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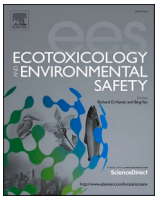

Efficient decolorization of recalcitrant dyes at neutral/alkaline pH by a new bacterial laccase-mediator system

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ABSTRACT

Laccases and laccase-mediator systems (LMS) are versatile catalysts that can oxidize a broad range of substrates coupled to the sole reduction of dioxygen to water. They possess many biotechnological applications in paper, textile, and food industries, bioethanol production, organic synthesis, detection and degradation of pollutants, and biofuel cell development. In particular, bacterial laccases are getting relevance due to their activity in a wide range of pH and temperature and their robustness under harsh conditions. However, the enzyme and the redox mediator's availability and costs limit their large-scale commercial use. Here we demonstrate that β-(10-phenothiazyl)-propionic acid can be used as an efficient and low-cost redox mediator for decolorizing synthetic dyes by the recombinant laccase SilA from *Streptomyces ipomoeae* produced in *E. coli*. This new LMS can decolorize more than 80% indigo carmine and malachite green in 1 h at pH = 8.0 and 2 h in tap water (pH = 6.8). Furthermore, it decolorized more than 40% of anthraquinone dye remazol brilliant blue R and 80% of azo dye xylidine ponceau in 5 h at 50 °C, pH 8.0. It supported at least 3 decolorization cycles without losing activity, representing an attractive candidate for a cost-effective and environmentally friendly LMS functional at neutral to alkaline pH.

1. Introduction

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are multicopper oxidoreductases ubiquitous in nature, found in bacteria, fungi, plants, and insects (Janusz et al., 2020). They are active on a wide range of phenolic and non-phenolic sub strates, which are oxidized in a mononuclear reactive center formed by a type I copper atom. Remarkably, substrates oxidation is solely coupled to reducing O_2 to H_2O in a trinuclear center composed of one type II copper atom and two type III copper atoms (Jones and Solomon, 2015). The presence of redox mediators can enhance the enzyme activity and range of oxidizable substrates. Redox mediators are low molecular weight substrates that could generate high redox potential intermediaries after oxidization by the laccase. These intermediaries act as electron carriers to non-enzymatically oxidize other compounds that are not direct substrates of the enzyme due to low kinetics or steric hindrance (Morozova et al., 2007; Cañas and Camarero, 2010). Due to their broad range of substrates, laccases and laccase-mediator systems (LMS) possess many biotechnological applications such as delignification of paper pulp (Singh and Arya, 2019), pre-treatment of biomass for biofuel production, biobleaching of textile dyes (Rodriguez-Couto, 2012), degradation of environmental organic and pharmaceutical pollutants (Arregui et al., 2019; Mlunguza et al., 2019), organic synthesis (Kunamneni et al., 2008), in beverage and food industries (Mayolo--Deloisa et al., 2020), and in the design of biosensors and biofuel cells (Zhang et al., 2018a). However, the success of the industrial use of laccases depends on optimizing the system's catalytic performance and the enzyme and mediator's production efficiency, which are the main barriers to LMS commercialization.

Many synthetic molecules such as 2,2′-azino-bis(3-

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ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 1-hydroxybenzotriazole (HBT), derivatives of lignin (syringaldehyde, vanillin, ferulic acid, acetosyringone, p-coumaric acid), and fungal metabolites were assayed as redox mediators for fungal and bacterial enzymes (Camarero et al., 2005; Morozova et al., 2007; Cañas and Camarero, 2010; Arregui et al., 2019; Singh and Arya, 2019). Despite this, a great effort is being made to find new and better compounds that meet the characteristics of an ideal redox mediator: readily available, non-toxic, affordable, and stable in successive redox cycles (Morozova et al., 2007; Cañas and Camarero, 2010). Phenothiazines represent a promising group of compounds that can act as redox mediators. Phenothiazine and its N-substituted derivatives are substrates of laccases (Kulys et al., 2000; Xu et al., 2000; Tetianec and Kulys, 2009) and follow an oxidation mechanism similar to that of ABTS (Blankert et al., 2005). However, only a few reports describe their use as efficient redox mediators for dye decolorization by fungal laccases (Soares et al., 2001; Camarero et al., 2005; Moldes and Sanromán, 2006).

Fungal laccases have been extensively explored as they were the first microbial laccases discovered. However, those of bacterial origin are gaining increasing importance due to their robustness under adverse conditions, and the possibility of being produced in industrial prokaryotic expression systems such as *E. coli* (Debnath and Saha, 2020). Bacterial laccases are usually stable at high temperatures, and many of them present tolerance to high chloride concentrations -up to 1 M- and to several organic solvents (Chauhan et al., 2017; Guan et al., 2018; Janusz et al., 2020). Furthermore, bacterial laccases show activity at neutral and alkaline pH, while fungal laccases are generally active only at acidic pH. These features make bacterial enzymes excellent candidates for the biobleaching of paper pulp and the treatment of textile effluents that usually show high salinity and alkaline pH (Arregui, 2019; Yaseen and Scholz, 2019). In the case of industrial effluents, the discharge of dyes into watercourses is a major environmental concern. They are usually toxic, mutagenic, carcinogenic, increase the biochemical and chemical oxygen demand, and reduce the light penetration in water, affecting the aquatic ecosystems (Lellis et al., 2019). Among the methods assayed for the treatment and bioremediation of effluents, the use of biological systems, particularly those based on laccases, are of the most promising for the removal and degradation of recalcitrant organic contaminants (Bilal et al., 2019; Katheresan et al., 2018).

CotA is a highly thermostable laccase (Martins et al., 2002) that has been characterized in diverse *Bacillus subtilis* strains and assayed for pollutant degradation (Cho et al., 2011; Guan et al., 2015; Zeng et al., 2016; Qiao et al., 2017; Wang and Zhao, 2017; Park et al., 2019; Qiao and Liu, 2019; Wang et al., 2019), organic synthesis (Sousa et al., 2019; Zhang et al., 2019) and development of biosensors (Fan et al., 2015). While SilA from *Streptomyces ipomoeae* belongs to the group of small or two-domain laccases found in *Actinobacteria* (Endo et al., 2002; Fernandes et al., 2014; Arregui et al., 2019). Less than ten small laccases from actinomycetes were characterized to date, but all showed outstanding thermostability and resistance to sodium azide and halides

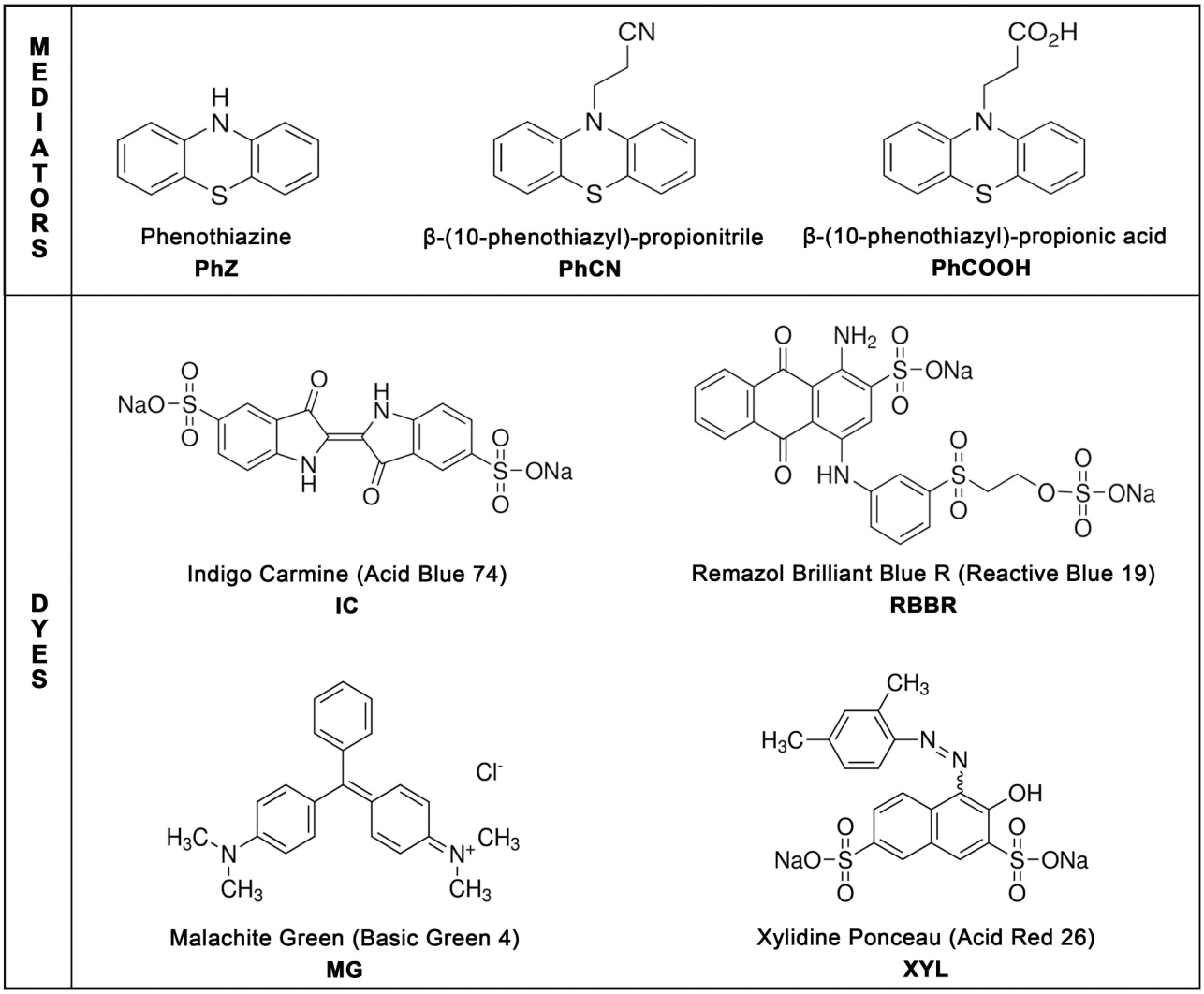

Fig. 1. Structures and acronyms of the dyes and mediators used this work.

(Fernandes et al., 2014). Small laccase SilA has shown to enhance the decolorization and detoxification of textile dyes in the presence of mediators such as acetosyringone and methyl syringate (Blánquez et al., 2019), degradation of fluoroquinolone based antimicrobials (Blánquez et al., 2016), biobleaching of eucalypt pulps (Eugenio et al., 2011), and delignification of lignocellulosic biomass (De La Torre et al., 2017).

Even though bacterial laccases represent a promising agent for clean oxidation processes (decolorization, bleaching, detoxification), their combination with phenothiazine-type compounds has not been explored to the best of our knowledge.

In this work, we combine phenothiazine and N-substituted derivatives as redox mediators with bacterial laccases to develop a LMS functional at neutral to alkaline pH. We present results using bacterial laccases CotA and SilA, along with phenothiazine and its N-substituted derivatives as redox mediators (Fig. 1, mediators) for decolorization of synthetic dyes containing indigoid, azo, anthraquinone, and triphenylmethane moieties (Fig. 1, dyes). Our results shows that the PhCOOH-SilA system can produce state-of-the-art decolorization yields using an affordable mediator and a recombinant laccase readily produced as a soluble and active enzyme in *E. coli*, a well-known industrial platform for protein expression. Remarkably, the decolorization process can be carried out using tap water without adjusting the pH at room temperature. This scalable laccase-mediator system can be explored in other industrial processes performed at alkaline pH, such as paper pulp biobleaching.

2. Material and methods

2.1. Materials

Phenothiazine ($\geq$ 98%) and acrylonitrile ($\geq$ 99%) were from Sigma-Aldrich. All other chemicals are analytical grade.

2.2. Synthesis of phenothiazine derivatives

β-(10-phenothiazyl)-propionitrile (PhCN) was synthesized as described by (Smith, 1950). The reaction was prepared by mixing phenothiazine and acrylonitrile cooled in an ice-bath and treated with 40% benzyltrimethylamonium hydroxide aqueous solution. The reaction mixture was warmed for one hour and then allowed to cool. The crystalline solid was recrystallized from acetone. ^{1}H NMR (500 MHz, *CDCl$_3$*): δ 2.85 (t,2 H), δ 4.28 (t,2 H), δ 6.85 – 7.21 (m,8 H).

β-(10-phenothiazyl)-propionic acid (PhCOOH) was synthesized by boiling a mixture of 2.5 g of PhCN, 2.5 g of sodium hydroxide, 7.5 ml of water, and 25 ml of methanol under reflux for 15 h. The product was added into cold MilliQ water acidified with 2 M hydrochloric acid solution until pH 7.0. The solid product was filtrated and recrystallized from ethanol. ^{1}H NMR (500 MHz, *CD$_3$OD*): δ 2.76(t,2 H), δ 4.20 (t,2 H), δ 6.90 – 7.24 (m,8 H).

Phenothiazine (PhZ) was recrystallized from benzene to obtain a yellow solid. ^{1}H NMR (500 MHz, *CDCl$_3$*): δ 6.85 – 7.30 (m,8 H).

2.3. Cloning and expression of recombinant CotA in Pichia pastoris

The coding sequence of laccase CotA was amplified by PCR using the total DNA of *Bacillus subtilis* ATCC 6633 as template and oligonucleotide primers CotFw (5′-GGATCCATATGACACTTGAAAAATTTG-3′) and CotRv (5′-AAGCTTATTTATGGGGATCAGTTATATC-3′). Total DNA of *B. subtilis* was extracted as described by Wilson (2001). Amplification primers were designed based on the consensus of the coding sequences for *B. subtilis* CotA laccase available at the GenBank database. PCR amplification was performed using 1 μg of DNA as the template in a 50 μl reaction mixture containing 1x Pfu DNA polymerase PCR buffer (Thermo Fisher Scientific), 2 mM MgSO4, 400 μM of each dNTP, 1 μM of each primer, and 2.5 units of Pfu DNA polymerase (Thermo Fisher Scientific). After the initial denaturation step at 95 °C for 5 min, amplification was performed by repeating 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min followed by a final extension step at 72 °C for 10 min. Blunt-ended PCR product of the expected size (1580 bp) was gel-purified and incubated 30 min at 70 °C in a 10 μl reaction containing 1X Taq DNA polymerase reaction buffer with $MgCl_2$ (Promega), 0.2 mM dATP, and 5 units of Taq DNA Polymerase (Promega) for A-tailing, and cloned in pGEM®-T Easy Vector (Promega). The identity of the inserted DNA fragment was confirmed by sequencing (Macrogen Inc Korea). The product of restriction with *Eco*RI and *Spe*I was cloned in sites *Eco*RI and *Avr*II of vector pPICNHIS (Niderhaus et al., 2018), to obtain vector pPICNHISCotA (Fig. S1). In this construction, the DNA coding sequence of laccase CotA is cloned in frame downstream of the signal secretion sequence from *Saccharomyces cerevisiae* α-mating factor, and the coding sequence for a six-histidine tag. Transcription is under the control of the AOX1 promoter (alcohol oxidase 1 from *P. pastoris*), which is inducible by methanol. Plasmid pPICNHISCotA was linearized with *Bgl*II restriction endonuclease and used for the transformation of *P. pastoris* strain SMD1168 (*his4*) (Invitrogen Life Technologies) by electroporation. Transformed *P. pastoris* cells are obtained by recombination between sequences AOX1 or HIS4 (histidinol dehydrogenase gene) in pPICNHISCotA and their homologous sequence counterparts present on the genome. Clones reverting histidine auxotrophy of *P. pastoris* strain SMD1168 were selected on minimal medium MD plates (0.34% yeast nitrogen base without amino acids, 10 g/l $(NH_4)_2SO_4$, 2% dextrose, 2% agar). Recombinant clones expressing and secreting active laccase were revealed by screening colonies showing green oxidation halos on minimal medium MM plates (0.34% yeast nitrogen base without amino acids, 10 g/L $(NH_4)_2SO_4$, and 2% agar) supplemented with 2 mM ABTS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonate)).

For the production of recombinant CotA, a single recombinant colony was grown in 5 ml of YPD medium (1% yeast extract, 2% peptone, 2% dextrose) for 16 h at 30 °C and 220 rpm and used to inoculate 25 ml of BMGY (1% yeast extract, 2% peptone, 0.34% yeast nitrogen base without amino acids, 10 g/l $(NH_4)_2SO_4$, 400 mg/l biotin, 1% glycerol, 100 mM potassium phosphate buffer, pH 6.0) in 250 ml shake flasks. After 48 h at 30 °C and 220 rpm, cells were harvested by centrifugation and resuspended in BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer, pH 6.0, 0.34% yeast nitrogen base without amino acids, 10 g/L $(NH_4)_2SO_4$, 400 mg/L biotin, 200 μM $CuSO_4$ and 3% sorbitol) to a final OD600 nm = 10. Cultures were performed in 1000 ml shake flasks at 28 °C and 220 rpm. Sterile methanol (0.5% final) was added every 24 h to maintain induction conditions (Campos et al., 2016). After 5 days of incubation in BMMY, the cultures were clarified by centrifugation at 1500 g for 10 min. The cell-free supernatant was concentrated by ultrafiltration (30 kDa MWCO, Amicon Ultra, Merck Millipore) and buffer exchanged to 50 mM sodium phosphate buffer, pH 8.0.

2.4. Cloning and expression of recombinant SilA in E. coli

The coding sequence of laccase SilA from *Streptomyces ipomoeae* (Genbank Acc number DQ832180) was synthesized by optimizing the codon usage for *E. coli* (Genescript) and incorporating restriction sites *Eco*RI and *Not*I at 5′ and 3′ends, respectively. *Eco*RI / *Not*I restriction product (990 bp) was cloned in the respective restriction sites into expression vector pHISTEV30a and used to transform *E. coli* BL21 (DE3) (Fig. S2). In this construction, the DNA coding sequence of SilA is cloned in frame with an N-terminal coding sequence for a six-histidine tag and the recognition sequence for tobacco etch virus protease (TEV). Transcription is under the control of the T7 promoter and the operator of the lac operon.

For SilA production, transformed *E. coli* cells were grown in LB broth at 37ºC containing 100 mg/L kanamycin until $OD_{600nm} = 0.6$. Induction was performed by adding 0.5 mM IPTG (isopropyl-b-D-thiogalactopyranoside) and 0.5 mM $CuSO_4$, overnight at 30 °C and 150 rpm. Cells were harvested by centrifugation, resuspended in 100 mM sodium phosphate

buffer pH 8.0, and disrupted by sonication. Cell debris was separated by centrifugation and supernatant was used as source of laccase.

2.5. Electrophoretic analysis of recombinant laccases

Protein extracts obtained in Sections 2.3 and 2.4 were separated by SDS-PAGE under reducing conditions and transferred to 0.45 μm nitrocellulose membranes (Bio-Rad Laboratories Inc, USA). Bands corresponding to recombinant laccases were revealed by Western blot using 0.1 μg/ml of polyclonal rabbit anti-HIS antibody (Genescript, USA) and phosphatase-linked goat anti-rabbit antibody (1:15000 dilution, Sigma Chemical Co., USA) as primary and secondary antibodies, respectively. Alkaline phosphatase activity was detected using 5 bromo-4 chloro-3 indolyl phosphate and nitroblue tetrazolium as substrates (Sigma Chemical Co., USA).

Native-PAGE was performed in 12% acrylamide gel at pH 8.8 under non-denaturing conditions. After running, laccase oxidative activity was revealed by incubation in 10 mM DMP solution in 100 mM sodium acetate pH 8.

2.6. Laccase activity assays

Laccase activity was determined by quantification of 5 mM 2,6 dimetoxyphenol (DMP) oxidation at 469 nm ($\varepsilon = 27.5\ mM^{-1}\ cm^{-1}$) in McIlvaine's buffer at pH 8.0 and room temperature (24 °C). One unit of enzymatic activity was defined as the amount of enzyme transforming 1 μmol of substrate per minute at the indicated pH and temperature.

2.7. Dye decolorization

Decolorization activity was determined by measuring the decrease in absorbance at the maximum wavelength in the visible spectrum of each dye: indigo carmine (Acid Blue 74, 610 nm); xylidine ponceau (Acid Red 26, 497 nm), remazol brilliant blue R (Reactive Blue 19, 590 nm) and malachite green (Basic Green 4, 617 nm) and expressed as:

$$\%\text{decolorization} = \frac{(Ac - Ar)}{Ac} x100$$

Where: Ac = absorbance of a control reaction without enzyme and Ar = absorbance of the decolorized reaction.

Reactions were performed in triplicates using 50 μM of dye in McIlvaine's buffer at room temperature (24 °C) or 50 °C. For indigo carmine decolorization, redox mediators were used at a ratio of 1:1 (50 μM) with dye, and the laccase final concentration was 0.1 UE/ml. For xylidine ponceau, remazol brilliant blue R and malachite green decolorization, the laccase concentration was 1 UE/ml, and redox mediators were used at a ratio of 5:1(250 μM) with dye.

For successive decolorization cycles, the decrease in the absorbance of 50 μM indigo carmine was determined in the presence of 50 μM of the different redox mediators and 0.1 UE/ml of laccase. After incubations of 1 h, A_{610nm} was determined, and concentrated dye was added to the reaction to reach the IC's initial concentration (50 μM).

UV–visible absorbance spectroscopic scans (200–800 nm) were carried out using a Nanodrop™ 2000 spectrophotometer for dye-decolorizations reactions after 24 h of incubation of 50 μM of each dye with 1 U/ml of SilA and 250 μM PhCOOH at pH 8.0 and 24 °C and control solutions of dyes and PhCOOH without the addition of the enzyme.

2.8. Redox potential of dyes and mediators

Solutions of 50 μM of IC were prepared in McIlvaine's buffer at pH 4.0 and 8.0, and redox potentials were determined by cyclic voltammetry at 25 mV/s using a system of three electrodes: glassy carbon, Ag/AgCl (3 M), and gold as working, reference and counter electrode, respectively. A solution of 50 μM was prepared for the mediators at the same conditions and with the same system. The redox potential was determined by differential pulse voltammetry with 2 mV step size, 0.25 s sample period, 0.05 s pulse time, and 50 mV pulse size.

2.9. Statistical analysis

All experiments were performed in triplicate, and the results were expressed as the mean ± standard deviation (SD). Statistical analysis was carried out with GraphPad 6.01 software (GraphPad Software Inc.) using a two-tailed unpaired *t*-test when the means of 2 groups were compared or a one-way ANOVA followed by Tukey's multiple comparisons test when the means of more than 2 groups were compared. Differences were considered statistically significant when p-value ≤ 0.05.

3. Results

3.1. Evaluation of phenothiazine and N-substituted derivatives as redox mediators for bacterial laccases

To test phenothiazine (PhZ) and the N-substituted derivatives β-(10-phenothiazyl)-propionic acid (PhCOOH) and β-(10-phenothiazyl)-propionitrile (PhCN) as redox mediators, we first evaluated their performance in decolorization of indigo carmine (IC), a dye commonly used in the textile industry to produce denim fabrics. We used two recombinant bacterial laccases produced in the course of this work: the three-domain CotA enzyme from *Bacillus subtilis* and the two-domain small laccase SilA from *Streptomyces ipomoeae*.

The CotA bacterial laccase was cloned from *B. subtilis* and expressed in *P. pastoris*. The recombinant enzyme's biochemical properties were identical to those previously reported for the enzyme expressed in *E. coli* (Martins et al., 2002), showing a molecular weight of 60–65 kDa (Fig S3a), and optimal conditions for the oxidation of DMP at pH 8.0.

The *S. ipomoeae* laccase SilA was expressed as a soluble intracellular protein in *E. coli* using microaerobic conditions to improve copper incorporation into the cell (Durão et al., 2008). The recombinant enzyme showed an apparent molecular weight of 39–40 kDa in denaturing conditions (Fig. S3b). Conversely, under non-denaturing conditions, only a band of 75–80 kDa was revealed by oxidation of DMP (Fig. S3b), corresponding to the dimeric active form as previously described (Molina-Guijarro and Pérez, 2009). Also, recombinant SilA showed optimal activity at pH 8.0 for the oxidation of DMP.

IC decolorization by SilA in the absence of redox mediators reached 19% after 5 h incubation at 24 °C, while in the presence of PhCOOH or PhZ, it reached 82% and 55%, respectively in the first hour (Fig. 2a).

On the other hand, CotA managed to decolorize 39% of IC after 5 h of incubation in the absence of redox mediators, while in the presence of PhCOOH or PhZ, it reached 90% and 75% in 1 h of incubation (Fig. 2b). These results show a striking improvement in performance and decolorization time when the mediators are present.

The compound PhCN did not significantly affect IC decolorization at pH 8.0 concerning the treatments with SilA or CotA alone ($p > 0.05$).

We performed three consecutive IC decolorization cycles to test the reusability of the bacterial LMS. Each cycle lasts 1 h at 24 °C and pH 8.0. Assays performed in the presence of PhCOOH show outstanding stability (Fig. 3). IC decolorization reached the same values after the three incubation cycles with SilA-PhCOOH (Fig. 3a) and after two incubation cycles with CotA-PhCOOH, with a slight decrease (4–5%) in the third cycle (Fig. 3b) ($p > 0.05$). These results show that PhCOOH could be acting as an ideal mediator, maintaining its redox capacity after at least 3 cycles of decolorization. On the other hand, in the presence of PhZ, IC decolorization efficiency significantly decreased by 50–60% after the first incubation cycle, while for PhCN, even though the decolorization yields were low, a stable behavior of the enzymes was observed.

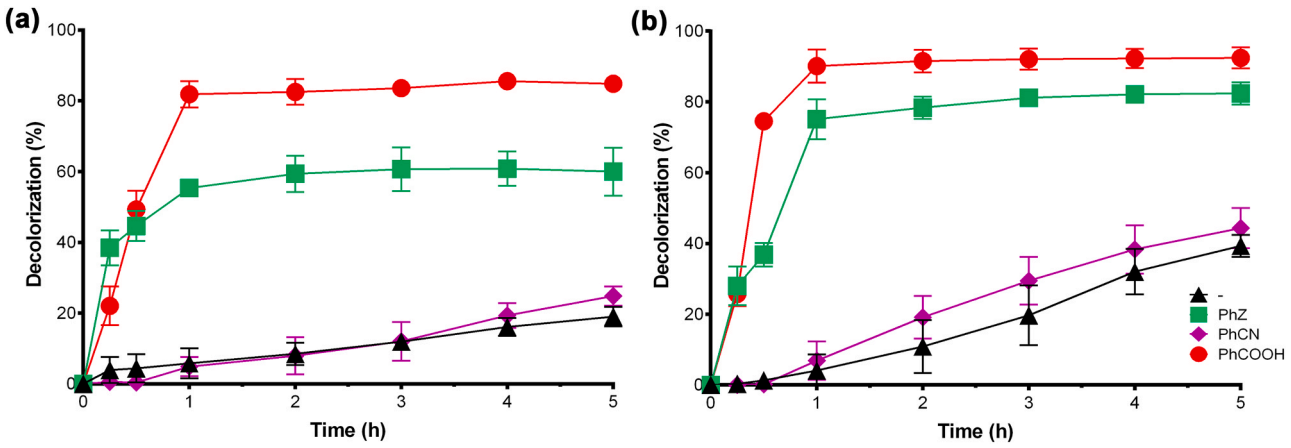

Fig. 2. Decolorization of IC by SilA (a) and CotA (b) without mediator (triangles) or in the presence of a mediator: PhZ (squares), PhCN (diamonds) and PhCOOH (circles) at 24 °C, pH 8.0.

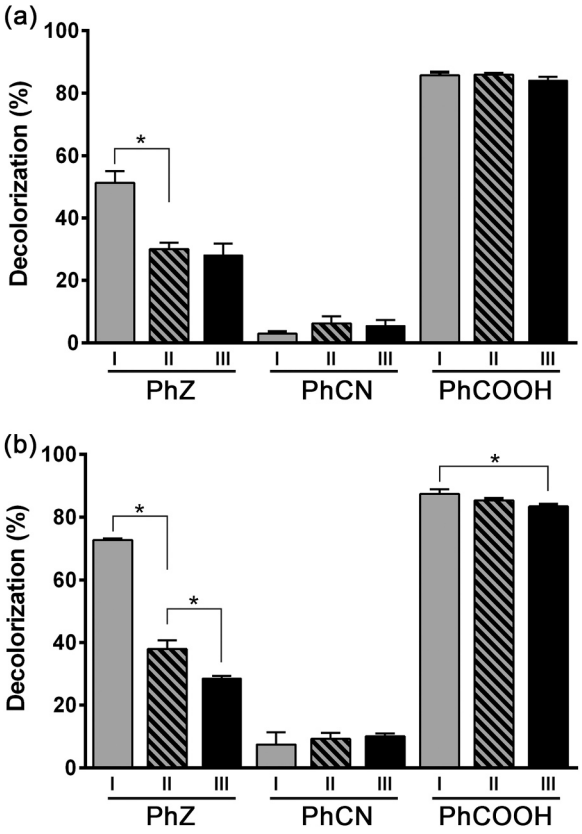

Fig. 3. Reusability of LMS. Decolorization of IC by SilA (a) and CotA (b) in the presence redox mediators at 24 °C, pH 8.0 in three consecutive cycles (I, II, III) of 1 h of incubation. An asterisk denotes statistically significant differences between cycles ($p \leq 0.05$).

3.2. IC oxidization mechanism mediated by phenothiazine and N-substituted derivatives

In Fig. 4, the oxidation potential for each mediator was quantified by differential pulse voltammetry, which allows determining the formal redox potentials of a species from the potential where the peak current appears. Both PhCOOH and PhCN presented formal potentials for the first oxidation process and a predominant form at pH 8.0 higher than the IC's formal potential at the same conditions (0.55 V vs. SHE, depicted with a line in Fig. 4.). On the other hand, the PhZ predominant form

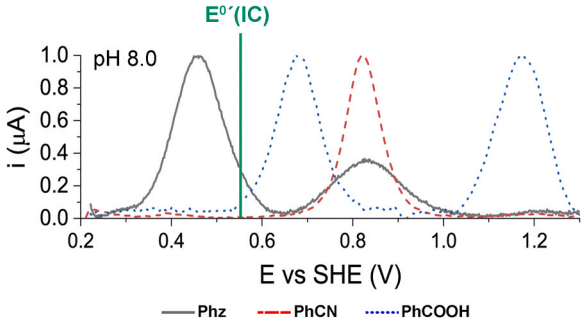

Fig. 4. Differential pulse voltammetries for PhZ (continuous line), PhCN (dashed line) and PhCOOH (dotted line). The peaks values represent the formal potentials for the different mediators and the vertical lines indicate the formal potential of IC at pH 8.0.

presented a formal potential lower than IC. However, PhZ and PhCOOH enhanced IC's decolorization by the laccases, while no effect was observed for PhCN. Additionally, PhZ and PhCOOH oxidation products showed redox potentials higher than the reported for the T1 copper of the laccases CotA ($E^\circ = 0.455$ V vs. SHE, Durão et al., 2006) and SilA ($E^\circ = 0.337$ V vs. SHE, Blánquez et al., 2019). These observations demonstrate that the decolorization of dyes using a combination of laccase and mediators is a complex mechanism that cannot be explained by the simple comparison of the formal redox potentials of the T1 Cu center, the mediator, and the dye (Bourbonnais et al., 1998; Zille et al., 2004).

The oxidation of phenothiazine derivatives involves the formation of radical species. Similar to the oxidative mechanism followed by the commonly used redox mediator ABTS (Bourbonnais et al., 1998), phenothiazines can undergo a two-electron process yielding a dication species with a high redox potential (Blankert et al., 2005) (Fig. S4). This redox potential depends on the N atom's protonation; thus, the oxidation products will depend on the derivative and the medium's pH (Cheng et al., 1978; Puzanowska-Tarasiewicz et al., 2005).

For PhZ, oxidized species with a formal potential of 0.46 V (PhZ^+/PhZ) can be formed at pH 8.0 and then further oxidized by the bacterial laccase generating a stronger oxidizing species (PhZ^{2+}) that can be responsible for IC decolorization. Even though the difference in formal redox potentials between the bacterial laccases and the PhZ^{2+}/PhZ^+ couple yields a negative value, the equilibrium for the equation.

$$Lac_{(ox)} + PhZ^+ \rightarrow Lac_{(red)} + PhZ^{2+}$$

can be displaced toward the products since the reduced laccase

immediately consumes the oxygen. At the same time, IC reacts with PhZ^{2+} regenerating the reactants. In this way, the ratio between products and reactants approaches zero in the Nernst thermodynamic equation:

$$\Delta E = \Delta E^0 - \frac{RT}{nF}\ln\frac{[PhZ^{2+}][Lac(red)]}{[PhZ^{+}][Lac(ox)]} \quad (1)$$

Therefore, ΔE adopts a positive value, and the reaction spontaneously occurs. A similar mechanism was observed for ABTS in the oxidation of veratryl alcohol catalyzed by a fungal laccase (Bourbonnais et al., 1998). Similarly, the differences between the redox potential of the laccases and PhCOOH can be overcome by the displacement in the oxidation reaction's equilibrium. Such differences are much higher for PhCN; consequently, no oxidation products are formed in the presence

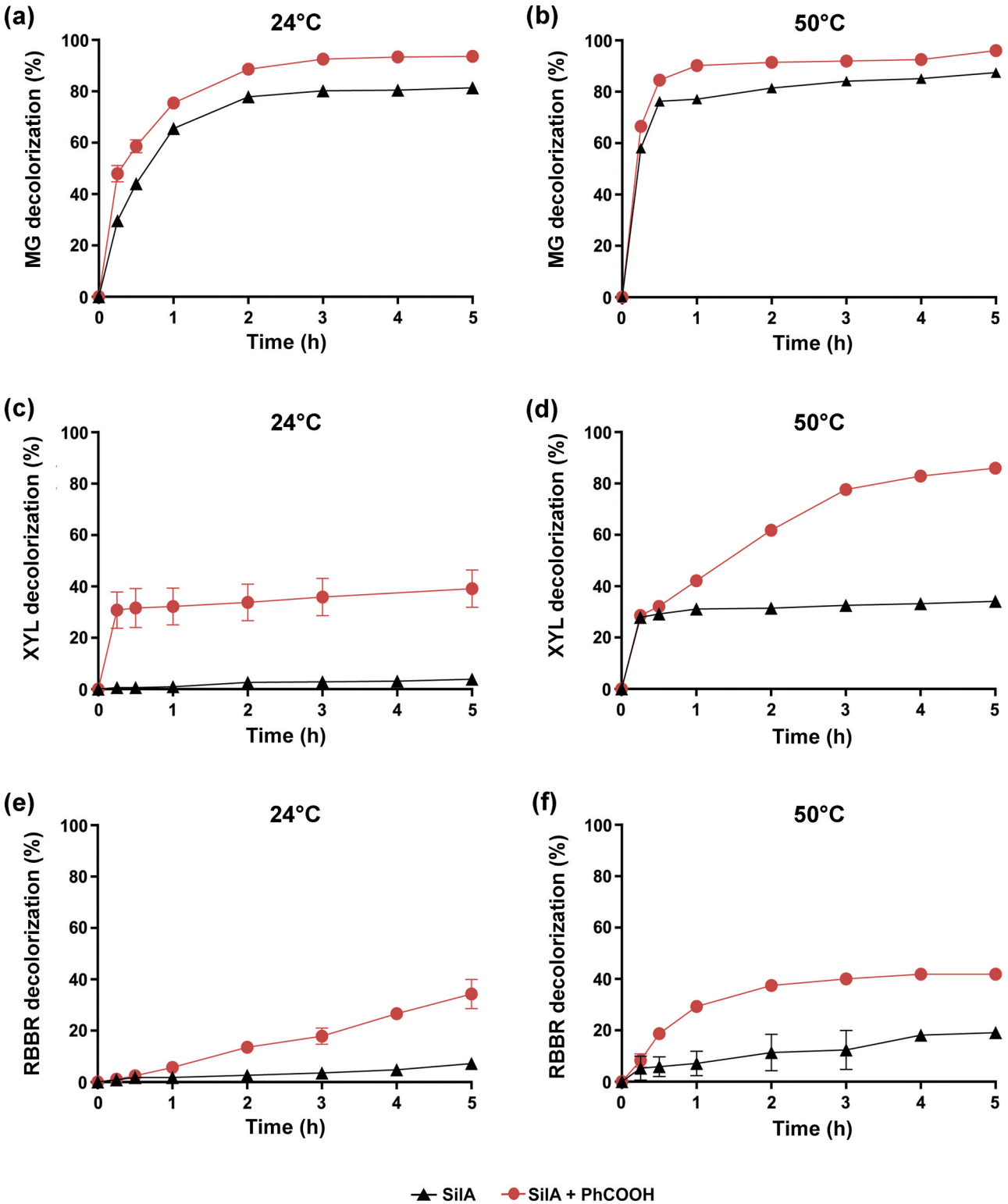

Fig. 5. Decolorization of recalcitrant industrial dyes at pH 8.0: MG (a and b) XYL (c and d), RBBR (e and f) by SilA in the absence (triangles) or in the presence of PhCOOH (circles) at 24 °C (a,c,e) or 50 °C (b,d,f).

of bacterial laccases.

3.3. Decolorization of recalcitrant dyes by the SilA-PhCOOH system at pH 8.0

We evaluated the decolorization of remazol brilliant blue R (RBBR, Reactive Blue 19), xylidine ponceau (XYL, Acid Red 26), and malachite green (MG, Basic Green 4) (depicted in Fig. 1), representing the three main groups of recalcitrant industrial dyes used worldwide: anthraquinone, azoic and triphenylmethane. Although both recombinant laccases showed similar efficiency and response to redox mediators for IC decolorization, CotA resulted in low production yields. Thus, we performed the following assays using SilA.

The decolorization efficiency was different for each dye, but in all cases, it showed a significant increase ($p \leq 0.05$) in the presence of PhCOOH and when the incubation temperature was raised to 50 °C, according to the optimal temperature range for SilA (Fig. 5).

Regarding triphenylmethane dyes, SilA was highly efficient for decolorization of MG, reaching 80% and 93% after 3 h of incubation at room temperature with the enzyme alone or in the presence of PhCOOH, respectively, with no significant ($p > 0.05$) increase in decolorization after 5 h of incubation (Fig. 5a). When incubations were performed at 50 °C, the reaction's rate was faster, with more than 75% decolorization reached in the first 30 min (Fig. 5b).

On the other hand, the enzyme alone could not decolorize more than 4% of XYL after 5 h at room temperature; however, it reached 34% of decolorization after 5 h of incubation at 50 °C. In the presence of PhCOOH an enhancement in decolorization was observed, reaching 86% after 5 h at 50 °C (Fig. 5d) and 37% after 5 h at room temperature (Fig. 5c), showing the ability of SilA-PhCOOH LMS to oxidize an azo compound.

Concerning RBBR less than 20% of decolorization was reached by SilA alone, either at room temperature or 50 °C. However, in the presence of PhCOOH, it increased to 33% and 50% after 5 h and 24 h at room temperature and 41% after 5 h at 50 °C (Fig. 5e,f).

3.4. Efficiency of dye decolorization by SilA-PhCOOH system in tap water at room temperature

We performed the treatments in tap water (pH 6.8) at room temperature (24 °C) to evaluate the SilA-PhCOOH system's efficiency for dye decolorization in mild conditions.

More than 80% of IC decolorization was reached in the first 15 min of incubation in the presence of PhCOOH (Fig. 6a), showing that our system has the potential to be used for IC degradation in mild conditions. Regarding MG, 91% decolorization was reached after 5 h of incubation in the presence of PhCOOH (Fig. 6b). For XYL and RBBR a decrease in the decolorization was observed in tap water compared to treatments at pH 8.0. However, there was still more than 60% and 50% of decolorization, respectively, after 24 h of incubation in mild conditions in the presence of PhCOOH (Fig. 6c,d).

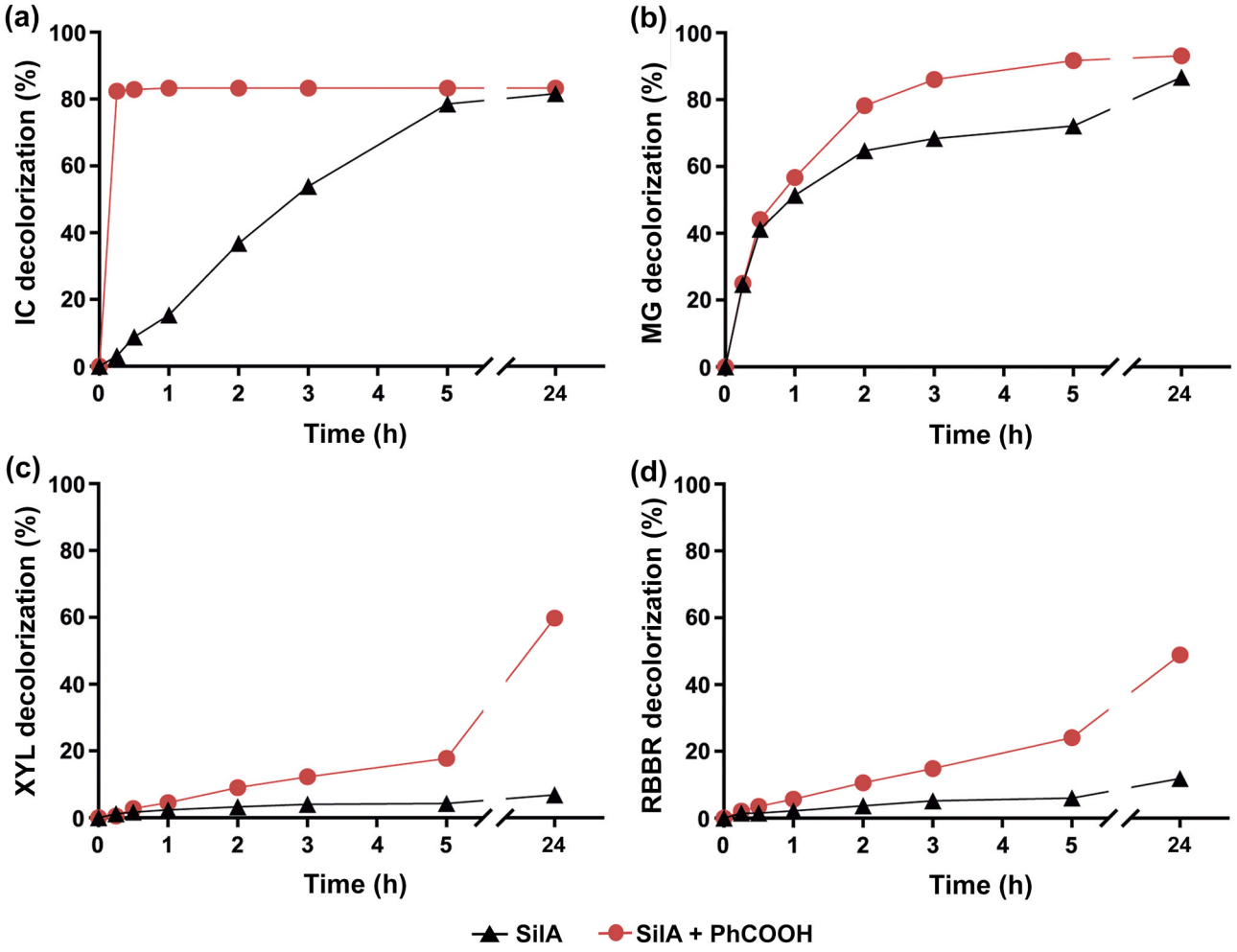

Fig. 6. Decolorization of IC and the recalcitrant dyes in tap water: IC (a), MG (b), XYL (c) and RBBR (d) by SilA in the absence (triangles) or the presence of PhCOOH (circles) at 24 °C.

3.5. UV–visible absorbance spectral analysis of decolorization products

The proposed mechanisms for the catalytic degradation of XYL, RBBR, and MG by laccase involve the formation of aromatic compounds of low molecular weight, which can be further degraded by chemical processes (Osma et al., 2010; Levin et al., 2012; Yang et al., 2015; Navas et al., 2020). As an approach to confirm these mechanisms in the SilA-PhCOOH LMS, we performed UV–visible absorption spectra of decolorizing reactions after 24 h of incubation. In all of the cases, we observed the disappearance of each dye's prominent absorbance peak due to cleavage of the chromophore, and the increase in the absorbance in the range of 200–400 nm, indicating the formation of aromatic compounds of lower molecular weight (Fig. S5). Also, for IC decolorization reactions, the disappearance of the characteristic peak at 610 nm was accompanied by the increase in the absorption at 250 nm, corresponding to the isatin-5-sulfonic acid released after the breakage of the C=C double bond in the dye molecule due to the oxidation by the LMS (Kandelbauer et al., 2008).

4. Discussion

Commercialization and industrial use of laccases face technical and economic challenges. The enzymes should be active and stable under industrial operating conditions, reusable, and their large-scale production and downstream processes have to be cost-effective (Agrawal et al., 2018; Osma et al., 2011). In these aspects, bacterial laccases are of particular interest since they can be active at neutral to alkaline pH, show thermal stability, and are often tolerant towards organic solvents and high salt concentrations. Additionally, they can be produced in prokaryotic hosts, such as *E. coli,* a well-known recombinant protein production platform (Rosano and Ceccarelli, 2014). However, efficient oxidation by bacterial enzymes often requires stable redox mediators, impacting the final cost of the process.

Here we tested PhZ and the N-substituted derivatives PhCN and PhCOOH as low-cost redox mediators for bacterial laccases. Both PhZ and PhCOOH enhanced IC decolorization by laccases CotA and SilA; however, PhCOOH was the most efficient. On the other hand, PhCN did not significantly improve IC decolorization by bacterial enzymes, despite being reported as a substrate for fungal laccases (Kulys et al., 2000).

These differences in mediator's responses could be explained by the combined effect of the relation between the redox potentials of the enzyme, the mediator, and the dye, and the reactants' concentrations. Thus, laccase can generate high redox potential PhZ^{2+} forms capable of oxidizing IC, despite PhZ first oxidation process and predominant form showed a redox potential lower than the dye.

This dissimilar behavior in the efficacy of PhZ and its N-substituted derivatives as redox mediators has also been reported by other authors using fungal enzymes. Degradation of anthracene by a *Trametes versicolor* laccase in the presence of PhZ showed no significant differences from incubation with the enzyme alone (Johannes et al., 1996).

Promazine (10-[3-dimethylaminopropyl] phenothiazine) is an efficient redox mediator used for decolorization of IC and aniline blue by the laccase from the basidiomycete *Pycnoporus cinnabarinus* at pH 5 (Camarero et al., 2005). Furthermore, promazine enhanced decolorization of IC and phenol red by two *T. versicolor* laccase isoenzymes at pH 4.5 (Moldes and Sanromán, 2006). However, neither promazine nor chlorpromazine was able to mediate the laccase-catalyzed oxidation of veratryl alcohol or delignification of pulp by fungal enzymes (Bourbonnais et al., 1997).

The LMS performance using PhCOOH was similar and even higher than the reported for IC decolorization by CotA and SilA in the presence of other redox mediators used at longer incubation times, higher temperature, and higher mediator:dye ratio (Table 1). PhCOOH combined with laccase SilA was also effective for IC decolorization in tap water at RT. PhCOOH has been reported to be the mediator present in the commercial formulation DeniLite® (Novozymes) used for IC bleaching. Since this system is based on a fungal recombinant laccase, its use is limited to pH < 6 and temperatures of 50 °C (Colomera and Kuilderd, 2015; Soares et al., 2001).

Table 1
Decolorization of IC by recombinant laccases SilA and CotA.

Laccase	Redox mediator	Decolorization / time	pH / T°	M:D ratio	References
SilA	–	27% / 24 h	8 / 35 °C	2:1	Blánquez et al. (2019)
	ASG	96% / 24 h			
	SYR	94% / 24 h			
	MeS	98% / 24 h			
	–	19% / 5 h	8.0 / 24 °C	1:1	This work
	PhZ	55% / 1 h			
	PhCOOH	82% / 1 h			
	PhCN	25% / 5 h			
	PhCOOH	81% / 15 min	6.8 / 24 °C	5:1	
CotA	ASG	100% / 6 h	6.2 / 30 °C	2:1	Qiao et al. (2017)
		100% / 6 h	9.0 / 30 °C		
	ASG	99% / 1 h	7 / 40 °C	2:1	Wang and Zhao (2017)
	–	39% / 5 h	8.0 / 24 °C	1:1	This work
	PhZ	75% / 1 h			
	PhCOOH	90% / 1 h			
	PhCN	44% / 5 h			

ASG: acetosyringone, SYR: syringaldehyde, MeS: methyl syringate
M:D ratio: mediator:dye ratio

Thus, our SilA-PhCOOH system could be an alternative for denim fabrics' biobleaching in neutral to alkaline pH conditions. Furthermore, laccases could reduce the undesired effect of re-deposition or back staining of indigo dye on white yarns after the bio-stone washing of denim garments by cellulases. Since this effect is enhanced at pH 4–6, but significantly reduced at neutral pH (Campos et al., 2001; Montazer and Sadeghian Maryan, 2010), the availability of LMS active at pH > 6 could contribute to the development of more efficient staining processes for the textile industry.

Additionally, we used the PhCOOH-SilA system to decolorize recalcitrant dyes, both at alkaline pH and in tap water. Finding efficient methods for removing these dyes is of paramount importance since reductive biotransformation of azo dyes results in the formation of toxic amines, while the anthraquinone dyes are resistant to degradation due to their fused aromatic ring structure (Ghaly et al., 2014; Singh et al., 2015). Furthermore, oxidation of MG could prevent its metabolic reduction to leucomalachite, an equally carcinogenic form but with more remarkable persistence in fish tissues (Hashimoto et al., 2011; Srivastava et al., 2004).

Although there are many MG degradation descriptions by microbial reductases or oxidation by fungal laccases at acidic pH, few describe the oxidation by bacterial LMS (Table 2). MG decolorization reached by SilA-PhCOOH was similar to the reported for laccase of *B. subtilis* that degraded 93% of the dye in 1 h of reaction in the presence of ASG (Wang and Zhao, 2017; Table 2). Furthermore, SilA-PhCOOH was highly efficient for MG decolorization in tap water at RT. Degradation of MG in mild conditions is crucial since it is used not only as a dye for silk, leather, and paper but mainly as a parasiticide and antifungal agent in aquaculture (Hashimoto et al., 2011).

Regarding the azoic dyes, we showed that SilA-PhCOOH was capable of decolorize monoazoic XYL. Previous reports showed that SilA could successfully decolorize other azoic dyes such as Reactive Black 5 and Orange II in the presence of acetosyringone or methyl syringate as redox mediators (Blánquez et al., 2019). On the other hand, there is only one recent report of XYL oxidation at alkaline pH. Laccase LAC-2.9 from *Thermus* sp 2.9 manage to decolorize 98% of XYL after 24 h of incubation at pH 9 and 60 °C, without the addition of redox mediators (Navas

Table 2
Decolorization of MG and RBBR by bacterial LMS.

	Laccase source	Redox mediator	Decolorization / time	pH / T°	Dye concentration	References
MG	*Bacillus vallismortis*	ABTS (100 mM)	79.6% / 24 h	6 / 37 °C	50 mg/L	Zhang et al. (2013)
		ASG (100 mM)	40% / 24 h			
		SYR (100 mM)	30% / 24 h			
	Bacillus safensis sp. S31	ABTS (0.015 mM)	20% / 2 h	8 / 30 °C	10 mg/L	Siroosi et al. (2018)
	Bacillus sp. KC2	SGZ (0.1 mM)	0% / 5 h	7 / 50 °C	25 mg/L	Asadi et al. (2020)
		ABTS (0.1 mM)	82.7% / 5 h			
	B. subtilis	ASG (0.1 mM)	93% / 1 h	7 / 40 °C	10 mg/L	Wang and Zhao (2017)
	E. coli (CueO)	ASG (0.1 mM)	98.5% / 24 h	7.5 / 55 °C	80 mg/L	Ma et al. (2017)
	S. ipomoeae (SilA)	PhCOOH (0.25 mM)	91% / 5 h	6.8 / 24 °C	18 mg/L	This work
			93% / 3 h	8 / 24 °C	(50 μM)	
			84% / 30 min	8 / 50 °C		
RBBR	*Bacillus licheniformis* LS04	ASG (0.1 mM)	80% / 6 h	6.2 / 40 °C	100 mg/L	Lu et al. (2013)
		ASG (0.1 mM)	97% / 6 h	9 / 40 °C		
	B. subtilis cjp3	ASG (0.1 mM)	62% / 6 h	6.2 / 30 °C	60 mg/L	Qiao et al. (2017)
			97% / 6 h	9 / 30 °C		
	Streptomyces coelicolor	ASG (1 mM)	21% / 5 h	9 / 45 °C	16 mg/L	Dubé et al. (2008)
		SYR (1 mM)	0% / 5 h			
	B. subtilis	ASG (0.1 mM)	34.5% / 1 h	7 / 40 °C	100 mg/L	Wang and Zhao (2017)
	S. ipomoeae (SilA)	PhCOOH (0.25 mM)	49% / 24 h	6.8 / 24 °C	31 mg/L	This work
			51% / 24 h	8 / 24 °C	(50 μM)	
			41% / 5 h	8 / 50 °C		

ASG: acetosyringone, SYR: syringaldehyde, SGZ: syringaldazine, ABTS: 2,2′-azino bis-(3-ethylbenzthiazoline-6-sulphonate)

et al., 2020), making this enzyme an attractive candidate to be evaluated with PhCOOH as redox mediator.

RBBR was the most recalcitrant to degradation by SilA-PhCOOH. The efficiency of degradation of RBBR by other bacterial laccases and LMS was very variable (Table 1). Small laccase SLAC from *Streptomyces coelicolor* showed a low decolorization rate even in the presence of redox mediators (Dubé et al., 2008). Conversely, laccases from *B. subtilis* cjp3, *B. licheniformis* LS04 reached almost 100% of decolorization in the presence of acetosyringone (Qiao et al., 2017; Lu et al., 2013). Thus, further optimization of reaction conditions and mediator:dye ratio could improve anthraquinonic dye decolorization by SilA-PhCOOH.

PhCOOH showed high efficiency as a redox mediator for bacterial laccases, was stable after successive decolorization cycles, representing an affordable mediator. Most reported dye decolorizations by LMS use ABTS, ASG, or syringaldehyde as redox mediators, while in the paper pulp production process, 1-hydroxybenzotriazole (HBT) is the most used. Based on the same supplier (Sigma Aldrich) prices, ABTS and ASG are the most expensive: ABTS is about 19,600 USD/mol, and ASG is USD 3500 USD/mol. On the other hand, we estimated a cost of 150 USD/mol for the synthesis of PhCOOH from PhZ (PhZ: 17 USD/mol), using analytical grade materials. This value is even lower than the cost of syringaldehyde (460 USD/mol) and HBT (230 USD/mol) species, adjusting to the concept of a low-cost mediator (Morozova et al., 2007).

Since the production of laccases in native organisms could be costly and incompatible with industrial fermentation processes (Debnath and Saha, 2020), we expressed *B. subtilis* CotA and *S. ipomoeae* SilA as recombinant proteins in *P. pastoris* and *E. coli*, respectively. Recombinant production of SilA in *E. coli* resulted in higher yields and was less laborious than the expression of CotA in *P. pastoris*. Laccase SilA was expressed as an intracellular soluble protein that was recovered in simple centrifugation and cell disruption steps, without the need to incorporate any additional purification process, simplifying the downstream processing and lowering the operational costs. Based on our previous experience in fungal laccase immobilization in a hierarchical meso/macroporous system in wrinkled-SiO2 spheres for dye decolorization (Fuentes et al., 2021) and in graphite screen printing electrodes containing multiwall carbon nanotubes for biosensor development (Borón et al., 2017); perspectives to improve the performance of this LMS include the immobilization of SilA on solid supports. Immobilization has proved to enhance the activity, stability, and reusability of the enzyme and is a widely explored strategy for laccase-based pollutant detection and removal from fresh and wastewater (Wong et al., 2019; Zhou et al., 2020; Datta et al., 2021). Diverse materials have been used as carriers for laccase immobilization, including inorganic solids, synthetic polymers, and biopolymers (Peng et al., 2019). New nanoscale supports such as inorganic nanoflowers, metal-organic frameworks, and magnetic nanoparticles have shown outstanding results for laccase immobilization (Wong et al., 2019; Zhou et al., 2020; Datta et al., 2021).

Additionally, other protein expression techniques, such as surface display in prokaryotic cells, could be explored (Guoyan et al., 2019). Expression of recombinant CotA fused to membrane proteins in *Bacillus* spores, or *E. coli* have shown to increase the copper incorporation, stability, and yields of the enzyme and was successfully applied for dye decolorization (Cho et al., 2011; Wan et al., 2017; Zhang et al., 2018b; Park et al., 2019).

Finally, we have shown that decolorization by SilA-PhCOOH involved the cleavage of the chromophore and the formation of low molecular weight aromatic compounds, according to the proposed mechanisms for the catalytic degradation of XYL, RBBR, and MG by laccase (Osma et al., 2010; Levin et al., 2012; Yang et al., 2015; Navas et al., 2020). These smaller molecules have shown lower toxicity levels than the dye (Legerská et al., 2016) and could be further degraded by chemical or biological processes. However, additional studies are needed to identify the decomposed substances generated by the SilA-PhCOOH system.

5. Conclusion

The comparative analysis of dye decolorization by laccase and the LMS shows the high variation in each system's efficiency. The LMS performance depends on the enzyme's source, the redox mediator, and the reaction conditions. This highlights the difficulty of predicting the oxidation of complex substrates and the need to carry out the systems' experimental optimization according to each process's main components. Our results demonstrate that PhCOOH can be efficiently used as a redox mediator for IC oxidation by bacterial laccases without inhibition of the enzyme or loss of activity in at least three decolorization cycles. In particular, the LMS based on SilA-PhCOOH allows the decolorization of recalcitrant synthetic dyes of indigoid, azo, anthraquinone, and triphenylmethane type in tap water and at pH 8.0. Low-cost of PhCOOH synthesis and production of recombinant SilA in *E. coli*, along with other properties of the enzyme, such as thermostability and tolerance to high salt concentrations, show the potential of this LMS to be applied in the removal of dyes in alkaline textile effluents, achieving state-of-the-art

decolorization yields. The SilA-PhCOOH system could also be valuable for other industrial processes, such as the delignification of alkaline paper pulp, biobleaching of indigo dye, reduction of back staining in the denim finishing process.

CRediT authorship contribution statement

Lucy L. Coria Oriundo: Investigation, Formal analysis, Writing - original draft preparation. **Fernando Battaglini**: Conceptualization, Resources, Funding acquisition, Writing - review & editing. **Sonia Wirth**: Methodology, Resources, Writing - original draft preparation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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The authors declare no financial or commercial conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112237.

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