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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 Cd^{+2}, As^{+5} and Ni^{+2} 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

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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 dyes 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 N_2 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 an 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 OD_{600} of 0.8 together with $CuSO_4$ (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 ($\mathcal{E} = 36{,}000\ M^{-1}cm^{-1}$) of oxidized ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulfonate]). Assays were performed at 60 °C and contained 1 mM $CuSO_4$ 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 $Cd(NO_3)_2$, $Cr(NO_3)_3$, $Pb(NO_3)_2$, $NiSO_4$ and Na_2HAsO_4 was added at final concentrations of 1, 10 and 100 mM, to the reaction conducted at pH 5.0 and containing 1 mM Cu SO_4, which was taken as control for 100% activity. The effect of $CuSO_4$ 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, $\lambda_{max} = 505$ nm) and Methyl Orange ($\lambda_{max} = 460$ nm); triphenylmethane dyes, Malachite Green ($\lambda_{max} = 618$ nm) and Gentian Violet ($\lambda_{max} = 590$ nm), the antraquinone dye, Remazol Brilliant Blue R (RBBR) ($\lambda_{max} = 590$ nm); and the indigoid dye Indigo Carmine ($\lambda_{max} = 608$ nm). Controls without laccase were conducted in parallel. All the reactions were performed in triplicate. Decolorization activity was calculated: % Decolorization = $[(I - F)/I] \times 100$, 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 cm^2 area), an Ag/AgClKCl 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. About the electrophoresis, separations were performed in a P/ACE MDQ (Beckman Coulter, Brea, CA, USA), equipped with a UV–Vis photodiode array detector. The software 32 KaratTM (Beckman Coulter) was used for data processing. All capillary electrophoresis experiments were performed at 25 °C. UV detection was carried out at 254, 460 and 505 nm. An untreated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 75 µm I.D. × 375 mm O.D. was used. Its total length was 62 cm and the effective length was 50 cm to the detector. The background electrolyte solution (BGE) consisted of 50 mM ammonium carbonate buffer (pH 9.5). After each run, the capillary was washed with 0.1 M NaOH for 5 min, then rinsed with distilled water for 3 min and BGE for 4 min. Aliquots of the sample solutions were introduced by 5 s at 3.5 kPa and the separation voltage was 20 kV. Finally, the capillary was flushed with water for 10 min at end of the day (Takeda et al. 1999). Mass spectrometric detection was performed using a Thermo Scientific TSQ Quantum Access MAX triple quadrupole system Mass Spectrometer (Thermo Scientific, CA, USA) with electrospray ionization. Ionization in negative ion mode was registered. The spray voltage was set at 3500 V. Nitrogen was employed as the nebulizer and auxiliary gas, set at 60 and 20 arbitrary units, respectively. The capillary temperature and vaporizer were set at 275 °C and 350 °C, respectively. For collision-induced dissociation, 99.997% argon (Grade 4.7) was used at 1.5 mTorr.

Chemicals

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 anthraquinonic 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.

Table 1 Percentage of dye decolorization after 6 and 24 h incubation with 0.15 U/ml LAC_2.9 at 60 °C with or without the addition of HBT or pHBA redox mediators. Values represent the mean of three replicates, with a standard error of less than 5%. Control values without laccase were subtracted in each case

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	$23.50 \pm 1.82^{a\#}$	$50.95 \pm 1.35^{*}$	$20.64 \pm 1.73^{\&a}$	24.36 ± 1.00^{d}	$19.58 \pm 1.74^{\$a}$	$33.45 \pm 2.14^{\Delta d}$
	LAC_2.9 + HBT	61.55 ± 2.24	68.03 ± 2.37	$24.10 \pm 2.04^{\&b}$	$36.06 \pm 1.05^{\bullet e}$	$28.67 \pm 2.01^{\$b}$	$39.19 \pm 1.44^{\Delta e}$
	LAC_2.9 + pHBA	$31.67 \pm 1.61^{\#}$	$52.92 \pm 2.59^{*}$	$26.76 \pm 3.79^{\&c}$	$37.22 \pm 3.00^{\bullet f}$	$27.60 \pm 3.44^{\$c}$	$37.32 \pm 2.42^{\Delta f}$
Xylidine	LAC_2.9	$34.31 \pm 2.34^{£}$	$59.96 \pm 1.15^{\pi}$	$52.72 \pm 5.10^{\Phi}$	$81.23 \pm 2.53^{\lambda}$	$72.06 \pm 1.93^{\varkappa}$	$97.93 \pm 2.69^{\sigma}$
	LAC_2.9 + HBT	47.19 ± 2.13^{g}	86.04 ± 3.94^{h}	$51.75 \pm 2.04^{\Phi g}$	$80.97 \pm 0.95^{\lambda h}$	$75.50 \pm 1.03^{\varkappa}$	$99.36 \pm 0.76^{\sigma}$
	LAC_2.9 + pHBA	$36.72 \pm 1.74^{£}$	$63.33 \pm 1.46^{\pi i}$	44.56 ± 2.23	68.28 ± 1.51^{i}	$69.04 \pm 0.69^{\varkappa}$	$91.10 \pm 0.71^{\sigma}$
Gentian Violet	LAC_2.9	12.46 ± 1.71^{j}	30.96 ± 1.50^{m}	$22.97 \pm 1.69^{\ddagger j}$	36.57 ± 1.12^{m}	$30.40 \pm 1.39^{₹}$	$40.07 \pm 2.02^{€m}$
	LAC_2.9 + HBT	55.35 ± 1.37	73.86 ± 2.05	$24.71 \pm 1.27^{\ddagger k}$	$50.70 \pm 2.10^{\dagger n}$	$28.92 \pm 0.98^{₹k}$	$44.26 \pm 0.59^{€n}$
	LAC_2.9 + pHBA	21.82 ± 1.16	54.14 ± 2.04	40.07 ± 1.70^{l}	$59.16 \pm 1.65^{\dagger}$	52.73 ± 0.75^{l}	$53.49 \pm 2.68^{€}$
Malachite Green	LAC_2.9	$52.31 \pm 2.68^{\infty}$	$71.59 \pm 2.40^{\int}$				
	LAC_2.9 + HBT	82.35 ± 0.72	$82.35 \pm 0.29^{\int}$				
	LAC_2.9 + pHBA	$52.47 \pm 2.27^{\infty}$	$73.21 \pm 1.82^{\int}$				
RBBR[X]	LAC_2.9	34.61 ± 1.82	51.28 ± 1.35^{t}	55.99 ± 1.84^{q}	66.48 ± 1.03	53.61 ± 1.93^{q}	54.04 ± 2.69^{t}
	LAC_2.9 + HBT	36.72 ± 1.24	56.10 ± 2.37^{u}	55.40 ± 2.78^{r}	64.22 ± 3.14^{u}	54.72 ± 1.03^{r}	58.44 ± 0.76^{u}
	LAC_2.9 + pHBA	34.36 ± 1.61	51.91 ± 2.59^{w}	41.56 ± 2.48^{s}	58.26 ± 2.99	48.05 ± 0.69^{s}	57.44 ± 0.71^{w}
Indigo Carmine[X]	LAC_2.9	97.90 ± 2.16					
	LAC_2.9 + HBT	99.10 ± 1.06					
	LAC_2.9 + pHBA	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 ($p < 0.05$)

[X]Means not significantly different among treatments ($p < 0.05$)

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, Xylidine 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 Xylidine 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 Xylidine

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 assayed indicates that the decolorization of dyes at these pH values 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 assessed at concentrations 1, 10 and 100 mM (Table 2). When assaying acidic cations such as Cr^{+3} and Pb^{+2} at high concentrations a precipitate was formed (Burriel Marti 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 Cd^{2+}, As^{5+} and Ni^{2+} respectively, but only 31% with Cu^{2+}. Earlier reports also showed that Cu^{2+} 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). 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 Mg^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+}, 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).

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

Metal ion	% Relative activity[a]		
	1 mM	10 mM	100 mM
Cu^{2+}	100.0	90.9 ± 9.2	31.2 ± 4.8
Cd^{2+}	100.7 ± 6.4	102.9 ± 6.0	98.9 ± 4.6
Cr^{3+}	100.1 ± 18.2	ND	ND
Pb^{2+}	105.6 ± 4.0	96.0 ± 5.4	ND
Ni^{2+}	97.3 ± 9.8	97.1 ± 6.7	74.7 ± 7.3
As^{5+}	107.0 ± 5.9	101.2 ± 5.6	85.8 ± 6.3

ND a precipitate was formed, which prevented spectrophotometric determination

[a]Laccase activity in the presence of 1 mM Cu^{2+} was set to 100%

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

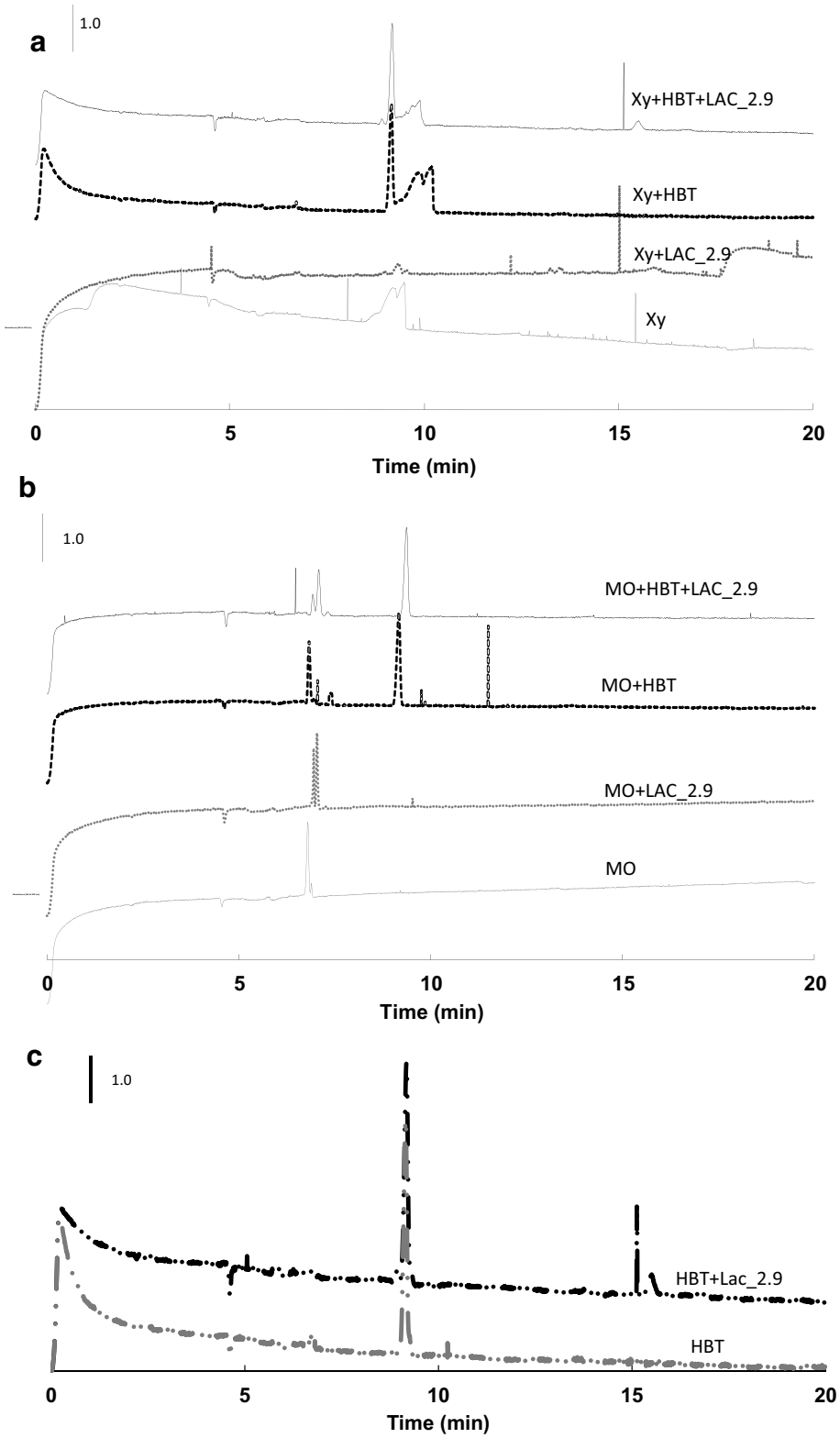

Fig. 1 Electropherograms of Xylidine (**a**) and Methyl Orange (**b**) at 254 nm: non-treated dye; dye treated with LAC_2.9; dye treated with only HBT; dye treated with HBT and LAC_2.9. Electropherograms of HBT alone and HBT + LAC_2.9 at 254 nm as controls in (**c**)

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

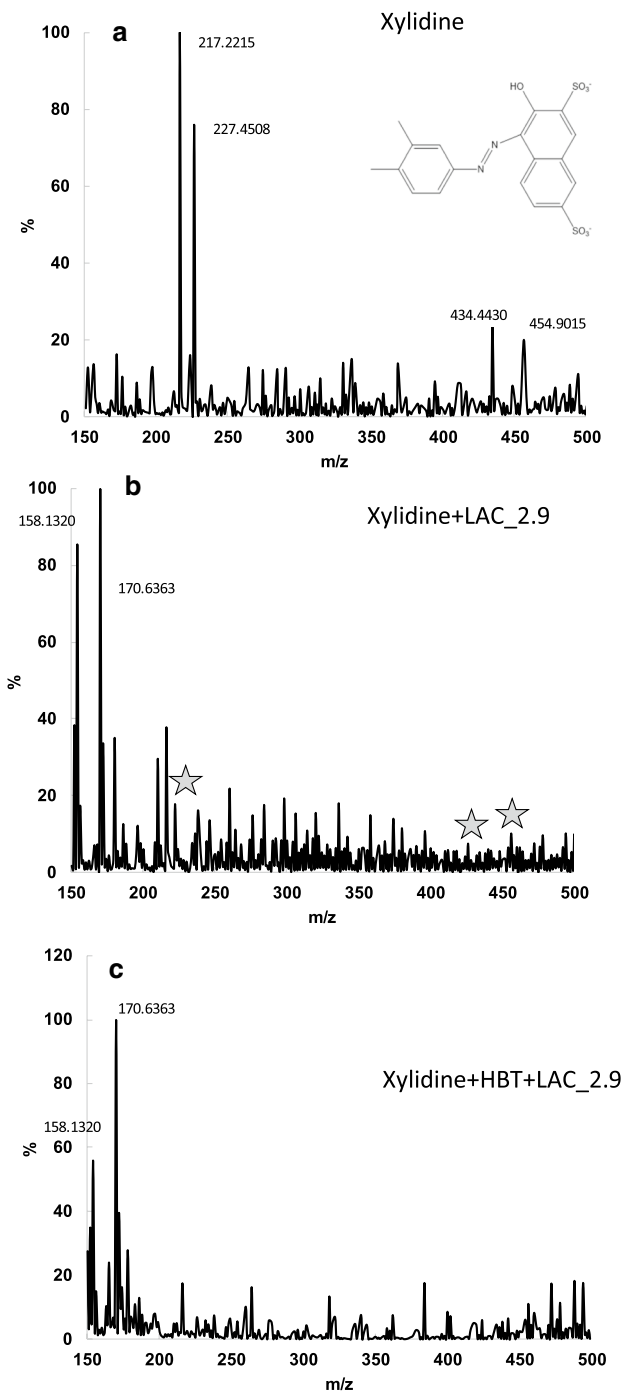

Fig. 2 Mass spectra of non-treated Xylidine (**a**), Xylidine treated with LAC_2.9 (**b**), Xylidine treated with HBT and LAC_2.9 (**c**). Stars indicate decreased signals

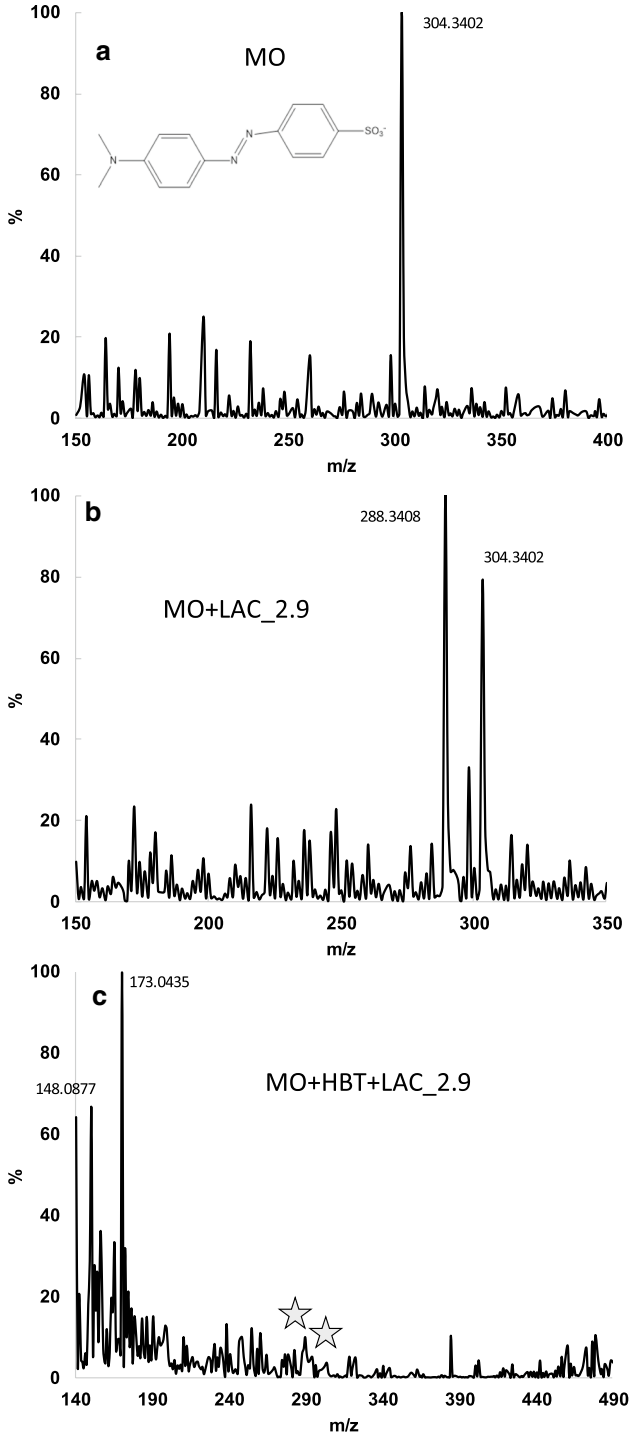

Fig. 3 Mass spectra of non-treated Methyl Orange (**a**), Methyl Orange treated with LAC_2.9 (**b**), Methyl Orange treated with HBT and LAC_2.9 (**c**). Stars indicate decreased signals

(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 conduce 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 phenoxyl radical (Almansa et al. 2004;

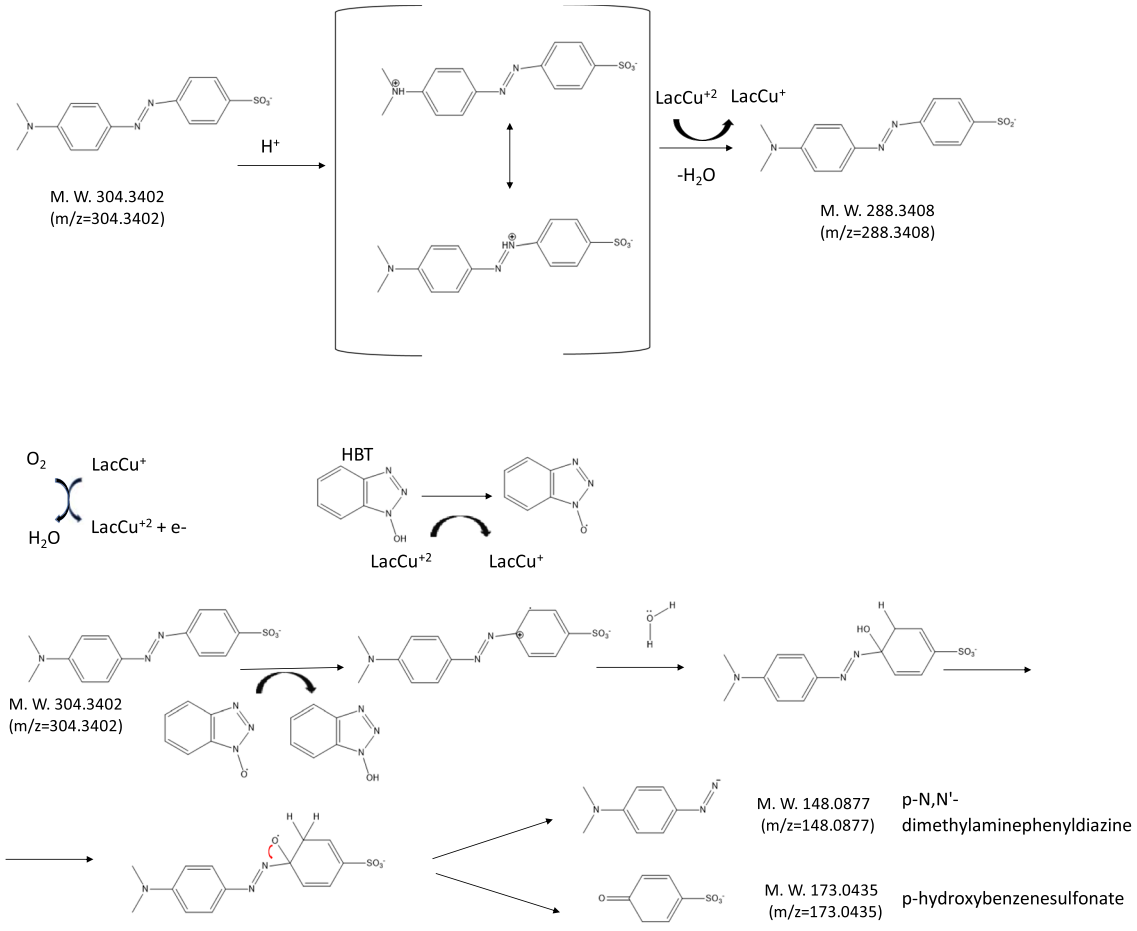

Fig. 4 Proposed degradation pathway for Methyl Orange by LAC_2.9 (**a**), and by LAC_2.9 in the presence of HBT (**b**)

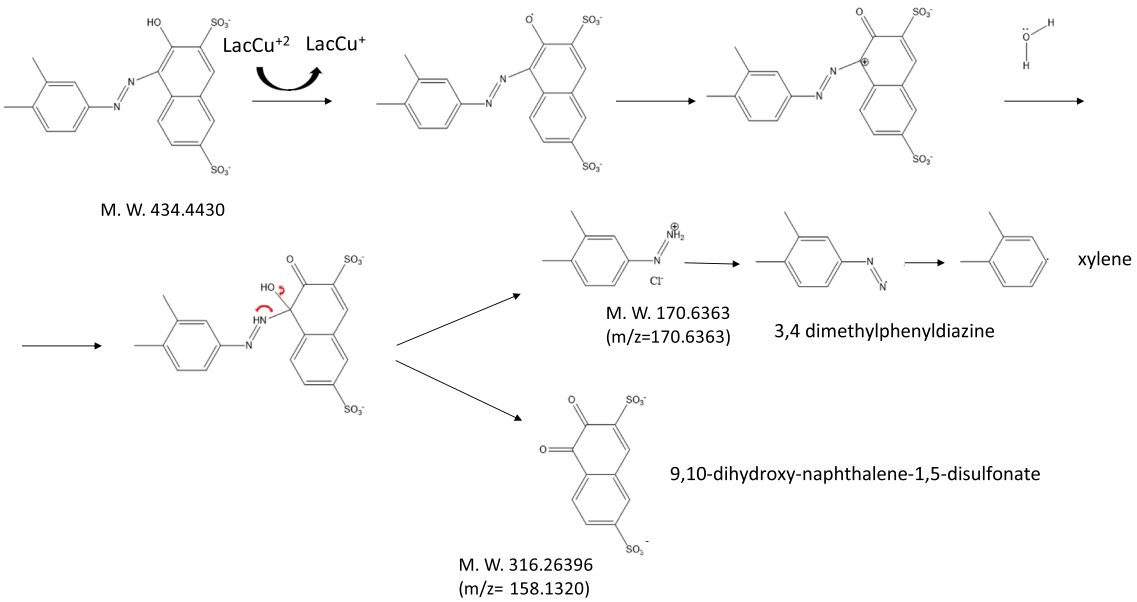

Fig. 5 Proposed degradation pathway for Xylidine by LAC_2.9 in the presence of HBT

Table 3 Degradation of azo dyes by different sources of laccases and main mechanism involved

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 N_2	Xia et al. (2019)
Stenotrophomonas maltophilia AAP56	Purified	Reactive Black 5	pHs 4.0–10.0, 50 °C, 0.2 mM $CuSO_4$, 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)

Table 3 (continued)

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-tetraol and 2-hydroxyl-6-oxalyl-benzoic 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 $CuSO_4$, 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						
Oudemansiella canarii	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)

Table 3 (continued)

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)

Table 3 (continued)

Laccase source	Applied enzyme form	Azo Dye	Reaction parameters	Decolorization results	Main putative mechanisms involved	References
Trichoderma atroviride F03	Whole culture	Reactive Black 5	pHs 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 immovilized	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 phenyldiazine intermediate and *p*-hydroxybenzene sulfonic acid intermediate	Mishra et al. (2011)

Enayatzamir et al. 2009) leading to the degradation of the chromophoric structure of the dye through the mechanism we propose in Fig. 5.

While the degradation of azo dyes has been extensively studied using cultures of a wide range of fungi and bacteria, work carried out using their purified enzymes is more limited. Table 3 summarizes the reports proposing a possible mechanism for the degradation of azo dyes obtained by using either a purified laccase as the sole enzyme or a culture fraction with laccase among other enzymatic activities. Possible degradation pathways for Methyl Orange were reported for fungal laccases (Telke et al. 2010; Mishra et al. 2011; Levin et al. 2012) as well as for the bacterium *Aeromonas* sp. DH-6 (Du et al. 2014). In this last case laccase as well as NADH-DCIP reductase and azo reductase activities contributed to azo dye degradation. Generally, the azoreductase catalyzes the initial step of bacterial azo degradation (reductive cleavage of the azo bond) and laccase is involved in the oxidation of sulfonated azo dyes. In the case of *Aeromonas* sp. DH-6, aromatic amines were obtained as intermediates in the biodegradation of Methyl Orange. As far as we know, these mechanisms have not been reported for purified bacterial laccases up to now. LAC_2.9 activity involved asymmetric cleavage of the azo bond obtaining *p*-hydroxybenzenesulfonate and *p*-*N*,*N*′- dimethylamine phenyldiazine as degradation products, which resemble those obtained by fungal laccases (Telke et al. 2010; Mishra et al. 2011; Levin et al. 2012). In this work, we show that a purified bacterial laccase could catalyze the decolorization of Methyl Orange without generation of aromatic amines. Additionally, we present insights into the mechanism of purified laccase action on the azo dye Xylidine. Culture filtrates from the white rot fungus *Trametes trogii* displaying laccase, Mn-peroxidase and cellobiose dehydrogenase activities were used for Xylidine decolorization and biodegradation products were determined (Levin et al. 2012). The same intermediaries were observed after LAC_2.9 treatment demonstrating that it catalyzes Xylidine degradation without the requirement of extra enzymes.

There are few reports on the detoxification of textile effluents with laccases, and no strict association was found between decolorization and detoxification (Adedayo et al. 2004; Anastasi et al. 2011; Legerská et al. 2018). The degradation of azo-dyes might generate compounds of increased toxicity (Zaharia and Suteu 2012). Toxicity changes after LAC_2.9 decolorization must still be evaluated. Nevertheless, recent decolorization studies involving bacterial laccases proved their ability to detoxify several azo dyes (Blánquez et al. 2019; Lade et al. 2015; Molina-Guijarro et al. 2009; Pereira et al. 2009; Xia et al. 2019).

Conclusions

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 *Thermus* 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.

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