Phenothiazines as efficient redox mediators for dye-decolorization at neutral / alkaline pH by bacterial laccases

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Abstract

Laccases are oxidoreductases with the outstanding ability to oxidize phenolic and non-phenolic substrates coupled to the reduction of O2 to H2O. Among them, bacterial enzymes are suitable biocatalysts for application in industrial processes under harsh conditions. However, to be active on high redox potential substrates, bacterial laccases requires of redox mediators: electron carriers between the laccase and other compounds not directly oxidizable by the enzyme. Here we demonstrate that β-(10-phenothiazyl)-propionic acid can be used as an efficient and low-cost redox mediator for decolorization of synthetic dyes by bacterial laccases. Using this laccase-mediator system, more than 80% of Indigo Carmine and Malachite Green decolorization was reached after 1 h or 2 h of incubation, respectively, both at pH 8 and in tap water (pH 6.8). Furthermore, more than 40% of Remazol Brilliant Blue R and 80% of Xylidine ponceau were decolorized after 5 h at pH 8 and 50°C. In addition, we showed this system supports at least 3 decoloration cycles without loss of activity, representing a promising biological process for cost-effective and environmentally friendly decolorization and degradation of synthetic dyes and for other industrial applications of laccases requiring neutral or alkaline pH.

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Laccases are oxidoreductases with the outstanding ability to oxidize phenolic and non-phenolic substrates coupled to the reduction of O_2 to H_2O. Among them, bacterial enzymes are suitable biocatalysts for application in industrial processes under harsh conditions. However, to be active on high redox potential substrates, bacterial laccases requires of redox mediators: electron carriers between the laccase and other compounds not directly oxidizable by the enzyme. Here we demonstrate that β-(10-phenothiazyl)-propionic acid can be used as an efficient and low-cost redox mediator for decolorization of synthetic dyes by bacterial laccases. Using this laccase-mediator system, more than 80% of Indigo Carmine and Malachite Green decolorization was reached after 1 h or 2 h of incubation, respectively, both at pH 8 and in tap water (pH 6.8). Furthermore, more than 40% of Remazol Brilliant Blue R and 80% of Xylidine ponceau were decolorized after 5 h at pH 8 and 50°C. In addition, we showed this system supports at least 3 decoloration cycles without loss of activity, representing a promising biological process for cost-effective and environmentally friendly decolorization and degradation of synthetic dyes and for other industrial applications of laccases requiring neutral or alkaline pH.

Keywords: Phenothiazine, Bacillus subtilis CotA, Streptomyces ipomoeae SilA, Dye Decolorization, Laccase.

Introduction

Laccases (benzenediol: oxygenoxidoreductases, EC 1.10.3.2) are multicopper oxidoreductases ubiquitous in nature being found in bacteria, fungi, plants, and insects (Janusz et al., 2020). They are active on a wide

range of phenolic and non-phenolic substrates that are oxidized in a mononuclear reactive center formed by a type I copper atom, followed by an intramolecular electronic transfer reaction of 4 electrons to a trinuclear center composed of one type II copper atom and two type III copper atoms, which perform the reduction of O_2 to H_2O (Jones and Solomon, 2015; Mate and Alcalde, 2017). Furthermore, the enzyme activity and range of oxidizable substrates can be enhanced by the presence of redox mediators. Redox mediators are low molecular weight substrates that, after oxidization by the laccase, could generate high redox potential intermediaries that act as electron carriers to non-enzymatically oxidize other compounds that are not direct substrates of the enzyme due to low kinetics or steric issues (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 pollutants (Arregui et al., 2019) and pharmaceuticals (Mluguza et al., 2019), organic synthesis (Kunamneni et al., 2008), in beverage and food industry (Mayolo-Deloisia et al., 2020), and the design of biosensors and biofuel cells (Zhang et al., 2018). However, the success for industrial use of laccases depends on not only on the optimization of the catalytic performance of the system but also of the production efficiency of the enzyme and mediator, which is one of the main barriers to commercialization of LMS.

Many synthetic molecules such as 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 1-hydroxybenzotriazole (HBT), derivatives of lignin such as syringaldehyde, vanillin, ferulic acid, acetosyringone, and p-coumaric acid and fungal metabolites were assayed as redox mediators for fungal and bacterial enzymes (Arregui et al., 2019; Camarero et al., 2005; Cañas and Camarero, 2010; Morozova et al., 2007; Singh and Arya, 2019). Also there are a few reports describing the use of phenoxazines and phenothiazines as a redox mediators for dye decolorization biobleaching of indigo carmine in the denim finishing process and oxidation of bisphenol A by fungal enzymes (Camarero et al., 2005; Ivanec-Goranina et al., 2015; Moldes and Sanromán, 2006; Soares et al., 2001). Despite this, a great effort is being made to find new and better compounds that meet the characteristics of an ideal redox mediator: it must be readily available, stable in its oxidized and reduced forms, non-toxic, affordable and support successive cycles of oxide-reduction without degradation (Morozova et al., 2007; Cañas and Camarero, 2010).

As the first microbial laccases discovered, fungal enzymes have been extensively explored for industrial use, however, bacterial laccases are gaining an increasing importance due to their robustness under harsh conditions. Bacterial laccases are usually more stable at high temperatures and many of them present tolerance to high chloride concentrations -up to 1M- and to diverse solvents (Chauhan et al., 2017; Guan et al., 2018; Janusz et al., 2020). Furthermore, bacterial enzymes show activity at alkaline pH, while fungal laccases are generally active only at acidic pH. These features make bacterial enzymes excellent candidates for the treatment of textile effluents, which usually show high salinity and alkaline pH (Arregui 2019; Yaseen and Scholz, 2019). The release of industrial dyes into watercourses is a major environmental concern. They are usually toxic, mutagenic, and carcinogenic and 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).

In order to find an efficient and environmentally friendly laccase-mediator system (LMS) functional at neutral to alkaline pH, we explored the use of bacterial laccases CotA from *Bacillus subtilis* and SilA from *Streptomyces ipomoeae* along with phenothiazine and N-substituted derivatives (Fig. 1) as redox mediators for decolorization of synthetic dyes containing indigoid, azo, anthraquinone and triphenylmethane moieties (Fig 1) at pH 8 and in tap water (pH 6.8). To the best of our knowledge, this is the first description for the use of phenothiazine and N-substituted derivatives as redox mediators for bacterial laccases. Our results show state-of-the-art decolorization yields using an affordable, scalabe LMS system.

1. **Material and Methods**
2. **Materials**

Trametes versicolor laccase was purchased from Sigma (product # 38429), as a lyophilized powder, resuspended in 1 ml of McIlvaine's buffer at pH 7, and preserved at -20^{o}C in aliquots.

Phenothiazine ([?]98%) and acrylonitrile ([?]99%) were from Sigma-Aldrich. All other chemicals are analytical grade.

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,2H), δ 4.28 (t,2H), δ 6.85 – 7.21 (m,8H).

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 hours. 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_3OD): δ 2.76(t,2H), δ 4.20 (t,2H), δ 6.90 – 7.24 (m,8H).

Phenothiazine (PhZ) was recrystallized from benzene to obtain a yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.85 – 7.30 (m,8H).

Production of recombinant laccases

Recombinant fungal LCC3 from *Trametes trogii* BAFC463 was produced in *Pichia pastoris* as previously described (Campos et al., 2016).

The coding sequence of laccase CotA was amplified from total DNA of*Bacillus subtilis* ATCC 6633 using oligonucleotides CotFw (5´-GGATCCATATGACACTTGAAAAATTTG-3´) and CotRv (5´-AAGCTTATTTATGGGGATCAGTTATATC-3´) and cloned into plasmid pGEMT-easy (Promega) for sequencing (Macrogen Korea). For CotA expression, coding sequence was cloned into vector pPICNHIS (Niderhaus et al., 2018), a derivative of plasmid pPIC9 (Invitrogen Life Technologies) in restriction sites EcoRI/AvrII to obtain vector pPICNHISCotA (Fig. S1a). Plasmid pPICNHISCotA was linearized with BglII and used for the transformation of *P. pastoris* strain SMD1168 (Invitrogen Life Technologies) by electroporation. 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). Production of recombinant CotA was performed 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) as previously described (Campos et al., 2016).

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 cloned in EcoRI/NotI restriction sites into expression vector pHISTEV30a (Fig.S1b) and used for transformation of *E. coli* BL21(DE3). For SilA production, transformed *E. coli* cells were grown in LB broth at 37^{o}C 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, and disrupted by sonication. Cell debris was separated from total proteins by centrifugation.

Purification of recombinant laccases was performed by Ni-NTA affinity chromatography as previously described (Campos et al., 2016).

2.4 Laccase activity assays

Laccase activity was determined by quantification of 5 mM 2,6 dimetoxyphenol (DMP) oxidation at 469 nm (ε = 27.5 mM^{-1} cm^{-1}) in McIlvaine's buffer at room temperature at pH 4 or pH 8 for fungal or bacterial enzymes, respectively. 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.

2.5 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 percentage of decolorization respect to control reactions without the addition of enzyme in the same conditions of pH and temperature. Reactions were performed in triplicates using 50 μM of dye in McIlvaine's buffer at 24°C or 50°C and laccase final concentration of 0.1 or 1 UE/mL. Redox mediators, PhZ, PhCN, and PhCOOH, were used at a ratio of 1:1 (50 μM) or 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 initial concentration of IC (50 μM).

Full UV-visible absorbance spectroscopic scans (200-800 nm) were carried out using a Nanodrop^{TM} 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 and 24°C and control solutions of dyes and PhCOOH without addition of the enzyme.

2.6 Redox potential of dyes and mediators

Solutions of 50 μM of IC were prepared in McIlvaine's buffer at pH 4 and 8, 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.

3 Results and Discussion

3.1 Evaluation of redox mediators for indigo carmine decolorization by laccases.

Since phenothiazine and β-(10-phenothiazyl)-propionic acid were previously reported as substrates and redox mediators for fungal laccases (Camarero et al., 2005; Soares et al., 2001), we decided to evaluate them as redox mediators for bacterial enzymes. Additionally, we tested β-(10-phenothiazyl)-propionitrile, which is an intermediate for the synthesis of PhCOOH and was also reported as a fungal laccase substrate (Kulys et al., 2000).

To test these three synthetic compounds, we first evaluated the improvement in decolorization of indigo carmine (IC), a dye commonly used in the textile industry to produce denim fabrics. We used two recombinant bacterial laccases with different redox potential in T1 Cu, produced in the course of this work: the three-domain CotA enzyme from*Bacillus subtilis* (E° = 0.455 V vs. SHE, Durão et al., 2006)) and the two-domain small laccase SilA from *Streptomyces ipomoeae* (E° = 0.337 V vs. SHE, Blánquez et al., 2019).

The CotA bacterial laccase was cloned from *B. subtilis* and expressed in *P. pastoris* . The biochemical properties of the recombinant enzyme were identical to those previously reported for the enzyme expressed in *E. coli* (Martins et al., 2002), being a 65 KDa monomeric protein, showing optimal conditions for the oxidation of DMP at pH 8.

The *S. ipomoeae* laccase SilA was expressed in *E. coli*using microaerobic conditions. The recombinant enzyme showed optimal activity at pH 8 for the oxidation of DMP and an apparent molecular weight of 75-80 KDa

under non-denaturing conditions. In contrast, in the Western blot of SDS-PAGE, only monomeric forms of 39-40 KDa were detected as previously described (Molina-Guijarro et al., 2009).

Decolorization of IC by bacterial laccases showed an increase in the presence of PhCOOH and PhZ, concerning treatments with the enzyme alone. In the absence of redox mediators, IC decolorization by SilA reached a 19% after 5 h incubation at 24°C, while in the presence of PhCOOH or PhZ in a 1:1 ratio with dye, it reached 84% and 55% in less than 1 h of incubation, respectively (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, it reached > 90% in less than 1 h of incubation. In the presence of PhZ, it showed a maximum of 75-80% of decolorization after 1 h of incubation (Fig. 2B).

The performance of the LMS using PhCOOH was similar and even higher to the reported for IC decolorization by CotA and SilA bacterial laccases in the presence of other frequently used redox mediators. Laccase SilA managed to decolorize 93% to 98% of IC at pH 8 in the presence of the lignin derivatives acetosyringone, syringaldehyde and methyl syringate but after longer incubation times (24 h) at higher temperature (35°C) and using a higher mediator:dye ratio (2:1) (Blánquez et al., 2019). Also, previous reports showed 99% of IC decolorization by *B. subtilis* CotA after 1 h incubation at pH 7 and 40°C in the presence of 100 µM acetosyringone (Wang and Zhao, 2017).

Regarding the compound PhCN, it did not show any effect on IC decolorization at pH 8 by SilA or CotA. A possible explanation could be the inhibition of the bacterial laccases by the nitrile moiety, since inhibition by cyanide has been reported for bacterial enzymes (Molina Guijarro et al., 2009; Endo et al., 2003). However, no differences in the decolorization process were observed between reactions with the enzymes alone or in presence of PhCN (Fig. 2). Therefore, the inability of PhCN to enhance IC decolorization is likely to be attributed to low kinetics between bacterial enzymes and mediator rather than to an inhibitory effect. Furthermore, assays performed using medium to high redox potential fungal laccases, showed that PhCN can function as an efficient redox mediator for IC decolorization by these enzymes. Both PhCN and PhCOOH, achieved more than 98% of IC decolorization in less than 30 min of incubation at pH 4 with the LcTv enzyme from *Trametes versicolor* and after 1 h of incubation in the presence of the thermostable LCC3 enzyme from *Trametes trogii* (Fig. S2).

To test the stability of bacterial laccase-mediator systems, we performed three consecutive cycles of IC decolorization. Although in the presence of PhZ, IC decolorization decreased by almost 50% after the first cycle, reactions in the presence of PhCOOH with CotA or SilA reached the same rates after each cycle (Fig. 3), indicating that this compound practically acts as an ideal mediatior, recovering its redox capacity after at least 3 cycles of decolorization.

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

Although bacterial laccases are reported to show lower redox potentials at T1 Cu than fungal enzymes, here we observed a similar IC decolorization efficacy by both groups of enzymes at their optimal pH in the presence of PhCOOH and PhZ. The fact that IC redox potential is pH dependant, decreasing its value as the pH increases, could in part, explain why at acidic pH it is directly oxidized only by fungal enzymes with medium to high redox potential at T1 Cu (E° 0.46-0.79 V vs. SHE, Mate and Alcalde, 2017) while at pH 8 it is susceptible of oxidation by laccases of bacterial origin showing T1 Cu $E^\circ < 0.46$ V vs. SHE (Mate and Alcalde, 2017). However, 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. The mechanism and the oxidation products depend on the type of derivative and the pH of the medium (Cheng et al., 1978; Puzanowska-Tarasiewicz et al., 2005). Similarly 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. 4).

PhZ, PhCN and PhCOOH were characterized by differential pulse voltammetry, an electrochemical technique

that allows in a simple way to establish the formal potentials of each species at the working conditions (Fig.5).

PhCOOH was the most efficient of the three species since it can mediate the oxidation of indigo carmine at both pH and with all the laccases. This could be related to the redox potential of the prevalent oxidized form at each pH: PhCOOH present formal potentials of 0.75 V and 0.68 V vs. SHE for the first oxidation process and predominant form at pH 4 and 8, respectively; in both cases close to or even higher than formal potentials of IC with values of 0.76 V and 0.55 V vs. SHE at pH 4 and 8, respectively.

For PhZ, even though it shows a formal potential of 0.64 V vs. SHE at pH 4, the incubation with the enzyme generates another colored compound with an absorption peak at 560 nm, that could be related to the major pathways observed in phenothiazine degradation once is oxidized, limiting its use for dye decolorization (Sackett et al., 1981). On the other hand, at pH 8 oxidized species with a formal potential of 0.46 V (PhZ^+/PhZ) can be formed and then further oxidized by the bacterial laccase generating a stronger oxidizing species (PhZ^{2+}). 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^+$$Lac_{(red)}$ + PhZ^{2+}

can be displaced toward products since the oxygen consume the reduced laccase immediately while the indigo carmine reacts immediately with the PhZ^{2+} regenerating the reactants. In this way, the Nernst thermodynamic equation:

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

presents a very small ratio between products and reactants; 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). Finally, PhCN resulted well suited for IC oxidation at pH 4, but not at pH8. For this compound, it seems that at pH 4 the predominant form is the second oxidation product with a formal potential of 0.85 V vs. SHE the responsible for IC oxidation. The redox potential difference between this mediator (PhCN) and the fungal laccase is around 0.1 V, its difference can be reverted by the compound concentrations involved in the reaction (eq. 1).

3.5 Decolorization of azoic, anthraquinone and triphenylmethane dyes by SilA-PhCOOH system at pH 8.

Since PhCOOH was the most suitable redox mediator for IC decolorization by bacterial enzymes, we evaluated it for the oxidation of remazol brilliant blue R (RBBR, Reactive Blue 19), xylidine ponceau (XYL, Acid Red 26) and malachite green (MG, Basic Green 4), representing the three main groups of recalcitrant industrial dyes used worldwide: azoic, anthraquinone and triphenylmethane. Finding efficient methods for removal of these dyes is of main 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; Shing et al., 2015a). Additionally, oxidation of MG could prevent its metabolic reduction to leucomalachite, an equally carcinogenic form but with greater persistence in fish tissues (Hashimoto et al., 2011; Srivastava et al., 2014).

Following the idea of developing a low-cost LMS, we conducted these dye decolorization assays using SilA laccase. SilA was expressed intracellularly as a soluble protein in *E. coli,* a well-known, fast and cost-efficient recombinant protein production platform (Rosano and Ceccarelli, 2014). The active enzyme was recovered in two simple steps of centrifugation and cell disruption without the need to incorporate any additional purification, since we observed the same decolorization performance with the enzyme purified by affinity chromatography or with the crude extracts, simplifying the downstream processing.

The decolorization efficiency was different for each dye, but in all cases it showed an increase in the presence of PhCOOH and at 50°C, according to the optimal temperature range for SilA. Laccase SilA alone was not able to significantly decolorize XYL at room temperature, however, it reached a 34% of decolorization after 5 h of incubation at 50°C (Fig. 6 a,d). In the presence of PhCOOH an enhancement in decolorization was

observed reaching 86% after 5 h at 50°C (Fig. 6d) and 66% after 24 h at room temperature (Fig. 6a), showing the ability of SilA-PhCOOH LMS to oxidize monoazo XYL. Although decolorization of XYL by different fungal laccases at acidic pH has been reported (Campos et al., 2016; Levin et al., 2005; 2012), there is only one recent report of oxidation by bacterial enzymes 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 et al., 2020), being an attractive candidate to be evaluated with PhCOOH. On the other hand, laccase SilA has been successfully used for decolorization of other azoic dyes such as Reactive Black 5 and Orange II (˜90% of decolorization after 24 h at pH 8 and 35°C) in the presence of 0.1 mM acetosyringone or methyl syringate as redox mediators, while for Acid Orange 63 o Tartrazine decolorization was not higher than 22% in the same incubation conditions. Also, in all these cases, the use of syringaldehyde was less efficient than acetosyringone and methyl syringate (Blánquez et al., 2019)\sout.

Of the dyes analyzed, RBBR resulted to be the most recalcitrant to degradation by SilA or SilA-PhCOOH. Less than 20% of decolorization was reached by the SilA alone either at RT or 50°C, however in the presence of PhCOOH it increased to 51% after 24 h at RT and 42% after 5 h at 50°C (Fig 6 b,e and Table S1). The efficiency of degradation of this dye by other bacterial laccases and LMS was very variable (Table S1). Small laccase SLAC from *Streptomyces coelicolor* reached only 23% of decolorization after 5 h at pH 9 and 45°C, without improvement in the presence of redox mediators (Dubé et al., 2008), while laccases from *B. subtilis* cjp3, *B. licheniformis* LS04 reached almost 100% of decolorization after 6 h of incubation at pH 9 in the presence of 0.1 mM acetosyringone as redox mediator (Qiao et al., 2017; Lu et al., 2013). On the other hand, laccases from thermophilic bacteria from *Thermus* genus showed high decolorization of RBBR even in the absence of redox mediators (Liu et al., 2015; Navas et al., 2020) (Table S1).

Regarding tryphenylmethane dyes, SilA was highly efficient for decolorization of MG reaching 80% and 93% after 3 h of incubation at RT with the enzyme alone or in the presence of PhCOOH, respectively (Fig. 6 c). When incubations were performed at 50°C, the final decolorization percentages were similar to RT, however the kinetics of the reaction was faster, since more than 80% of decolorization was reached in the first 30 min (Fig. 6 f). Although there are many descriptions of MG degradation by microbial reductases or oxidation by fungal laccases at acidic pH, few describe the oxidation by bacterial laccases. As observed for other dyes, the decolorization efficiency was variable, depending on the enzyme source and the redox mediator used (Table S1). While the laccase of *Klebsiella pneumoniae* reached a fast MG decolorization at pH 7.5 and 70°C even in the absence of redox mediators (Lui et al., 2017), laccases from *Bacillus vallismortis*or *Bacillus* sp. KC2 reached about 80% of MG decolorization only in the presence of ABTS but less than 40% in the presence of other redox mediators and less than 10% with the enzyme alone (Asadi et al., 2020; Zhang et al., 2013) .

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

In order to evaluate the efficiency of SilA-PhCOOH system for dye decolorization in mild conditions, we performed the treatments in tap water (pH 6.8) at room temperature (24°C).

More than 80% of IC decolorization was reached in the first 5 min of incubation in tap water at 24°C in the presence of PhCOOH (Fig 7a), showing that our system has the potential to be used for IC degradation in mild conditions. PhCOOH has been reported to be the mediator present in a the commercial formulation DeniLite® of Novozymes for bleaching of indigo carmine. Since it is composed of a fungal recombinant laccase, its use is recommended at pH below 6 and temperatures of 50°C (Colomera and Kuilderd, 2015; Soares et al., 2001), thus, our SilA-PhCOOH system could be an alternative for the biobleaching of denim fabrics in neutral to alkaline pH conditions. Furthermore, it was reported that the use of laccases could reduce the undesired effect of re-deposition or backstaining of indigo dye on white yarns after the bio-stone washing of denim garments with cellulases. Both acid and neutral cellulases are alternatively used for stone washing, but the combined use of cellulases with laccases functional at neutral pH could contribute to reduce the problem of backstaining as this process is high at pH 4-6 but significantly lower at neutral pH (Campos et al., 2001; Montazer and Sadeghian, 2010).

Regarding MG, 92% decolorization was reached after 5 h of incubation in the presence of PhCOOH (Fig

7b). This result is of remarkable importance since MG is not only used as a dye for silk, leather, and paper, but also as a parasiticide and antifungal agent in aquaculture (Hashimoto et al., 2011).

For XYL and RBBR a decrease in the decolorization was observed in tap water compared to treatments at pH 8, 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. 7 c,d).

3.7 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 lower molecular weight, which can be further degraded by chemical processes (Levin et al. 2012; Navas et al., 2020; Osma et al., 2010; Yang et al., 2015). As an approach to confirm this mechanisms in SilA-PhCOOH LMS, we performed full scan of UV-visible absorption spectra of decolorized reactions after 24 h of incubation. In all of the cases, we observed the disappearance of the main absorbance peak of each dye due to cleavage of the chromophore, and the increase in the absorbance in the range of 200 to 400 nm indicating the formation of aromatic compounds of lower molecular weight (Fig S3). 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 de LMS (Kandelbauer et al., 2008; Wang et al., 2017).

Although these smaller molecules formed after oxidation by the laccase have shown lower toxicity levels than the dye (Legerska, 2016), further studies are needed to identify the decomposed substances generated by the SilA-PhCOOH system.

3.8 Comparative costs of redox mediators

Most of reported dye decolorizations by bacterial LMS use ABTS, ASG or syringaldehyde as redox mediators, while in paper pulp proceses the use of 1-hydroxybenzotriazole (HBT) is most common, however the costs of each compound are quite different.

Based on the prices by the same supplier (Sigma Aldrich), ABTS and ASG are the most expensive: ABTS is about 19,600 USD/mol and ASG is USD 3,500 USD/mol. On the other hand we estimated a cost of 150 USD/mol for the synthesis of PhCOOH from PhZ (PhZ: 17USD/mol), using analytical grade materials. This value is even lower that the cost of the other commonly used redox mediators syringaldehyde (460 USD/mol) and HBT (230 USD/mol), adjusting to the concept of low-cost mediator (Morozova et al 2007).

Conclusions

The comparative analysis of dye decolorization by laccase and LMS shows the high variation in the efficiency of each system, depending on the source of the enzyme, the redox mediator and the reaction conditions, highlighting the difficulty in predict the oxidation of complex substrates and the need to carry out an experimental optimization of the systems, according to the main components of each industrial process or effluent to be treated. Our results demonstrate that PhCOOH can be efficiently used as redox mediator for IC oxidation by bacterial laccases at alkaline pH without inhibition of the enzyme or loss of activity in at least 3 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. 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 (see Table S1). In addition, the SilA-PhCOOH system could be valuable for other industrial processes such as delignification of alkaline paper pulp, biobleaching of indigo dye and reduction of backstaining in denim finishing process and for the design of biosensors and biofuel cells functional at alkaline or physiological pH.

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Figure captions

Fig. 1. Structures and acronyms of phenothiazine and N-substituted derivatives used as redox mediators and dyes tested in this work.

Fig. 2. Decolorization of 50 μM Indigo Carmine by of 0.1UE/mL of laccase SilA (A), and CotA (B) was assayed without mediator (triangles) or the presence of 50 μM PhZ (squares), PhCN (diamonds) or PhCOOH (circles) at 24°C in McIlvaine's buffer pH 8. Decolorization is represented as % respect to absorbance at t_0. Error bars correspond to the standard deviation for triplicates.

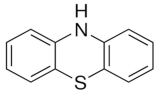
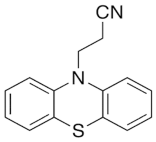
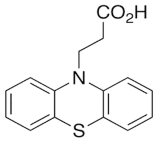
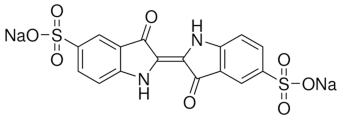
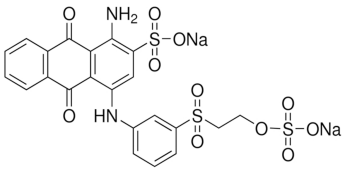
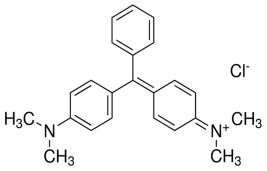
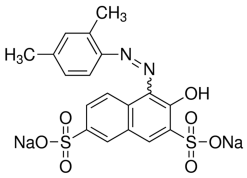
Fig. 3. Decolorization of 50 μM Indigo Carmine by of 0.1UE/mL of laccase SilA (a) and CotA (b) in the presence of 50 μM of each redox mediator at 24°C in McIlvaine's buffer pH 8. Decolorization reactions were performed in three consecutive cycles (I, II, III) of 1 h of incubation by adding concentrated fresh dye solutions to reach initial absorbance at the beginning of each cycle. Decolorization is represented as % respect to absorbance at t_0. Error bars correspond to the standard deviation for triplicates.

Fig. 4. Oxidation process of phenothiazine derivatives.

Fig. 5. Differential pulse voltammetries for PhZ (continuous line), PhCN (dashed line) and PhCOOH (dotted line). Experiments carried out in McIlvaine's buffer at pH 4 (a) and pH 8 (b).The vertical lines indicate the formal potential of IC at the corresponding pH.

Fig. 6 Decolorization of 50 μM XYL (a and d), RBBR (b and e), and MG (c and f) by 1UE/mL of laccase SilA was assayed in the absence (triangles) or the presence of 250 μM PhCOOH (circles) at 24°C (a,b,c) or 50°C (d,e,f) in citrate-phosphate buffer pH 8. Decolorization is represented as % respect to absorbance at t_0. Error bars correspond to the standard deviation for triplicates.

Fig. 7. Decolorization of 50 μM IC (a), MG (b), XYL (c) and RBBR (d) by of 1UE/mL of laccase SilA was assayed in the absence (triangles) or the presence of PhCOOH (circles) at 24°C in tap water. Decolorization is represented as % respect to absorbance at t_0. Error bars correspond to the standard deviation for triplicates.

MEDIATORS	 Phenothiazine **PhZ**	 β-(10-phenothiazyl)-propionitrile **PhCN**	 β-(10-phenothiazyl)-propionic acid **PhCOOH**
DYES	 Indigo Carmine (Acid Blue 74) **IC**	 Remazol Brilliant Blue R (Reactive Blue 19) **RBBR**	
	 Malachite Green (Basic Green 4) **MG**	 Xylidine Ponceau (Acid Red 26) **XYL**