Fast decolorization of azo dyes in alkaline solutions by a thermostable metal-tolerant bacterial laccase and proposed degradation pathways

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Abstract
Biocatalytic decolorization of azo dyes is hampered by their recalcitrance and the characteristics of textile effluents. Alkaline pH and heavy metals present in colored wastewaters generally limit the activity of enzymes such as laccases of fungal origin; this has led to an increasing interest in bacterial laccases. In this work, the dye decolorization ability of LAC_2.9, a laccase from the thermophilic bacterial strain *Thermus* sp. 2.9, was investigated. Its resistance towards different pHs and toxic heavy metals frequently present in wastewaters was also characterized. LAC_2.9 was active and highly stable in the pH range of 5.0 to 9.0. Even at 100 mM Cd²⁺, As⁵⁺ and Ni⁰⁺ LAC_2.9 retained 99%, 86% and 75% of its activity, respectively. LAC_2.9 was capable of decolorizing 98% of Xylidine, 54% of RBBR, 40% of Gentian Violet, and 33% of Methyl Orange after 24 h incubation at pH 9, at 60 °C, without the addition of redox mediators. At acidic pH, the presence of the mediator 1-hydroxybenzotriazole generally increased the catalytic effectiveness. We analyzed the degradation products of laccase-treated Xylidine and Methyl Orange by capillary electrophoresis and mass spectrometry, and propose a degradation pathway for these dyes. For its ability to decolorize recalcitrant dyes, at pH 9, and its stability under the tested conditions, LAC_2.9 could be effectively used to decolorize azo dyes in alkaline and heavy metal containing effluents.

Keywords Thermostable bacterial laccase · *Thermus* sp. 2.9 · Azo dye · Decolorization

Abbreviations
RBBR Remazol Brilliant Blue R
HBT 1-Hydroxybenzotriazole
ABTS 2,2’-Azino-di-[3-ethylbenzthiazoline sulfonate]
pHBA Para-hydroxybenzoic acid
CE Capillary electrophoresis

Introduction
Laccases are multicopper oxidases (MCO) that couple the oxidation of a wide range of substrates with the reduction of molecular oxygen to water. Due to their ability to oxidize phenolic compounds, laccases can act on a variety of persistent environmental pollutants present in the waste of several industrial processes such as dyes from textile effluents (Singh et al. 2015; Unuofin et al. 2019). The colored effluents discharged into water bodies can cause an increase in biochemical oxygen demand (BOD) and chemical oxygen demand (COD) and decrease the transmittance of light, reducing the photosynthetic activity in these ecosystems. Additionally, the release of dyes into the environment is toxic to living organisms (Collivignarelli et al. 2019). The
degradation processes in the treatment plants generally remove a low percentage of dye content and often result in by-products of greater toxicity, such as carcinogenic amines from azo dyes (Collivignarelli et al. 2019; Zaharia and Suteu 2012). Thus, the application of laccases for decolorization and detoxification of effluents from the textile industries is particularly attractive. The use of low-molecular weight compounds as redox mediators can broaden their oxidation range by forming stable radicals that may continue oxidizing large molecules and non-phenolic substrates (Blánquez et al. 2019).

There are many reports on fungal extracts and purified laccases combined with synthetic or natural mediators capable of decolorizing several groups of textile dyes (Unuofin et al. 2019). Fungal laccases are typically maximally active at acidic pH, which limits their use in textile dyes-containing effluents that are generally alkaline due to the presence of auxiliary dyeing compounds (Kokol et al. 2007). In these conditions, bacterial laccases show more potential compared with fungal laccases, but still need further research (Blánquez et al. 2019; Guan et al. 2018; Ma et al. 2018). Decolorization of azo dyes by bacteria has been previously described (Blánquez et al. 2019; Garg and Tripathi 2016; Mandic et al. 2019) and potential degradation pathways have been identified (Thakur et al. 2014; Xia et al. 2019). Despite this, information on the mechanism of azo dye degradation by purified bacterial laccases is limited. Pereira et al. (2009) proposed a mechanism for decolorization of the azo dye, Sudan Orange G, by CotA-laccase from Bacillus subtilis which uses radical coupling reactions that result in oligomers and potential polymers. Degradation of the azo dye Reactive blue 52 by laccase 12B from Bacillus amyloliquefaciens expressed in Escherichia coli was monitored by HPLC–DAD and the resulting precipitate was analyzed by FTIR spectroscopy. The solution contained a unique product peak without chromophore, while the aggregates (assigned to a polymeric form of the dye) retained the blue color (Loncar et al. 2013). Recently the mechanism for Evans blue decolorization by a mutant of CotA-laccase from Bacillus pumilus expressed in Pichia pastoris was analyzed through liquid chromatography–mass spectrometry; the azo bond (–N=N–) was transformed into N₂ instead of toxic aniline compounds, in which water was the only by-product in the degradation process. Moreover, biological toxicity test showed that this laccase could efficiently detoxify Evans blue (Xia et al. 2019).

We have previously reported the expression and biochemical characterization of a bacterial laccase (LAC_2.9) from the thermophilic strain Thermus sp. 2.9 and evaluated its ability to modify lignocellulosic biomass (Navas et al. 2019). In this work, we investigated the decolorization ability of LAC_2.9 on different types of dyes, and the effect of two redox mediators in the process. We also characterized the resistance of the enzyme towards different pHs, and toxic heavy metals frequently present in wastewaters. Additionally, insights into the mechanism of LAC_2.9 action on the azo dyes Xylidine and Methyl Orange are presented.

**Materials and methods**

**Enzyme preparation**

Recombinant LAC_2.9 was expressed in E. coli and purified as described previously (Navas et al. 2019). Briefly, genomic DNA from Thermus sp. 2.9 was used to amplify the gene coding for LAC_2.9 fused to a N-terminal 6xHis tag by PCR. The product was cloned into vector pJexpress 404 (DNA2.0) and the recombinant plasmid was transformed into E. coli. Expression of LAC_2.9 was induced by adding IPTG (1 mM final) to the LB medium when the culture reached an OD₆₀₀ of 0.8 together with CuSO₄ (0.5 mM final). The soluble LAC_2.9 was purified using a Ni–NTA affinity column. Purified LAC_2.9 activity was detected colorimetrically measuring the absorbance at 436 nm (Ɛ = 36,000 M⁻¹cm⁻¹) of oxidized ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]). Assays were performed at 60 °C and contained 1 mM CuSO₄ and 3 mM ABTS in Britton-Robinson buffer pH 5.0 (Mongay and Cerdà 1974). One unit (U) of laccase activity was defined as the amount of enzyme needed to oxidize 1 μmol of ABTS in 1 min.

**pH stability and effect of metal ions on activity of LAC_2.9**

To evaluate the effect of cations on LAC_2.9, each one of Cd(NO₃)₂, Cr(NO₃)₃, Pb(NO₃)₂, NiSO₄ and Na₂HAsO₄ was added at final concentrations of 1, 10 and 100 mM, to the reaction conducted at pH 5.0 and containing 1 mM Cu SO₄, which was taken as control for 100% activity. The effect of CuSO₄ at 10 and 100 mM was also examined. The pH stability of LAC_2.9 was evaluated by determining the residual activity of the enzyme after 24 h incubation at 25 °C in Britton-Robinson buffer at pHs 5.0, 7.0 and 9.0, using ABTS as substrate.

**In vitro decolorization of dyes by LAC_2.9**

The decolorization assays were performed in Britton-Robinson buffer at three pH values (5.0, 7.0 and 9.0) in 0.5 ml volume containing the dye (at the concentration required for each dye to obtain 1.0 absorbance units at its maximum wavelength), 1 mM CuSO₄, and 0.075 U of purified LAC_2.9. When added to the reaction, the redox mediator [1-hydroxybenzotriazole (HBT) or para-hydroxybenzoic acid (pHBA), Sigma] was set to 0.5 mM. Reactions were incubated at 60 °C. Absorbance was measured after
6 and 24 h for reactions containing the following dyes, at the indicated wavelengths: azo dyes, Xylidine (Ponceau 2R, \(\lambda_{\text{max}} = 505 \text{ nm}\)) and Methyl Orange (\(\lambda_{\text{max}} = 460 \text{ nm}\)); triphenylmethane dyes, Malachite Green (\(\lambda_{\text{max}} = 618 \text{ nm}\)) and Gentian Violet (\(\lambda_{\text{max}} = 590 \text{ nm}\)), the antraquinone dye, Remazol Brilliant Blue R (RBBR) (\(\lambda_{\text{max}} = 590 \text{ nm}\)); and the indigoid dye Indigo Carmine (\(\lambda_{\text{max}} = 608 \text{ nm}\)). Controls without laccase were conducted in parallel. All the reactions were performed in triplicate. Decolorization activity was calculated: % Decolorization = 
\[
\frac{(I - F)}{I} \times 100,
\]
where \(I\) = initial absorbance and \(F\) = absorbance of decolorized reaction (Parshetti et al. 2006).

**Electrochemical measurements**

Electrochemical experiments were carried out with a potentiostat (TEQ, Argentina), provided with a digital signal generator for implementation of different electrochemical techniques. A glassy carbon working electrode (0.25 cm² area), an Ag/AgCl/KCl 3 M reference electrode (BAS) and a platinum wire auxiliary electrode were used for differential pulse polarography (DPP). DPP experiments were carried out using a potential ramp starting at 0.0 V and going up to +0.800 V or +0.900 V (vs Ag/AgCl) in a pulsed manner. The following experimental parameters were employed: potential step = 0.5 mV, pulse width = 0.06 s, pulse amplitude = 50 mV, and pulse period = 0.2 s.

**Capillary electrophoresis–mass spectra analysis**

Laccase or laccase/HBT decolorized azo dyes (after 24 h incubation at pH 7) were analyzed by capillary electrophoresis (CE) and mass spectra (MS) and compared with the control without the enzyme. Initial dye concentration was respectively 33.6 mg l⁻¹ and 18.3 mg l⁻¹ for Xylidine and Methyl Orange after 24 h incubation without the addition of redox mediators at pH 9. Activity at this pH demonstrates the potential for treatment of wastewater effluents.

Indigo Carmine was from ICN (Costa Mesa, CA, USA), Malachite Green from Mallinckrodt (Phillipsburg, NJ, USA). All other chemicals were from Sigma (St. Louis, MO, USA).

**Statistical analysis**

The data presented are mean values of triplicate assays with a standard error of less than 5%. Infostat software (Di Rienzo et al. 2017) was used to analyze the variance. The significant differences among treatments were compared by Tukey’s multiple range test at 5% level of probability.

**Results and discussion**

**In vitro decolorization of dyes by LAC_2.9**

In this paper, the decolorization of two azo dyes with diverse structural configurations, two triphenylmethane dyes, an antraquinonic dye and an indigoid dye was investigated at three different pHs using purified LAC_2.9. The dyes were decolorized with variable efficiency depending on their structural class and the pH of the reaction (Table 1). Results at pHs 7 and 9 are not shown for Indigo Carmine and Malachite Green as both dyes were decolorized at these pHs in the control reactions without enzyme. LAC_2.9 fully decolorized the dye Indigo Carmine without the addition of redox mediators after 6 h at pH 5. The optimal pH for the decolorization activity of LAC_2.9 depended on the dye assayed. For example, LAC_2.9 displayed higher activity at acidic pH with Methyl Orange, and at alkaline pH with Xylidine. The results showed that at acidic pH, the presence of HBT increased the catalytic effectiveness better than pHBA, which only improved LAC_2.9 Gentian Violet decolorization efficiency at pHs 7 and 9 (Table 1). Nevertheless, LAC_2.9 was capable of decolorizing 98% of Xylidine, 54% of RBBR, 40% of Gentian Violet, and 33% of Methyl Orange after 24 h incubation without the addition of redox mediators at pH 9. Activity at this pH demonstrates the potential for treatment of wastewater effluents.
The purified recombinant bacterial laccase SilA from *Streptomyces ipomoeae* CECT 3341 was also active over wide temperature and pH ranges and it decolorized dyes of diverse chemical structures: 30, 14 and 10% of the azoic dyes Reactive Black 5, Orange II and Tartrazine, and 12% of the triphenylmethane dye Cresol Red after 24 h incubation at 35 °C and pH 8 (Blánquez et al. 2019), but this enzyme could not decolorized the azoic dye Acid Orange 63 and the anthraquinonic Acid Black 48 without the addition of redox mediators. The recombinant laccase from *Streptomyces cyaneus* was also incapable of decolorizing the azo dye Orange II (Moya et al. 2010). However, a thermostable laccase from *Thermus thermophilus* SG0.5JP17-16 expressed in *P. pastoris* decolorized 100%, 94%, 94% of the azoic dyes Congo Red, Reactive Black B and Reactive Black WNN, respectively, and 73% of the anthraquinonic RBBR, after 24 h incubation at 70 °C and pH 7.5 (Liu et al. 2015). The purified laccase from *Geobacillus thermocatenulatus* decolorized 60% of Congo Red after 6 h of incubation and up to 99% after 32 h of incubation (Verma and Shirkot 2014).

Regarding the mono-azo dyes with different molecular configuration that were assayed, Xyline was decolorized more efficiently than Methyl Orange by purified LAC_2.9, with 98% decolorization attained at pH 9 after 24 h of incubation (Table 1). Decolorization of Methyl Orange was most efficient at pH 5, in presence of HBT (68% after 24 h). The structural heterogeneity influences the charge density distribution of the dye’s molecules and hence their redox potentials (Ciullini et al. 2008). In turn, the relationship between the redox potential of the laccase and the dye substrate is a critical factor in the decolorization process of these compounds. Fungal laccases with high redox potential can oxidize a wide range of substrates; but bacterial laccases usually possess low and medium redox potentials [i.e. SilA of *S. ipomoeae*, 337 mV (Blánquez et al. 2019); *B. subtilis* CotA, 455 mV (Pereira et al., 2009)] limiting their oxidative ability. Nevertheless, the redox potential of the *Stenotrophomonas maltophilia* AAP56 laccase was determined at 638 mV (Galai et al. 2014). The best efficiency in Xyline decolorization at alkaline pHs can be explained by the shift of dye redox potential from 750 mV (at pH 5) to 530 mV (at pHs 7 or 9), thus decreasing the need of HBT addition (HBT redox potential 710 mV) (Supplementary Material, Fig. 1a-f). Therefore, the enzymatic oxidation of Xyline

| Dye                  | Treatment     | pH 5 | pH 7 | pH 9 |
|----------------------|---------------|------|------|------|
|                      |               | 6 h  | 24 h | 6 h  | 24 h | 6 h  | 24 h |
| Methyl Orange        | LAC_2.9       | 23.50±1.82 | 50.95±1.35 | 20.64±1.73 | 24.36±1.00 | 19.58±1.74 | 33.45±2.14 |
|                      | LAC_2.9 + HBT | 61.55±2.24 | 68.03±2.37 | 24.10±2.04 | 36.06±1.05 | 28.67±2.01 | 39.19±1.44 |
|                      | LAC_2.9 + pHBA| 31.67±1.67 | 52.92±2.59 | 26.76±3.79 | 37.22±3.00 | 27.60±3.44 | 37.32±2.42 |
| Xyline               | LAC_2.9       | 34.31±2.34 | 59.96±1.15 | 52.72±5.10 | 81.23±2.53 | 72.06±1.93 | 97.93±2.69 |
|                      | LAC_2.9 + HBT | 47.19±2.13 | 86.04±3.94 | 51.75±2.04 | 80.97±0.95 | 75.50±1.03 | 99.36±0.76 |
|                      | LAC_2.9 + pHBA| 36.72±1.74 | 63.33±1.46 | 44.56±2.23 | 68.28±1.51 | 69.04±0.69 | 91.10±0.71 |
| Gentian Violet       | LAC_2.9       | 12.46±1.71 | 30.96±1.50 | 22.97±1.69 | 36.57±1.12 | 30.40±1.39 | 40.07±2.02 |
|                      | LAC_2.9 + HBT | 55.35±1.37 | 73.86±2.05 | 24.71±1.27 | 50.70±2.10 | 28.92±0.98 | 44.26±0.59 |
|                      | LAC_2.9 + pHBA| 21.82±1.16 | 54.14±2.04 | 40.07±1.70 | 59.16±1.65 | 52.73±0.75 | 53.49±2.68 |
| Malachite Green      | LAC_2.9       | 52.31±2.68 | 71.59±2.40 | 22.97±1.69 | 36.57±1.12 | 30.40±1.39 | 40.07±2.02 |
|                      | LAC_2.9 + HBT | 82.35±0.72 | 82.35±0.29 | 24.71±1.27 | 50.70±2.10 | 28.92±0.98 | 44.26±0.59 |
|                      | LAC_2.9 + pHBA| 52.47±2.27 | 73.21±1.82 | 40.07±1.70 | 59.16±1.65 | 52.73±0.75 | 53.49±2.68 |
| RBBRX                | LAC_2.9       | 34.61±1.82 | 51.28±1.35 | 55.99±1.84 | 66.48±1.03 | 53.61±1.93 | 54.04±2.69 |
|                      | LAC_2.9 + HBT | 36.72±1.24 | 56.10±2.37 | 55.40±2.78 | 64.22±3.14 | 54.72±1.03 | 58.44±0.76 |
|                      | LAC_2.9 + pHBA| 34.36±1.61 | 51.91±2.59 | 41.56±2.48 | 58.26±2.99 | 48.05±0.69 | 57.44±0.71 |
| Indigo CarmineX      | LAC_2.9       | 97.90±2.16 | 99.10±1.06 | 97.90±2.16 | 99.10±1.06 | 99.60±0.98 | 99.60±0.98 |
|                      | LAC_2.9 + HBT | 99.10±1.06 | 99.60±0.98 | 97.90±2.16 | 99.10±1.06 | 99.60±0.98 | 99.60±0.98 |

The significant differences among treatments with laccase or laccase-mediator systems (1); and among pHs assayed (2) were compared by Tukey’s multiple range test at 5% level of probability

1) Means superscripted with the same symbol are not significantly different (p < 0.05)
2) Means superscripted with the same letter are not significantly different (p < 0.05)
XMeans not significantly different among treatments (p < 0.05)
is favored when Xylidine ortho hydroxyl group is deprotonated. Conversely, when Methyl Orange is oxidized by LAC_2.9, the redox mediator contributes by reducing the redox potential of the substrate (from 580 to 530 mV) (Supplementary Material, Figure 1g, h).

**pH-stability of LAC_2.9 and effect of metal ions on activity**

The pH optimum of activity of LAC_2.9 varies depending on the substrate, e.g. 5.0 for ABTS, and 6.0 for 2,6-dimethoxyphenol (DMP) (Navas et al. 2019). To evaluate the pH stability of LAC_2.9 the activity of the enzyme was determined after 24 h incubation at room temperature at three different pHs (5.0, 7.0, 9.0). Percentages of relative activity were respectively 90.3 ± 8.3, 101.8 ± 2.1 and 94.4 ± 17.0. The high residual activity of the enzyme at the three pHs was not affected by enzyme instability in the reaction through incubation time.

The effect on the activity of LAC_2.9 of several metal ions frequently present in textile effluents was also evaluated. Each of these was assayed at concentrations 1, 10 and 100 mM (Table 2). When assaying acidic cations such as Cr³⁺ and Pb²⁺ at high concentrations a precipitate was formed (Burriel Martí et al. 2002), which prevented spectrophotometric determination. Neither of the other metal ions tested showed a remarkable inhibitory effect on laccase activity up to a concentration of 10 mM. Even at 100 mM LAC_2.9 retained 99%, 86% and 75% of its activity with Cd²⁺, As⁵⁺ and Ni²⁺ respectively, but only 31% with Cu²⁺. Earlier reports also showed that Cu²⁺ can inhibit substrate conversion by laccase (Kim and Nicell 2006) possibly through interruption of the enzyme electron transport system (Torres et al. 2003).

These results supply more evidence of LAC_2.9 robustness for industrial applications. The thermal stability of LAC_2.9 was assessed previously (Navas et al. 2019).

### Table 2 Effect of metal ions on the activity of LAC_2.9 laccase

| Metal ion | % Relative activity¹ |
|-----------|----------------------|
|           | 1 mM | 10 mM | 100 mM |
| Cu²⁺      | 100.0 | 90.9±9.2 | 31.2±4.8 |
| Cd²⁺      | 100.7±6.4 | 102.9±6.0 | 98.9±4.6 |
| Cr³⁺      | 100.1±18.2 | ND | ND |
| Pb²⁺      | 105.6±4.0 | 96.0±5.4 | ND |
| Ni²⁺      | 97.3±9.8 | 97.1±6.7 | 74.7±7.3 |
| As⁵⁺      | 107.0±5.9 | 101.2±5.6 | 85.8±6.3 |

ND a precipitate was formed, which prevented spectrophotometric determination

¹Laccase activity in the presence of 1 mM Cu²⁺ was set to 100%

After 16 h at 60 °C LAC_2.9 showed no decrease of activity, and retained 80% activity after 16 h at 70 °C and after 6 h at 80 °C. The effect of Mg²⁺, Mn²⁺, Zn²⁺, Ca²⁺, sodium dodecyl sulfate (SDS), ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT) and NaCl on the activity of LAC_2.9 was also evaluated by Navas et al. (2019). Each of these reagents was tested at a concentration of 1 mM and NaCl also at 10 and 100 mM. With respect to metal ions, LAC_2.9 was more resistant than LacTT from *T. thermophilus* SG0.5JP17-16 (Liu et al. 2015).

**Proposed mechanism for Xylidine and Methyl Orange decolorization by LAC_2.9**

We performed two analytical methodologies to evaluate the mechanism involved in the decolorization of the azo dyes at pH 7: capillary electrophoresis (CE) and mass spectrometry (MS). The results of CE are shown in Fig. 1 (absorbance detected at 254 nm) and Supplementary Material, Fig. 2 (wavelengths in the visible spectrum). The electrophoretic profile of Xylidine appeared between 8 and 9.5 min and was reduced considerably after laccase treatment without mediator (Fig. 1a, grey dotted line). The reaction with laccase and HBT showed somewhat less reduction in the Xylidine signal (Fig. 1a, upper line), which is in accordance with the results for decolorization (Table 1). In the presence of HBT alone the Xylidine signal remained unchanged (Fig. 1a, bold black broken line). In the mass spectra of Xylidine (Fig. 2a) structures representing the dye (m/z 434.4430, m/z 227.4508 and m/z 454.9015) almost completely disappeared after LAC_2.9 treatment, and two degradation products appeared (m/z 158.1320, 170.6363) (Fig. 2b). In the presence of HBT, no new peaks were recorded (Fig. 2c and Supplementary Material, Fig. 3), indicating that HBT is not mediating the degradation of this dye at pH 7.

Figure 1b shows the CE-UV/DAD for Methyl Orange treated or not with LAC_2.9 in presence and absence of HBT at pH 7. The dye profile appeared at 7 min. After LAC_2.9 treatment, this peak shifted its retention time slightly and it is shown as a split peak or two unresolved peaks (Fig. 1b, grey dotted line). This may be due to loss of water for Methyl Orange treated with LAC_2.9 (as confirmed by later MS experiments); however, it does not cause a significant change in electrophoretic mobility or signal intensity, and hence, it is not evident a process of degradation. In presence of HBT, a new peak around 6.5 min appeared and the peak at 7 min decreased (Fig. 1b, bold black broken line). These results suggest that in presence of HBT a different mechanism of Methyl Orange decolorization takes place. By mass spectra, we observed that Methyl Orange (m/z 304.3402) (Fig. 3a) decreased after laccase treatment, while a new structure (m/z 288.3408) that could be attributed to dehydrated...
Methyl Orange appeared (Fig. 3b). This structure, with a lower conjugation degree, might be responsible for the observed change in color intensity (Table 1), but no evidence of degradation is shown. When HBT was supplied to the reaction, we observed differences in the mechanism of decolorization: a decrease in dye structure intensity at m/z 304.3402 and 288.3408, accompanied by at least two peaks of lower m/z, possibly degradation products.
Extremophiles

(Fig. 3c). These peaks were not observed with HBT without laccase (Supplementary Material, Fig. 4). Taking together, these results indicate that HBT acts as mediator in the degradation of Methyl Orange by LAC_2.9. A proposed mechanism is shown in Fig. 4, where HBT forms a radical cation which co-oxidizes the dye. The necessity of a redox mediator to conduct degradation of Methyl Orange could be due to the lack of a phenolic –OH in the vicinity of the azo bond (–N=N–). In contrast, the –OH close to the azo bond in the Xylidine molecule would allow the generation of a phenoxy radical (Almansa et al. 2004;
Fig. 4  Proposed degradation pathway for Methyl Orange by LAC_2.9 (a), and by LAC_2.9 in the presence of HBT (b)

Fig. 5  Proposed degradation pathway for Xylidine by LAC_2.9 in the presence of HBT
| Laccase source | Applied enzyme form | Azo Dye | Reaction parameters | Decolorization results | Main putative mechanisms involved | References |
|----------------|---------------------|---------|---------------------|------------------------|----------------------------------|------------|
| Bacterial      |                     |         |                     |                        |                                  |            |
| Recombinant SilA from *S. ipomoeae*, expressed in *E. coli* | Purified | Acid Orange 63, Reactive Black 5, Orange II, Tartrazine, | pH 8, 35 °C, 0.1 mM acetosyringone (AS), syringaldehyde (SA) and methyl syringate (MeS) as mediators, 24 h | 13.63, 94.11, 88.86 and 20.97% decolorization, respectively. Enhanced by the addition of MeS mediator | The oxidation of MeS (which has the weakest acceptor group at the para-position) gives an stable phenoxy radical | Blánquez et al. (2019) |
| *Aeromonas* sp. DH-6 | Whole culture (laccase, NADH-DCIP reductase, and azo reductase activities detected) | Methyl Orange | pHs 3.0–12.0, 5–60 °C | 100% decolorization at pHs 3.0–7.0 after 12 h of incubation. It decreased below 40% at pH values beyond 8.0. Mostly complete decolorization was observed at 35 °C after 4 h of incubation. The decolorization decreased rapidly at temperatures over 45 °C | N,N-Dimethyl-p-phenylenediamine and 4-aminobenzene-sulfonic acid were identified as the main intermediates of Methyl Orange biodegradation | Du et al. (2014) |
| Recombinant CotA mutant GWLF from *B. pumilus*, expressed in *P. pastoris* | Purified | Congo Red, Trypan Blue, Evans Blue, Reactive Red 152, Reactive Orange 13, Reactive Black 5 | pH 10.0, 37 °C, 0.3 mM ABTS as mediator, 24 h | 80.35% decolorization of Evans Blue, 71.44% of Reactive Orange 13, and 64.72% of Reactive Black 5 | The azo bond was transformed into N₂ | Xia et al. (2019) |
| *Stenotrophomonas maltophilia* AAP56 | Purified | Reactive Black 5 | pHs 4.0–10.0, 50 °C, 0.2 mM CuSO₄, 0.5 mM ABTS, HBT, AS, SA as mediators | Optimal decolorization was obtained at pHs 7.0–8.0, depending on the mediators used. With ABTS 80% dye removal was detected within 15 min (99% after 60 min), AS 75%, SA 65% and HBT 13% | Analysis of degradation products showed reduction of the azoic bridge with a partial mineralization | Galai et al. (2014) |
| Recombinant CotA from *B. subtilis* expressed in *E. coli* | Purified | Sudan Orange G | pHs 5.0–10.0, 37 °C, ABTS, VA and HBT (1–100 µM) as redox mediators | 80% of Sudan Orange G was decolorized after 24 h without mediator. A two-fold increase in the rate of biotransformation in the presence of 10 µM of ABTS was observed. pH 8 was reported as optimal | The enzymatic oxidation of Sudan Orange G resulted in the production of oligomers and, possibly polymers, through radical coupling reactions | Pereira et al. (2009) |
| Laccase source | Applied enzyme form | Azo Dye | Reaction parameters | Decolorization results | Main putative mechanisms involved | References |
|----------------|---------------------|---------|---------------------|------------------------|-----------------------------------|------------|
| *Pseudomonas desmolyticum* NCIM 2112 | Whole culture (lignin peroxidase, laccase and tyrosinase activities detected) | Reactive Red 141 | pHs 6.8–7.8, 30 °C | Complete decolorization after 96 h | GC–MS identification of 8-amino-naphthalene-1,3,6,7-tetrol and 2-hydroxyl-6-oxalylbenzoic acid as final metabolites supports the degradation of Reactive Red 141 by desulfonation before and after ring cleavage | Kalme et al. (2007) |
| *P. desmolyticum* NCIM 2112 | Purified | Direct Blue-6, Reactive Green 19A, Reactive Red 141 | pH 4.8, 30 °C | Direct Blue-6 and Reactive Red 141DB6 were completely decolorized within 16 h whereas Reactive Green 19A within 12 h | The degradation products of Reactive Green 19A were identified as 4-amino, 6-hydroxynaphthalene, 2-sulfonic acid by HPLC | Kalme et al. (2009) |
| Recombinant LAC_2.9 from *Thermus* sp. 2.9, expressed in *E. coli* | Purified | Xylidine, Methyl Orange | pH 5.0, 7.0 and 9.0, 60 °C, 1 mM CuSO₄, 0.5 mM HBT or pHBA as redox mediator | 98% decolorization of Xylidine and 33% of Methyl Orange after 24 h incubation at pH 9, without the addition of redox mediators | Methyl Orange: HBT formed a radical cation which co-oxidized the dye (Fig. 4). Xylidine: the –OH close to the azo bond allowed the generation of a phenoxyl radical leading to the degradation of the dye (Fig. 5). | This study |
| Fungal | Cell suspension | Congo red | pH 5.5, 30 °C, 24 h | 80% decolorization | Laccase acted not only on the dye chromophore group, but also cleaved different covalent bonds, causing an effective fragmentation of the molecule | Iark et al. (2019) |
| Laccase source     | Applied enzyme form | Azo Dye                          | Reaction parameters          | Decolorization results             | Main putative mechanisms involved                                                                 | References       |
|-------------------|---------------------|----------------------------------|------------------------------|-----------------------------------|---------------------------------------------------------------------------------------------|------------------|
| *Trametes villosa*| Purified            | Synthetized dye I and dye II     | pH 5.0, 25 °C, 24 h          | 70% decolorization of dye I and 90% of dye II | The products of degradation participated in linking reactions with unreacted and reacted dye. The formation of polymerized products stopped the degradation process leading to incomplete decolorization of the dye solutions | Zille et al. (2005) |
| *Aspergillus ochraceus* NCIM-1146 | Purified | Methyl Orange, Reactive Golden Yellow HER | pH 4.0, 40 °C, 4 h          | 56% decolorization of Methyl Orange, 90% of Reactive Golden Yellow HER | Biodegradation of Methyl Orange involved asymmetric cleavage of the azo bond, resulting in formation of a p-N,N'-dimethylamine phenyldiazine intermediate and a p-hydroxybenzene sulfonic acid intermediate | Telke et al. (2010) |
| *T. trogii*       | Culture filtrates (laccase, Mn-peroxidase and cellobiose dehydrogenase activities detected) | Xylidine, Methyl Orange       | pH 4.5, 30 °C, 24 h          | 75% decolorization of Methyl Orange and 96% of Xylidine | Xylidine decolorization involved colored quinones reduction by CDH, rendering naphthalene sulfonate and xylene as final products | Levin et al. (2012) |
| *Trametes pubescens* | Purified | Congo Red                       | pH 5.0, 50 °C, 72 h          | 80.53% decolorization             | The first step was the reduction of the –N=N– bond. The degraded metabolites were identified as naphthalene amine, biphenyl and naphthalene diazonium | Si et al. (2013)  |
| Laccase source | Applied enzyme form | Azo Dye               | Reaction parameters | Decolorization results | Main putative mechanisms involved | References               |
|---------------|---------------------|-----------------------|---------------------|------------------------|-----------------------------------|--------------------------|
| *Trichoderma atroviride* F03 | Whole culture       | Reactive Black 5      | pH 3.0–8.0, 20–35 °C | 87.2% decolorization at pH 5 and 27 °C after 8 days | The Reactive Black 5 biodegradation was initiated by the cleavage of the bis-azo bond and followed by deamination and hydroxylation which were mediated by laccase to produce naphthalene-1,2,8-triol and sulphuric acid mono-[2-(toluene-4-sulfonyl)-ethyl] ester | Adnan et al. (2015) |
| *T. pubescens* Puriﬁed and immobilized | Congo Red, Acid Black 172 | pH 5, 50 °C, 48 h | 54.24% and 68.84% decolorization of Congo Red and Acid Black 172, respectively | 2-Nitronaphthalene, 1-Naphthalene diazonium, 6-nitro-2-naphthol, and 2-naphthol are the main degradation products of Acid Black 172 | Zheng et al. (2016) |
| White rot fungus isolate | Whole culture | Methyl Orange | 12 days of fermentation at pH 5.0 and 30 °C | Strain showed decolorization in late exponential or early stationary phase in UV/visible spectra of the synthetic dye medium | The biodegradation of Methyl Orange involved asymmetric cleavage of the azo bond, resulting in formation of a p-N,N'-dimethylamine phenyl-diazine intermediate and p-hydroxybenzene sulfonic acid intermediate | Mishra et al. (2011) |
There is growing interest in assessing the potential of bacterial laccases for industrial and environmental applications especially in hostile conditions. LAC_2.9 laccase from *Therms* sp. 2.9 was capable of partially decolorizing chemically diverse dyes such as the azoic Xylidine and Methyl Orange, the antraquinonic RBBR, and the triphenylmethane Gentian Violet, without the addition of redox mediators even at pH 9 and 60 °C. Due to the thermal properties of LAC_2.9 and its tolerance to alkaline pHs and high heavy metals concentrations, this enzyme could be used in the decolorization of textile effluents, a process usually performed at high temperature (> 55 °C) and neutral/alkaline pH. Degradation pathways are suggested for the action of LAC_2.9 on Xylidine and Methyl Orange azo dyes, not reported for purified bacterial laccases previously.

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**Author contributions** LN obtained and purified the enzyme and performed the decolorization experiments. RC performed the CE and MS analysis and the statistical analysis of decolorization experiments. LL and MB conceived the study and MB supervised the project. All authors have contributed to the writing and revising of the manuscript and approved the final version to be submitted.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

**References**

Adedayo O, Jawapour S, Taylor C, Anderson WA, Moo-Young M (2004) Decolourisation and detoxification of methyl red by aerobic bacteria from a wastewater treatment plant. J Microbiol Biotechnol 20:545–550. https://doi.org/10.1023/b.wibi.0000043150.37318.5f

Adnan LA, Sathishkumar P, Mohd Yusoff AR, Hadibarata T (2015) Metabolites characterisation of laccase mediated Reactive Black 5 biodegradation by fast growing ascomycete fungus *Trichoderma atroviride* F03. Int Biodeterior Biodegrad 104:274–282. https://doi.org/10.1016/j.ibiod.2015.05.019

Almansa E, Kandelbauer A, Pereira L, Cavaco-Paulo A, Gubitz GM (2004) Influence of structure on dye decoloration with laccase mediator systems. Biocatal Biotransform 22:315–324. https://doi.org/10.1080/01416430400024508

Anastasi A, Parato B, Spina F, Tiganii V, Prigione V, Varese GC (2011) Decolourisation and detoxification in the fungal treatment of textile effluents, a process usually performed at high temperature (> 55 °C) and neutral/alkaline pH. Degradation pathways are suggested for the action of LAC_2.9 on Xylidine and Methyl Orange azo dyes, not reported for purified bacterial laccases previously.
textile wastewaters from dyeing processes. N Biotechnol 29:38–45. https://doi.org/10.1016/j.nbitech.2011.08.006
Blanquex A, Rodríguez J, Brisso V, Mendes S, Martins LO, Ball AS, Arias ME, Hernández M (2019) Decolorization and detoxification of textile dyes using a versatile Streptomyces lividans RD-1 laccase-natural mediator system. Saudi J Biol Sci 26:913–920. https://doi.org/10.1016/j.sjbs.2018.05.020
Burriel Martí F, Lucena Conde F, Arribas Jimeno S, Hernández Mendoza J (2002) Química analítica cualitativa. International Thomson Editores Spain, Madrid
Ciullini I, Tili S, Scozzafava A, Briganti F (2008) Fungal laccase, cellobiose dehydrogenase, and chemical mediators: combined actions for the decolorization of different classes of textile dyes. Bioresour Technol 99:7003–7010. https://doi.org/10.1016/j.biortech.2008.01.019
Collivignarelli M, Abbá A, Carnevale Miino M, Damiano S (2019) Treatments for color removal from wastewater: state of the art. J Environ Manage 236:727–745. https://doi.org/10.1016/j.jenvman.2018.11.094
Di Rienzo JA, Casanoves F, Balzarini MG, Gonzalez L, Tablada M, Collivignarelli M, Abbà A, Carnevale Miino M, Damiano S (2019) Decolorization of textile dyes by a soil-derived white-rot fungus. Bioresour Technol 289:121655. https://doi.org/10.1016/j.biortech.2019.121655
Efeytazm K, Tabanbdeh F, Yakhchali B, Alikhani HA, Rodríguez Couto S (2009) Assessment of the joint effect of laccase and cellobiose dehydrogenase on the decoloration of different synthetic dyes. J Hazard Mater 169:176–181. https://doi.org/10.1016/j.jhazmat.2009.03.088
Galá S, Korri-Youssef H, Harzouki MN (2014) Characterization of yellow bacterial laccase SmLac/role of redox mediators in azo dye decolorization. J Chem Technol Biotechnol 89:1741–1750. https://doi.org/10.1002/jctb.4254
Garg SK, Tripathi M (2016) Microbial strategies for discoloration and detoxification of azo dyes from textile effluents. Res J Microbiol 12:1–19. https://doi.org/10.3923/rjm.2017.1.19
Guan ZB, Luo Q, Wang HR, Chen Y, Xiao XR (2018) Bacterial laccases: promising biological green tools for industrial applications. Cell Mol Life Sci 75:3569. https://doi.org/10.1007/s00018-018-2883-z
Iark D, dos Reis Buzzo AJ, Garcia JAA, Corrêa VG, Helmi CV, Corrêa JS, Helmi CV (2019) Microbial degradation and detoxification of azo dye Congo red by a new laccase from Oudemansiella canarii. Bioresour Technol 289:121655. https://doi.org/10.1016/j.biortech.2019.121655
Kalme S, Ghodake G, Govindwar S (2007) Red HE7B degradation using desulfonation by Pseudomonas desmolyticum NCIM 2112. Int Biodeterior Biodegrad 60:327–333. https://doi.org/10.1016/j.ibiod.2007.05.006
Kalme S, Jadhav S, Jadhav M, Govindwar S (2009) Textile dye degradative laccase from Pseudomonas desmolyticum NCIM 2112. Enzyme Microb Technol 44:65–71. https://doi.org/10.1016/j.enzmictec.2008.10.005
Kim Y, Nicell JA (2006) Impact of reaction conditions on the laccase catalyzed conversion of bisphenol A. Bioresour Technol 97:1431–1442. https://doi.org/10.1016/j.biortech
Kokol V, Doliška A, Eichlerová I, Baldrian P, Nerud F (2007) Decolorization of textile dyes by whole cultures of Ischnoderma resinosum and by purified laccase and Mn-peroxidase. Enzyme Microb Technol 40:1673–1677. https://doi.org/10.1016/j.enzmictec.2006.08.015
Lade H, Kadam A, Paul D, Govindwar S (2015) Biodegradation and detoxification of textile azo dyes by bacterial consortium under sequential microaerophilic/aerobic processes. EXCLI J 14:158–174. https://doi.org/10.17179/excli2014-642
Legerská B, Chmelová I, Battíková M, Ondrejovič M (2018) Decolorization and detoxification of monooazo dyes by laccase from the white-rot fungus Trametes versicolor. J Biotechnol 285:84–90. https://doi.org/10.1016/j.jbiotec.2018.08.011
Levin L, Grassi E, Carballo R (2012) Efficient azoic dye degradation by Trametes trogii and a novel strategy to evaluate products released. Int Biodeterior Biodegrad 75:214–222. https://doi.org/10.1016/j.ibiod.2012.04.005
Liu H, Cheng Y, Du B, Tong C, Liang S, Han S, Zheng S, Lin Y (2015) Overexpression of a novel thermostable and chloride-tolerant laccase from Thermus thermophilus SG0.5JP17-16 in Pichia pastoris and its application in synthetic dye decolorization. PLoS One 10(3):e0119833. https://doi.org/10.1371/journal.pone.0119833
Lončar N, Božič N, Lopez-Santin J, Vujčić Z (2013) Bacillus amyloliquefaciens laccase—from soil bacteria to recombinant enzyme for wastewater decolorization. Bioresour Technol 147:177–183. https://doi.org/10.1016/j.biortech.2013.08.056
Ma S, Liu N, Jia H, Dai D, Zang J, Cao Z, Dong J (2018) Expression, purification, and characterization of a novel laccase from Setosphaeria turcica in Escherichia coli. J Basic Microbiol 58:68–75. https://doi.org/10.1002/jobm.201700212
Mandic M, Djokic L, Nikolavits E, Prodanovic R, O’Connor K, Jeremić S, Topakas E, Nikolodinovic-Runic J (2019) Identification and characterization of new laccase biocatalysts from Pseudomonas species suitable for degradation of synthetic textile dyes. Catalysts 9:629. https://doi.org/10.3390/catal9070629
Mishra A, Kumar S, Pandey A (2011) Laccase production and simultaneous detoxication of synthetic dyes in unique inexpensive medium by new isolates of white rot fungus. Int Biodeterior Biodegrad 65:487–493. https://doi.org/10.1016/j.ibiod.2011.01.001
Molina-Guijarro JM, Pérez J, Muñoz-Dorado J, Guillén F, Moya R, Hernández M, Arias ME (2009) Detoxification of azo dyes by a novel pH-versatile, salt-resistant laccase from Streptomyces ipomoea. Int Microbiol 12:13–21. https://doi.org/10.2436/20.1501.01.77
Mongay C, Cerda V (1974) A Britton–Robinson buffer of known ionic strength. Ann Chim 64:409–412
Moya R, Hernández M, García-Martín AB, Ball AS, Arias ME (2010) Contributions to a better comprehension of redox-mediated decolorization of azo dyes by a laccase produced by Streptomyces cyanus CECT 3335. Bioresour Technol 101:2222–2229. https://doi.org/10.1016/j.biortech.2009.11.061
Navas LE, Martínez FD, Taverna ME, Fetherolf MM, Eltis LD, Nicolau V, Estenzos D, Campos E, Benintende GB, Berretta MF (2019) A thermostable laccase from Thermus sp. 2.9 and its potential for delignification of Eucalyptus biomass. AMB Express 9(1):24. https://doi.org/10.1186/s13568-019-0748-y
Parshetti GK, Kalme SD, Saratade GD, Govindwar SP (2006) Biodegradation of malachite green by Kocuria rosea MTCC 1532. Acta Chim Slov 53:492–498
Pereira L, Coelho AV, Viegas CA, dos Santos MMC, Robalo MP, Martins LO (2009) Enzymatic biotransformation of the azo dye Sudan Orange G with bacterial CoA-laccase. J Biotechnol 139:68–77. https://doi.org/10.1016/j.jbiotec.2008.09.001
Si J, Peng F, Cui B (2013) Purification, biochemical characterization and dye decolorization capacity of an alkali-resistant and metal-tolerant laccase from Trametes pubescens. Bioresour Technol 128:49–57. https://doi.org/10.1016/j.biortech.2012.10.085
Singh RL, Singh PK, Singh RP (2015) Enzymatic decolorization and degradation of azo dyes—a review. Int Biodeterior Biodegrad 104:21–31. https://doi.org/10.1016/j.ibiod.2015.04.027
Takeda S, Tanaka Y, Nishimura Y, Yamane M, Siroma Z, Wakida S (1999) Analysis of dyestuff degradation products by capillary electrophoresis. J Chromatogr A 853:503–509. https://doi.org/10.1016/s0021-9673(99)00579-8

Telke AA, Kadam AA, Jagatap SS, JadHAV JP, Govindwar SP (2010) Biochemical characterization and potential for textile dye degradation of blue laccase from Aspergillus ochraceus NCIM-1146. Biotechnol Bioprocess Eng 15:696–703. https://doi.org/10.1007/s12257-009-3126-9

Thakur JK, Paul S, Dureja P, Annapurna K, Padaria JC, Gopal M (2014) Degradation of sulphonated azo dye Red HE7B by Bacillus sp. and elucidation of degradative pathways. Curr Microbiol 69:183–191. https://doi.org/10.1007/s00284-014-0571-2

Torres E, Bustos-Jaimes I, Borgne SL (2003) Potential use of oxidative enzymes for the detoxification of organic pollutants. Appl Catal B-Environ 46:1–15. https://doi.org/10.1016/s0926-3373(03)00228-5

Unuofin JO, Okoh AI, Nwodo UU (2019) Aptitude of oxidative enzymes for treatment of wastewater pollutants: a laccase perspective. Molecules 24:2064. https://doi.org/10.3390/molecules24122064

Verma A, Shirkot P (2014) Purification and characterization of thermostable laccase from thermophilic Geobacillus thermocatenulatus MS5 and its applications in removal of textile dyes. Sch Acad J Biosci 2:479–485

Xia J, Wang Q, Luo Q, Chen Y, Liao XR, Guan ZB (2019) Secretory expression and optimization of Bacillus pumilus CotA-laccase mutant GWLF in Pichia pastoris and its mechanism on Evans blue degradation. Process Biochem 20:33–41. https://doi.org/10.1016/j.procbio.2018.12.034

Zaharia C, Suteu D (2012) Textile organic dyes. Characteristics, polluting effects and separation/elimination procedures from industrial effluents. A critical overview. In: Tomasz P (ed) Organic pollutants ten years after the Stockholm Convention, Environmental and analytical update. IntechOpen. https://doi.org/10.5772/32373

Zheng F, Cui BK, Wu XJ, Meng G, Liu HX, Si J (2016) Immobilization of laccase onto chitosan beads to enhance its capability to degrade synthetic dyes. Int Biodeterior Biodegrad 110:69–78. https://doi.org/10.1016/j.ibiod.2016.03.004

Zille A, Gornacka B, Rehorek A, Cavaco-Paulo A (2005) Degradation of azo dyes by Trametes villosa laccase over long periods of oxidative conditions. Appl Environ Microbiol 71:6711–6718. https://doi.org/10.1128/aem.71.11.6711-6718.2005

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