacks functional groups suitable for covalent attachment. To allow a meaningful comparison between natural mediators and ABTS within the same immobilization

framework, the study therefore shifted toward encapsulation-based approaches, in which both enzyme and mediator could be retained without chemical modification.

The first encapsulation strategy involved immobilization of enzymes and mediators within alginate beads, a commonly used biopolymer system for enzyme entrapment[7,8]. Alginate bead formation relies on ionic crosslinking with divalent metal cations; accordingly, Ca^{2+}-alginate and Fe^{2+}-alginate beads were prepared by dropwise addition of sodium alginate solutions containing enzyme and mediator into the corresponding metal salt solutions, resulting in the formation of spherical hydrogel beads[9]. Both bead types were evaluated to assess whether the nature of the crosslinking cation could influence enzyme activity.

However, activity tests revealed that enzymes encapsulated in alginate beads exhibited strongly reduced or negligible catalytic activity. Enzyme performance was assessed using a standard ABTS oxidation assay by incubating approximately 50 mg of enzyme-loaded beads in 3 mL of ABTS solution and monitoring color development spectrophotometrically. In most tested formulations, no detectable ABTS oxidation was observed. Only beads prepared using the highest enzyme loading (1 U/mL enzyme in the alginate solution) showed a measurable response, corresponding to an absorbance of approximately 0.05 compared to the blank, which is considered very low. Similar trends were observed for both laccase and HRP, and for both Ca^{2+}- and Fe^{2+}-crosslinked beads, indicating that enzyme deactivation or severe mass-transfer limitations likely occurred. These effects may be attributed to restricted substrate diffusion and partial shielding of the enzyme active sites by the dense alginate matrix[10].

Given the limited enzymatic activity observed in alginate-based systems, alternative encapsulation platforms were explored. Building on prior experience with metal-organic frameworks (MOFs) and their demonstrated ability to retain high enzyme activity, an iron-based MOF was selected as a more suitable host material. An Fe-based system was particularly attractive due to the possibility of room-temperature, aqueous synthesis and the use of relatively benign reagents. In this study, MIL 100(Fe) was synthesized following a reported green protocol[11]. Briefly, trimesic acid (H_3BTC) was dissolved in aqueous NaOH to form Solution 1, while $FeCl_2 \cdot 4H_2O$ was dissolved in water to form Solution 2. Solution 1 was added dropwise to Solution 2 under continuous stirring, and the mixture was left to react overnight at room temperature. The resulting precipitate was collected by centrifugation (7000 g) and washed thoroughly with water and ethanol.

For enzyme immobilization, 200 mg of MIL 100(Fe) was incubated with 5 mL of laccase solution. To determine the adsorption capacity of the MOF, a range of enzyme concentrations (approximately 0.1-1 U/mL) was initially tested. The results showed high enzyme uptake, with approximately 90% adsorption even at the highest tested concentration (1 U/mL) (Figure 4). Since near-complete adsorption was achieved at 1 U/mL, and the resulting immobilized enzyme retained high catalytic activity, this concentration was selected for subsequent experiments.

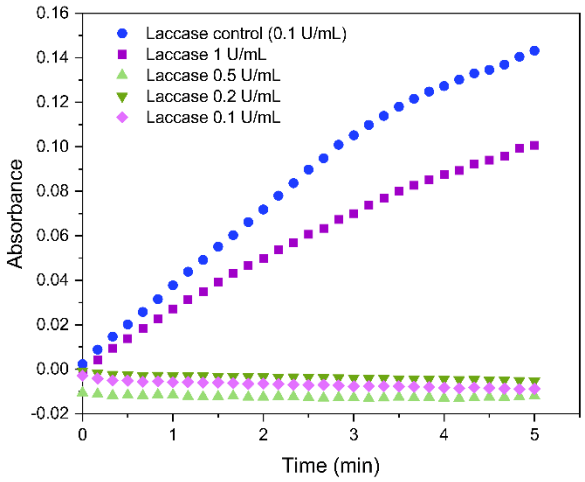

Figure 4. Adsorption of laccase onto MIL 100(Fe) MOF. Although each adsorption curve was obtained using its corresponding enzyme-only control, only the control at 0.1 U/mL laccase concentration is displayed for clarity.

4. Stability and reusability of laccase immobilized on MIL-100 MOF

The operational stability of the immobilized biocatalyst was evaluated by monitoring enzyme activity over multiple reuse cycles. Laccase_MIL 100(Fe) (50 mg) was incubated with 3 mL of ABTS solution, and catalytic activity was assessed spectrophotometrically. After each activity measurement, the material was recovered and washed by suspension in water on an orbital shaker for 1 h to remove residual substrate and reaction products. This procedure was repeated for 10 consecutive cycles, with activity measured once per day.

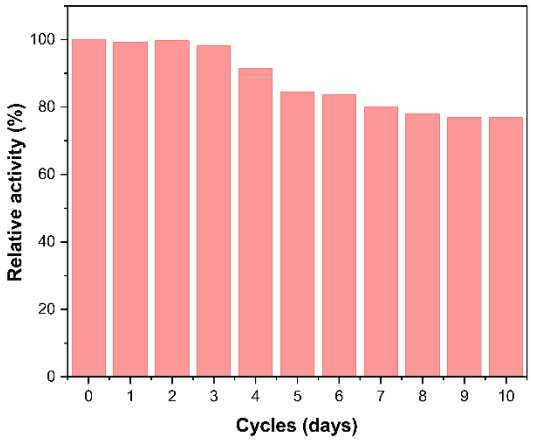

Figure 5. Reusability of enzyme_MOF biocatalyst measured through ABTS oxidation test.

The immobilized laccase exhibited high operational stability, retaining about 80% of its initial activity after 10 cycles. The small loss in activity demonstrates that MIL 100(Fe) MOF provides an effective microenvironment for enzyme stabilization, likely by restricting enzyme leaching and protecting the protein structure during repeated use[12]. These results confirm the suitability of MIL 100(Fe) as a robust support for enzyme immobilization in aqueous catalytic systems.

5. Immobilization of mediator molecules in MIL-100(Fe)

Following the confirmation of high operational stability for laccase immobilized in MIL 100(Fe), subsequent experiments focused on the incorporation of mediator molecules into the MOF

structure. Based on the solution-phase screening results, vanillin (natural mediator) and ABTS (synthetic mediator) were selected, as both demonstrated enhancement of laccase activity compared to other tested phenolic compounds.

Mediator immobilization was performed by incubating 100 mg of MIL 100(Fe) with aqueous solutions of vanillin or ABTS (1 mg/mL) under gentle agitation overnight. The resulting materials were washed at least five times with water to remove non-adsorbed mediator molecules. In parallel, laccase was immobilized on MIL 100(Fe) using the same procedure described previously. After immobilization, both laccase_MIL 100(Fe) and mediator_MIL 100(Fe) powders were dried under a nitrogen atmosphere.

For co-immobilized systems, 50 mg of laccase-MIL 100(Fe) was physically mixed with 50 mg of mediator_MIL 100(Fe) to obtain composite biocatalysts containing both components within the same material system. As controls for subsequent experiments, bare MIL 100(Fe) and mediator-loaded MIL 100(Fe) without enzyme were prepared and tested under identical conditions.

6. Degradation of organic pollutants by immobilized Laccase-mediator systems

To evaluate the applicability of the immobilized laccase-mediator systems toward chemically distinct contaminants, diclofenac (DFC), bisphenol A (BPA), and acid fuchsin (AF) were selected as model pollutants. Initial experiments with DFC (20 µg/mL) showed that laccase_MIL-100(Fe) was already highly active toward this substrate, and no clear enhancement from mediator incorporation could be resolved under the tested conditions (Figure 6). Therefore, subsequent experiments focused on BPA (≈40 µg/mL), a recalcitrant phenolic compound, and AF (125 µg/mL), which was poorly degraded in the earlier free-enzyme screening, thereby enabling clearer assessment of mediator effects.

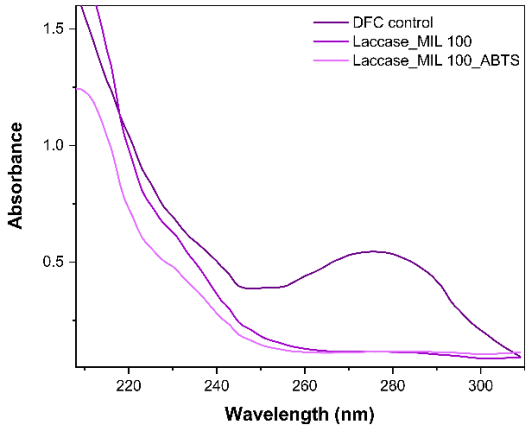

Figure 6. Removal of DFC by Laccase_MIL 100 and Laccase_MIL 100_ABTS systems.

All pollutant tests were conducted using 50 mg of laccase_MIL 100(Fe) mixed with 50 mg of either MIL 100(Fe) (no mediator) or mediator-loaded MIL 100(Fe); the MOF-only control contained 100 mg MIL 100(Fe). For BPA, the removal increased from 55% with MIL 100(Fe) alone to 76% with laccase_MIL 100(Fe), indicating a substantial enzymatic contribution beyond adsorption. Incorporation of mediators further improved BPA removal, reaching 82% for vanillin and 88% for ABTS, consistent with mediator-assisted electron transfer facilitating oxidation of phenolic substrates (Figure 7a).

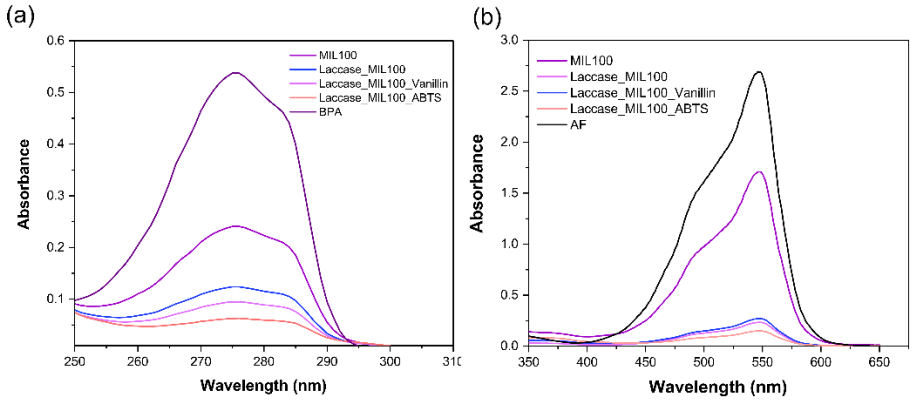

Figure 7. Degradation of BPA (a) and AF (b) by immobilized Laccase and Laccase_mediator systems.

For AF, MIL 100(Fe) alone removed 36%, confirming a significant adsorption contribution (Figure 7b). Notably, all laccase-containing systems achieved very high removal (89-95%), with the order laccase_MIL 100(Fe)_ABTS (95%) > laccase_MIL 100(Fe) (91%) ≈ laccase_MIL 100(Fe)_vanillin (89%). The relatively small differences among the laccase-based formulations suggest that, under these conditions, AF removal is dominated by the immobilized laccase system in combination with MOF-assisted effects (substrate preconcentration at/within the porous support and enhanced local reaction rates), while mediator addition provides only a modest further improvement. Overall, these results demonstrate that mediator benefits are substrate-dependent: clear enhancement was observed for BPA, whereas AF showed high removal already in the immobilized laccase system, and DFC was readily degraded by laccase without requiring mediator assistance.

Conclusions and future perspectives

With the grant awarded for this project (500 000 sek in total, for 6 months), we systematically investigated enzyme-mediator systems as a sustainable strategy for the degradation of emerging organic contaminants in water, with particular emphasis on mediator selection, immobilization, and operational stability. Solution-phase screening demonstrated that mediator efficiency is strongly enzyme- and substrate-dependent, with ABTS consistently providing the highest activity enhancement, while natural phenolic mediators such as vanillin showed moderate but relevant effects. Immobilization studies revealed that conventional alginate-based encapsulation led to severe activity losses, whereas the iron-based MOF MIL 100(Fe) provided an effective platform for enzyme immobilization, enabling high enzyme loading, excellent stability, and reusability over multiple cycles. Importantly, immobilized laccase retained high catalytic activity and enabled efficient degradation of structurally diverse pollutants, including phenolic compounds and dyes, with mediator addition further enhancing performance in a substrate-dependent manner. The combined effects of adsorption, enzyme stabilization, and localized catalysis within the MOF framework were shown to play a key role in pollutant removal, particularly for compounds that were poorly degraded by free enzymes in solution.

Future work should focus on decoupling adsorption, enzymatic transformation, and MOF-assisted effects through targeted control experiments and advanced analytical techniques to fully elucidate degradation mechanisms. The co-immobilization strategy can be further optimized by controlling mediator loading, spatial distribution within the MOF, and enzyme-mediator proximity to maximize catalytic synergy while minimizing leaching. Extending the system to real wastewater

matrices will be essential to assess robustness in the presence of competing substrates and inhibitors. In addition, long-term stability studies and continuous-flow experiments would provide valuable insight into the scalability of the proposed biocatalysts. Finally, expanding the range of natural mediators and exploring other water-stable MOFs could further improve sustainability and performance, supporting the development of efficient and environmentally benign enzyme-based water treatment technologies.

References

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