Journal of Molecular Catalysis B: Enzymatic 68 (2011) 216–222

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Induction, purification, and characterization of a laccase isozyme from *Pleurotus sajor-caju* and the potential in decolorization of textile dyes

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ARTICLE INFO

Article history:
Received 11 August 2010
Received in revised form 4 November 2010
Accepted 5 November 2010
Available online 11 November 2010

Keywords:
Pleurotus sajor-caju
Laccase
Induction
Industrial dyes
Decolorization

ABSTRACT

Ferulic acid causes a strain of *Pleurotus sajor-caju* to excrete large amounts of one laccase isozyme, that was purified to homogeneity. Ferulic acid was the best inductor for laccase in this fungal strain. Laccase molecular weight, copper, and sugar content were characterized. Its catalytic activity was studied using a wide range of phenolics and aromatic amines; the purified laccase was found to be able to oxidize catechols, quinols, methoxyphenols, some aromatic amines and their methyl derivatives, and also resorcinol and phloroglucinol. In addition, its thermostability and activity in the presence of some organic solvents were evaluated. The ability of the enzyme to decolorize some textile dyes was studied, also in the presence of the co-oxidizer hydrogen peroxide, and compared to that of some peroxidases.

1. Introduction

Laccases (LCs, benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are cuproproteins which are widespread among higher plants [1,2], bacteria [3], and ligninolytic ("white-rot") fungi [4,5]. Typically, LCs are blue proteins that contain four redox-active cupric ions. These cupric ions pertain to defined spectroscopic types. One type I cupric ion (blue copper) is devoted to accept an electron from oxidizable substrates. One type II ion (non-blue copper) is connected to an antiferromagnetic, epr-silent type III couple where molecular oxygen is bound and reduced to two water molecules [6]. Fungal LCs are acidic, extracellular glycoproteins [4,7,8]. LCs are usually inducible enzymes, for which a number of inductors have been found [9,10]. Fungal LCs are involved in lignin biodegradation. Some decades ago, it was determined that LC-deficient mutants of white-rot fungi are unable to delignify wood [11]. Phenoxy radicals (arising from LC action on phenolics contained in wood) are involved as redox mediators during wood delignification by white-rot fungi. Their involvement during wood delignification by white-rot fungi became clear because purified LC is substantially unable to degrade isolated lignin [12]. Recently, new substrates have been discovered, according to a specificity of LC far beyond lignin-related phenolics [13]. These enzymes are of potential technological interest not only in the pulp and paper industry [14,15], but also in the fields of green chemistry [16], bioethanol production, food treatment [17], biosensors [18], and bioremediation of many effluents, which also contain industrial dyes [19,20].

In the current study, the edible white-rot fungus, *Pleurotus sajor-caju* (Fries) Singer (also known as *Lentinus sajor-caju* (Fries)), has been chosen as a source of LC, because of its good virulence, tolerance of relatively high temperatures, and ease of cultivation [21]. A wide variety of aromatic molecules, including phenolics, aromatic amines, and nonphenolics have been evaluated as possible LC inductors. This allowed for the building up of a production protocol. In this way we obtained large amounts of the enzyme, which was purified and characterized for its biochemical properties and operational features. Substrate specificity, catalytic activity under different pH and temperature conditions, stability against organic solvents, and the ability to decolorize some industrial dyes are reported.

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); FA, ferulic (*p*-hydroxy-*m*-methoxycinnamic) acid; LC, laccase, EC 1.10.3.2; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; MnP, manganese peroxidase, E.C. 1.11.1.13; LiP, lignin peroxidase, E.C. 1.11.1.14; HRP, horseradish peroxidase; ARS, alizarin red S; PNS, phenosafranine; XO, xylenol orange; MG, methyl green; MB, methyl blue; MO, methyl orange.

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doi:10.1016/j.molcatb.2010.11.008

2. Experimental

All the reagents used were of the highest quality available. These reagents were used as purchased, without further purification. In particular, syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde azine) was purchased from Sigma (Cat. No. S-7896), and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid, ABTS) was purchased from Fluka (Cat. No. 11557).

P. sajor-caju strain ACR-16 (Cattedra di Chimica Biologica Collection) was maintained using periodic transfer onto malt/agar plates.

2.1. LC induction and production

The growth medium for *P. sajor-caju* contained 2% *w*/*v* malt extract, 0.5% *w*/*v* yeast extract, 10 mM potassium phosphate buffer pH 6 and 0.1 mM $CuSO_4$ (Cu source for LC production). When present, inductors were used at a final concentration of 10 mM. Cultures were grown in 500 mL Erlenmeyer flasks. Both flasks and culture media were sterilized at 121 °C for 20 min.

During growth, the temperature was set at 25 °C and relative humidity was set at 70%. A small volume of the culture (100 μL) was aseptically removed after every 5 days up to 30 days in order to measure LC activity.

2.2. Enzyme activity and protein content

2.2.1. Spectrophotometric assays

One LC E.U. was defined as the amount of enzyme capable of oxidizing 1 μmol of syringaldazine per minute at pH 6 and 25 °C. All assays were performed in air-saturated solutions.

LC activity was monitored spectrophotometrically (UltroSpec 2100 pro, Amersham Bioscience, Milan, Italy); syringaldazine oxidation was followed by measuring absorbance at 525 nm (ε_{525} = 65,000 M^{-1} cm^{-1} [22]). The assay mixture contained 50 mM potassium phosphate buffer pH 6, 50 μM syringaldazine and a suitable enzyme amount in a final volume of 3 mL. In control experiments, ABTS oxidation was monitored at 420 nm (ε_{420} = 36,000 M^{-1} cm^{-1} [23]). The assay mixture contained 50 mM potassium phosphate buffer pH 6, 100 μM ABTS and a suitable enzyme amount in a final volume of 3 mL.

2.2.2. Oxymetric assays

LC activity was also measured at 25 °C by monitoring oxygen uptake, which was determined using a Clark-type electrode coupled to an OXYG1 Hansatech oxygraph (Hansatech Instruments Ltd., King's Lynn, Norfolk, England).

2.2.3. Use of redox mediators

For certain experiments, some well known redox mediators were spectrophotometrically tested: a substrate (ARS, final concentration 300 μM) and a non-substrate (veratryl alcohol, final concentration 1 mM) were tested in the absence (control experiments) and presence of 1 mM of three mediators, for example, *N*-hydroxybenzotriazole, *N*-hydroxysuccinimide, and 4-hydroxyTEMPO. ARS bleaching was determined following the decrease in absorbance at 520 nm (ε_{520} = 7200 M^{-1} cm^{-1} [24]), and veratraldehyde formation was determined at 310 nm (ε_{310} = 9300 M^{-1} cm^{-1} [25]).

2.2.4. Protein content determination

Protein content was determined using the Blue Brilliant Coomassie G250 method [26], with bovine serum albumin as a standard.

2.3. LC purification

In a typical purification procedure, the culture medium from a 500 mL flask was collected and ultrafiltered using a Sartocon apparatus (Sartorius, Milan, Italy) equipped with polysulfone membranes (10,000 Da cutoff) at 4 °C. In order to achieve complete removal of low MW substances, 5 L of 50 mM potassium phosphate buffer and 50 mM 6-aminohexanoic acid (as a protease inhibitor) was added. At the end, LC solution was concentrated to approximately 500 mL.

Then the enzyme was prepared in 0.25 M NaCl and mixed with freshly prepared calcium phosphate gel [27] at 4 °C. In brief, the gel was prepared by slowly mixing 0.1 mol Na_3PO_4 and 0.2 mol $CaCl_2$ in 50 mL H_2O each. The pH was adjusted to 6 using 85% phosphoric acid. The gel was then collected by removing the supernatant after centrifugation at 8000 × *g* for 30 min.

The slurry was gently stirred for 30 min and the supernatant was recovered by centrifugation at 8000 × *g* for 30 min. NaCl was removed from the enzyme solution by ultrafiltration, using the same apparatus described above.

The resulting solution was then made in 0.2 M NaCl and loaded onto a DEAE-cellulose column (15 cm × 5 cm), which was pre-equilibrated with 50 mM potassium phosphate buffer pH 6 with 0.2 M NaCl. Three mL fractions were collected and analyzed using a syringaldazine assay and by measuring absorbance at 280 nm.

NaCl was removed from LC-containing fractions, as described above. Active fractions were pooled and loaded onto a DEAE-sepharose column (10 cm × 2.5 cm) that had been pre-equilibrated with 50 mM potassium phosphate buffer pH 6. Unbound proteins were removed by washing with the same buffer. LC elution was performed using a 50 mM potassium phosphate buffer with a linear gradient of NaCl (0–0.3 M).

LC-containing fractions were desalted and concentrated by ultradialysis using 10,000 Da molecular weight cut-off membranes (Sigma–Aldrich, Milan, Italy).

The last purification step was size exclusion chromatography using a HiLoad 16/60 Superdex 200 column (GE Healthcare, Milan Italy). FPLC Akta Prime (Amersham Bioscience, Milan, Italy) was used with a 0.4 mL/min constant flow equipped with a UV detector. Column equilibration and sample elution were performed with 50 mM potassium phosphate buffer pH 6 with 0.15 M NaCl. Collected fractions containing LC were then concentrated and desalted by ultradialysis and finally stored at −20 °C until used.

Purity was verified by native PAGE and SDS-PAGE using 12% polyacrylamide gels and the Laemmli protocol [28]. Isoelectric focusing, IEF, was performed using a 5% polyacrylamide gel and a pH range of 3–10 (Ampholine, Sigma, Milan Italy). Comparison with pI standards allowed to determinate the pI of LC.

2.4. LC characterization

The copper content of LC was determined using the method described by Kocharekar and Thakkar [29], in which the cupric complex of isonitrosopropiophenone thiosemicarbazone is spectrophotometrically quantified. The standard curve was prepared using copper (II) nitrate.

The sugar content was determined using the phenol/sulfuric acid method described by Dubois et al. [30] using glucose as the standard sugar.

The activity at different pH values was determined repeating the catalytic assay in the presence of different 25 mM McIlvaine buffers ranging from pH 3 to 9. The stability of the purified LC under different pH conditions was determined by incubating for 1 h the enzyme with 25 mM McIlvaine buffers ranging from pH 3 to 9, with increments of one pH unit. Afterwards, the pH was adjusted to 6 (with 1 M McIlvaine buffer pH 6) and the remaining catalytic activ-

ity was spectrophotometrically determined as described above. Thermostability was assayed by incubating the enzyme at the indicated temperature (±1 °C). Enzyme samples were quickly brought to 25 °C and the remaining catalytic activity was assayed as previously described.

Michaelis–Menten kinetic parameters were calculated using R 2.5.1 software (*R Foundation for Statistical Computing, Vienna*).

2.5. Mass spectroscopy

RP-HPLC–electrospray ionization-MS (RP-HPLC–ESI-MS) analyses were carried out using a Surveyor HPLC system (ThermoFisher, San Jose, CA, USA) connected by a T splitter to a photodiode array detector and the ESI/ion trap mass spectrometer LCQ Advantage (ThermoFisher).

The chromatographic column was a Vydac C4 (Grace Vydac, Hesperia, CA, USA) medium-bore column (2.1 mm × 150 mm, 5 μm particle size, 300 Å pore size). The following solutions were used for RP-HPLC–ESI-MS analysis: (Eluent A) 0.056% trifluoroacetic acid (TFA); (Eluent B) acetonitrile–water (80:20) containing 0.050% TFA. A linear gradient from 0 to 100% in 30 min, at a flow rate of 0.20 mL/min was applied. The T splitter addressed a flow-rate of 0.14 mL/min toward the diode array detector and 0.06 mL/min toward the ESI source. Electronebulization was performed at a flow rate of 10 μL/min with a sheath gas flow rate of 34 (arbitrary units). MS spray voltage was 4 kV and the capillary temperature was 280 °C. The ion-trap analyzer operated in positive mode in the 300–2000 *m/z* range and spectra were acquired every 3 ms. The photodiode array detector was set at 214 and 276 nm.

Molecular mass of purified LC as determined by deconvolution of average ESI mass spectra performed automatically using either the software provided with the MagTran 1.0 software [31].

2.6. Decolorization of dyes

LC decolorization of dyes was performed spectrophotometrically. Six different dyes, including Alizarin Red S (ARS, MW 342.2, λ_{max} 520 nm), phenosafranine (PNS, MW 322.80, λ_{max} 520 nm), xylenol orange (XO, MW 782.56, λ_{max} 435 nm), methylene blue (MB, MW 319.86, λ_{max} 660 nm), methyl green (MG, MW 472.51, λ_{max} 632 nm), and methyl orange (MO, MW 327.34, λ_{max} 464 nm) were used in this study. In a final volume of 3 mL, 50 mM potassium phosphate buffer, pH 6 and 1 E.U. of purified LC were incubated with 120 μM of ARS, 30 μM of PNS, 200 μM of XO, 15 μM of MB, 150 μM of MG, or 65 μM of MO.

A series of experiments was also performed by adding 8.8 mM hydrogen peroxide to the reaction mixture.

When horseradish peroxidase (HRP) was used, up to 20 E.U. were present in a final volume of 1 mL of 25 mM potassium phosphate buffer pH 7 and H_2O_2 8.8 mM. In the case of LiP, 0.2 E.U. were present in the final volume of 1 mL, and the H_2O_2 concentration was 0.176 mM.

Moreover, in order to mimick MnP action, other experiments were carried out using Mn(III) as the putative oxidizing agent. To this purpose, 1 mM manganese triacetate was dissolved in 50 mM sodium malonate buffer, pH 6.5 [32].

3. Results and discussion

3.1. LC induction and purification

A number of different compounds have been tested as putative LC inductors; their effects are reviewed in Fig. 1. Among the tested molecules, ferulic acid (FA) is, by far, the most efficient inductor. It is worth noting that the structure of FA is similar to that of coniferyl alcohol, the most abundant monolignol of the three lignin precursors. However, other guaiacyl compounds such as vanillyl alcohol and vanillic acid did not exhibit an inducing effect comparable to FA. This suggests that the C_6C_3 backbone of the phenylpropanoids may be required to effectively induce the LC isozyme(s) involved in lignin degradation. This finding is different from that of Murugesan et al. [33] who singled out 2,5-xylidine as the best inductor for laccase in *P. sajor-caju*. Clearly, a different fungal strain was used in that study, and unfortunately a comparison between the two described laccases is still not possible. Activity staining with syringaldazine of a native electropherogram of the crude culture medium revealed the presence of only two detectable isozymes. The more negative was by far more abundant while the other was lost since the calcium phosphate gel treatment. Therefore that minor component of the enzymic pattern was not longer present along the subsequent purification steps.

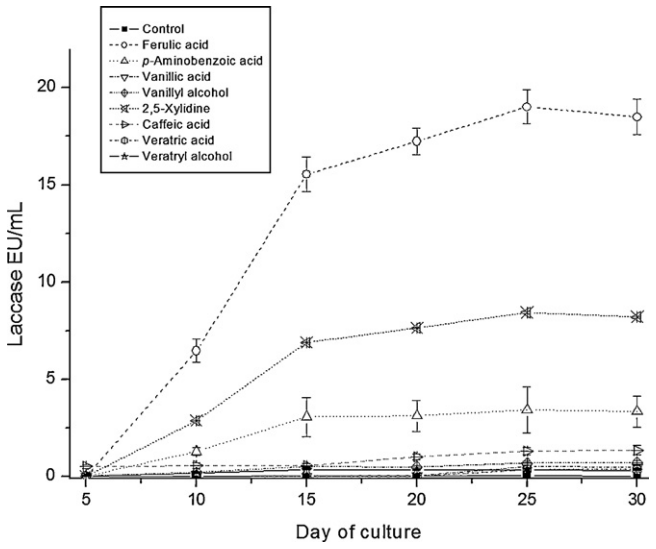

Fig. 1. Effect of inductors on LC production by *P. sajor-caju*. Ferulic acid was the most effective inductor among the tested chemicals because a more than 2 order of magnitude increase in LC production was measured when compared with a control culture. Each inductor was present at a final concentration of 10 mM.

Other than FA, *p*-aminobenzoic acid is one of the best LC inductors tested. This observation suggests that in addition to taking part in lignin metabolism, LC may be involved in other xenobiotic detoxification processes.

Surprisingly, 2,5-xylidine [33,34], one of the first discovered LC inductors, did not exhibit outstanding results in our strain of *P. sajor-caju*. In fact, it inhibited fungus growth to a certain extent (not shown). Therefore, we discontinued using this aromatic amine as an inductor.

Our primary goal was not to find the quickest response to an inductor, but to single out the molecule capable of inducing the maximum enzyme activity. This will be important for future LC production at industrial fermentor scale. Therefore, unlike other published studies, we tested enzyme activity in the cultures for 30 days (a maximum of production was observed after 24 days, as noted in another study [35]. The same (10 mM) inductor concentrations were tested for all the (putative) inductors. Once FA was determined to be the most effective inductor, other experiments were carried out to determine the optimal concentration of FA. Cultivation in the presence of 10 mM (approximately 2‰, *w/v*) FA for 20–25 days resulted in fast fungal growth with the highest LC production. Higher concentrations of FA resulted in fungal growth inhibition and a drop in LC production.

The enzyme was then purified as shown in Table 1. The first step, a batch treatment with calcium phosphate gel, was necessary to remove a large fraction of brown-blackish pigments that arose

Table 1
Summary of LC purification from the culture media of *Pleurotus sajor-caju*.

Purification step	V_{tot} (mL)	Total activity EU	Total protein (mg)	Specific activity	Purification fold	Percent recovery
Ultrafiltrated culture media	460	8369	10,506	0.80	1	100
Calcium phosphate gel	523	7689	769	10.0	12.5	92
DEAE cellulose	502	6660	507	13.1	16.3	80
DEAE sepharose	100	3191	4.00	798	998	38
Ultradialysis	6	2380	1.44	1653	2066	28
Superdex	3	1185	0.36	3290	4113	14

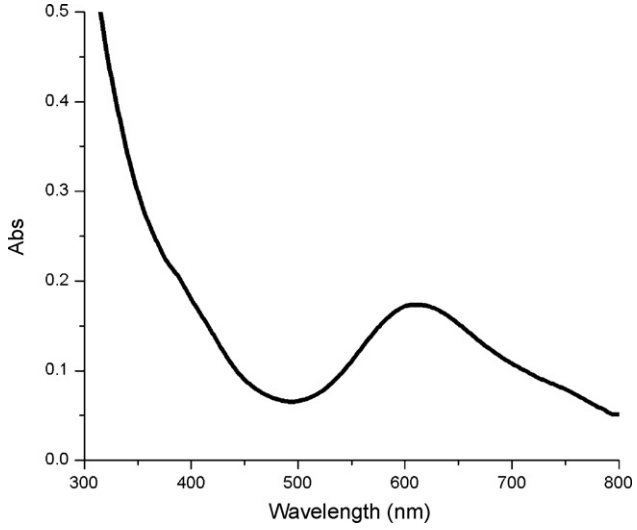

Fig. 2. UV–Vis absorbance spectrum of the purified LC. An absorbance peak at 610 nm and a broad shoulder around 330–350 nm suggest the presence of type I and type III copper metal ions [33].

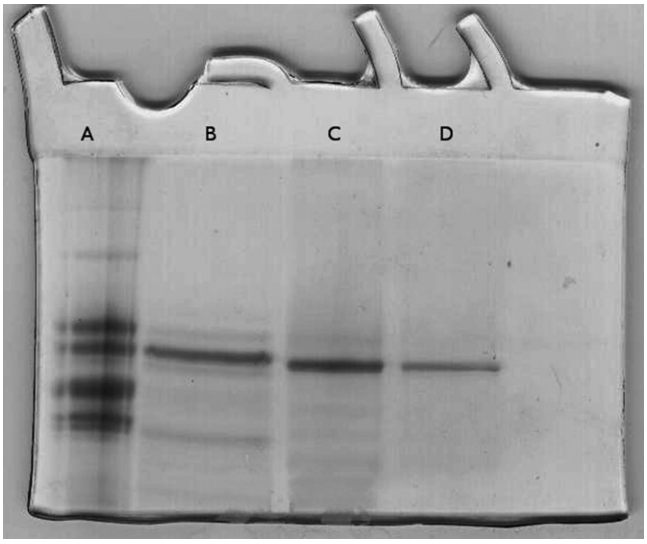

Fig. 3. SDS-PAGE of *P. sajor-caju* LC during purification steps. LC is visualized in Lane A after calcium phosphate gel treatment; Lane B after DEAE-cellulose ion exchange chromatography; Lane C after DEAE-sepharose ion exchange chromatography; and Lane D after Superdex size exclusion chromatography. Gels were performed following the Laemnli protocol [28] (stacking gel 4% and separating gel 12%) and Coomassie stained.

from FA oxidation by the produced LC. Further steps allowed the removal of the remaining pigments and contaminating proteins.

The purified enzyme is blue, similar to most LCs, with a relative maximum absorbance at 620 nm (Fig. 2), which is typical for type I cupric ions. The shoulder observed at approximately 330 nm is indicative of a type III dicupric cluster [33]. Type II copper cannot be observed by UV/Vis spectroscopy because its absorption is very weak, but it is present in all of the fungal LCs studied [5] and is in accordance with the copper content of the enzyme (*vide ultra*). Even if some characterization tests could be carried out on partially purified enzyme preparations from the DEAE–Sepharose column, the last gel filtration step (to eliminate a minor peak that eluted just prior to the enzyme) was essential to obtain stable LC preparations. This observation suggests that the minor peak corresponded to a proteolytic enzyme that caused gradual activity loss, even at low temperatures. This was confirmed by SDS-PAGE. In samples where this contaminating protein had not been removed, a number of low molecular weight spots arose with time (not shown).

When completely purified, the enzyme could be frozen and stored for months at −20 °C without appreciable loss of activity.

3.2. Molecular weight, pI, copper and sugar content

The enzyme is monomeric, with a MW*r* of 61 kDa (SDS-PAGE, Fig. 3) or 55.340 ± 0.005 kDa (RP-LC–ESI-MS). This is similar to other LCs from white-rot fungi [4].

P.sajor-caju LC is an acidic protein, similar to other fungal LCs and has a pI of 3.2.

As expected, *P. sajor-caju* LC is a glycoprotein that contains 6.7 ± 0.3% sugar (expressed as glucose). This is similar to other LCs from white-rot fungi [4], which usually exhibit a sugar content lower than LCs from plants.

The copper content was determined to be 0.00435% (corresponding to 3.8 cupric ions per protein molecule), in a good agreement with the theoretical 4 cupric ions per molecule.

3.3. Substrate specificity

Activity assays using a large variety of putative substrates confirmed the wide specificity of the enzyme, that has been reported previously for other fungal LCs [36]. Because it is well known that many substrates give rise a complex mixture of products, which are often unknown, with very different and not comparable ε values, oxygen consumption was measured in the current study to obtain more reliable and coherent results (Table 2). However, because one cannot exclude the non-enzymatic oxygen consumption by unstable intermediates arising from LC action on the primary substrates, a certain margin of error still exists for oxymetric measurements. However, at pH 6 non-enzymatic oxygen consumption by such putative intermediates should be rather low. In spite of this, oximetry represents a substantial improvement over the largely arbitrary photometric analysis.

Table 2 shows that syringaldazine is not only the most useful substrate because of its exceptionally high ε value, but it is also the preferred substrate of the enzyme, closely followed by ABTS (results were confirmed by determination of kinetic constants; as shown in Table 3, syringaldazine has a quite low K_M [33]). Besides being a very efficient enzyme inductor, FA is also a good substrate. Syringic (4-hydroxy-3,5-dimethoxybenzoic) and caffeic (*m*,*p*-dihydroxycinnamic) acids also are fairly good substrates, as is *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine. Simple polyphenols, such as pyrogallol (1,2,3-trihydroxybenzene), cate-

Table 2
Substrate oxidizing activity of purified LC. Each substrate was present at a final concentration of 1 mM. Enzyme activity was measured by oxygen uptake determined using an oxygraph.

Substrate	Relative activity %
Syringaldazine	100
ABTS	96
Ferulic acid	50
Syringic acid	47
NNN′N′-Tetramethyl-*p*-phenylenediamine	41
Caffeic acid	37
Catechol	36
Pyrogallol	34
N,N-Diethyl-*p*-phenylenediamine	34
p-Phenylenediamine	33
Gallic acid	31
4-Methyl-catechol	31
Vanillic acid	31
4-*tert*-Butylcatechol	20
Hydroquinone	20
Protocatechuic acid	17
Phloroglucinol	14
Resorcinol	13
o-Phenylenediamine	5

Table 3
Determination of kinetic parameters for LC purified from *P. sajor-caju*. Syringaldazine and ABTS were used as substrates, and non-linear regression was performed using R 2.5.1 software (*R Foundation for Statistical Computing, Vienna*). $n = 5$.

Substrate	Kinetic parameter	Value
Syringaldazine	K_M	2.96 ± 0.45 μM
	k_{cat}	3.42 ± 0.12 s^{-1}
	k_{cat}/K_M	1.16 ± 0.22 μM^{-1} s^{-1}
ABTS	K_M	88.9 ± 4.2 μM
	k_{cat}	3.29 ± 0.22 s^{-1}
	k_{cat}/K_M	0.037 ± 0.004 μM^{-1} s^{-1}

chol (1,2-dihydroxybenzene) and quinol (1,4-dihydroxybenzene), were oxidized at reasonable rates. However, methyl and especially *tert*-butyl substituents on the aromatic rings adversely affected reactivity. Surprisingly, *m*-polyphenols, such as resorcinol (1,3-dihydroxybenzene) and phloroglucinol (1,3,5-trihydroxybenzene), were also oxidized by this LC. Of the aromatic amines tested, *o*-phenylenediamine is very slowly oxidized. As expected, methoxybenzenes, such as veratrole (1,2-dimethoxybenzene) and veratric (3,4-dimethoxybenzoic) acid, behave as nonsubstrates. On the whole, this LC exhibits a wide substrate specificity and could be a powerful tool for many technological applications.

With respect to dyes, the degradative ability of LCs from some white-rot fungi has been described previously [37,38].

Because of enzyme-promoted dye bleaching future technical applications, the action of *P. sajor-caju* LC was studied and compared to that of some peroxidases. Diverse behavior has been observed for different enzymes and dyes (Table 4).

Table 4
Bleaching of textile dyes by LC. For comparison, the effect of other ligninolytic enzymes is reported.

Dye	Conversion 1 h%				
	Lc	Lc + H_2O_2	HRP	LiP	Mn^{3+}
ARS	76	85	5	23	42
PNS	1	4	0	57	3
XO	29	22	15	2	14
MB	4	8	12	15	2
MG	50	75	99	37	67
MO	6	1	18	17	9

Table 5
The effect of well-known redox mediators on LC oxidation of ARS and VA. Results are expressed in μM/min.

	ARS	VA
No redox mediator	77.4	0.20
1-Hydroxybenzotriazole	62.3	0.00
4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl	83.1	0.20
N-Hydroxysuccinimide	81.9	0.41

ARS is a hydroxyquinone dye, bearing a catechol motif that is probably the target of LC action. This dye is efficiently degraded by the enzyme: the putative diquinone intermediate easily undergoes degradation (following nucleophilic attack by water) without the need of a further LC intervention. Addition of H_2O_2 greatly enhances LC action because hydrogen peroxide speeds up diquinone degradation [20].

The HRP redox potential might be too low for an efficient ARS attack. LiP is somewhat more effective but Mn^{3+} is more active, suggesting that MnP could be used for ARS bleaching.

PNS (a phenylphenazinium dye) is quite resistant to LC, regardless of the presence of H_2O_2. The only enzyme that exhibits a detectable effect on this inert compound is LiP, which is quickly inactivated during its action [39].

As a complex, substituted *o*-aminophenol, that XO is a fair LC substrate is not surprising. Moreover, among the tested enzymes, LC is the best bleaching catalyst for this compound. However, the adverse effect of hydrogen peroxide remains to be explained.

Under the experimental conditions, MB and MO were both poor LC substrates, although the addition of H_2O_2 speeds their degradation. However, they are degraded (although not efficiently) by both HRP and LiP.

In spite of being a dication, which should make electron extraction by any oxidizing agent unlikely, MG is rapidly degraded under all the experimental conditions tested. The addition of H_2O_2 also significantly enhanced the rate of degradation.

In conclusion, in selected cases, LC could be a potential biotechnological tool for industrial dye bleaching. Because of its wide substrate specificity, LC is more promising than other ligninolytic enzymes.

The use of redox mediators that widen the specificity of LCs to nonsubstrates such as nonphenolic lignin substructures and/or other compounds lacking the phenolic (or aromatic amine) functional group has become very popular in recent years because it could open new fields for LC applications [12,37].

In the current study, some water-soluble N–OH type redox mediators were chosen from those available and tested in the presence of ARS (a good LC substrate) and veratryl alcohol (VA, a nonsubstrate, although commercial samples usually contain a trace amount of the substrate vanillyl alcohol, which accounts for an apparent slow activity), as shown in Table 5. On the whole, their action is not very pronounced. Moreover the widely used *N*-hydroxybenzotriazole decreased the catalytic activity of the enzyme. Because LC activity remained unchanged or was reduced by their use, the use of redox mediators was rather disappointing. Only *N*-hydroxysuccinimide gave more promising results during VA oxidation. This is in good agreement with the fact that the employment of N–OH type redox mediators during lignin and lignin-model compound oxidation is well known [40].

3.4. LC stability

The enzyme is moderately thermostable, and its catalytic activity remained almost unaffected after an 8 h incubation at 30 °C. In addition, approximately 60% of the original activity was still present after 8 h at 40 and 50 °C, whereas approximately 40% activity sur-

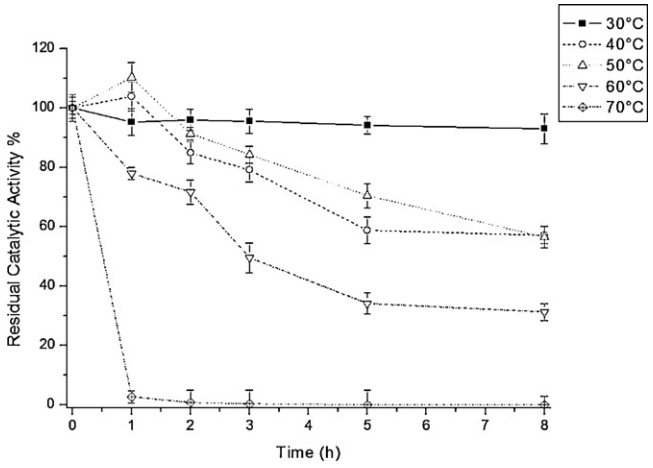

Fig. 4. Thermal stability of LC purified from *P. sajor-caju*. $n = 5$.

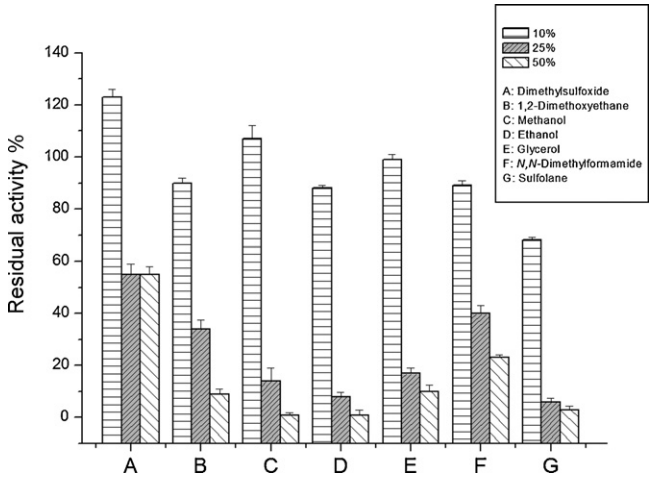

Fig. 6. Stability of LC purified from *P. sajor-caju* in the presence of various organic solvents. $n = 5$.

vived after 8 h at 60 °C. LC was completely inactivated within 1 h at 70 °C (Fig. 4).

In spite of being a quite acidic protein, enzyme activity was negligible at pH 3–4, a sharp maximum was found at pH 6, and activity was nearly undetectable at pH 8. In contrast, the enzyme remained rather stable within the pH range 3–9, with a slight preference for alkaline conditions (Fig. 5). In conclusion, purified LC exhibited a remarkable temperature and pH stability, making it a promising industrial prospect.

LC activity was also assayed in the presence of water-miscible organic solvents that could help dissolve some substrates and therefore widen the application field of the enzyme. These results are summarized in Fig. 6. Sulfolane had an adverse action on the enzyme, even at the lowest concentration tested. LC was sensitive to methanol and ethanol: its activity, which was nearly unchanged at the lowest concentration, was virtually abolished in 1:1 water/alcohol mixtures. More activity was retained in the presence of dimethoxyethane and glycerol. Dimethylformamide and especially dimethylsulfoxide proved to be the most compatible with LC.

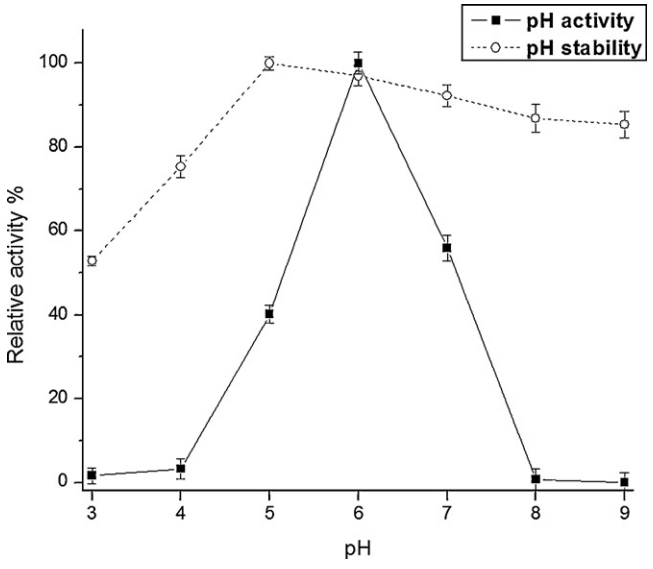

Fig. 5. Determination of optimal pH activity and pH stability of purified LC. $n = 3$.

4. Conclusion

Induction of LC production in *P. sajor-caju* by FA was found to be a convenient way to obtain large amounts of the enzyme. This enzyme has shown broad substrate specificity, high levels of activity within a reasonable pH range, and is stable at moderately high temperatures. Purified LC also exhibited a promising potential in the degradation of some recalcitrant textile dyes. Further studies are in progress in order to exploit the potential of this LC in this field and in the remediation of phenolics in wastewater.

Acknowledgments

This research was funded in part by the Italian Ministry of University and Research (FIRB Project RBAU01CJP9) and by the Regione Autonoma Sardegna (PO Sardegna FSE 2007–2013, L.R.7/2007 "Promozione della ricerca scientifica e dell'innovazione tecnologica in Sardegna"). These had no any role for the conduct of the research and/or preparation of the article; in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

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