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Heterologous production, characterization and dye decolorization ability of a novel thermostable laccase isoenzyme from *Trametes trogii* BAFC 463

Paula A. Campos[a], Laura N. Levin[b], Sonia A. Wirth[a,*]

[a] *Laboratorio de Agrobiotecnología, Departamento de Fisiología, Biología Molecular y Celular, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, IBBEA-CONICET-UBA, Piso 2, Pabellón 2, Ciudad Universitaria, C1428EGA Buenos Aires, Argentina*
[b] *Laboratorio de Micología Experimental, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, PROPLAME-PRHIDEB-CONICET, Piso 4, Pabellón 2, Ciudad Universitaria, C1428EGA Buenos Aires, Argentina*

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ABSTRACT

Laccases are multicopper polyphenol oxidases that are able to catalyze the oxidation of a wide range of phenolic compounds with the simultaneous reduction of O_2 to H_2O. Despite their promising industrial uses, feasible incorporation of laccases in harsh processes requires the bioprospecting and/or engineering of enzymes to be stable and active in acidic or alkaline pHs, high temperatures, oxidative conditions and tolerant to high salinity and/or organic solvents. Here we used a PCR-based screening to clone two novel laccase coding sequences from the white-rot basidiomycete *Trametes trogii*. Recombinant expression of *lcc3* gene in *Komagataella* (=*Pichia*) *pastoris* showed that it encodes a thermo active and thermostable laccase with an optimum temperature of 50 °C and with a half-life of 45 min at 70 °C and a stability higher than 3 h at 60 °C. Furthermore, recombinant LCC3 was capable of decolorizing between 50% and 100% of indigoid, triarylmethane, azoic and anthraquinonic synthetic dyes in the presence of the natural redox mediator acetosyringone within 2 h of incubation at pH 6 and 70 °C.

1. Introduction

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are multicopper polyphenol oxidases that are able to catalyze the oxidation of a wide range of phenolic compounds and aromatic amines with the concomitant four-electron reduction of O_2 to H_2O [1,2]. This property makes them excellent candidates for biotechnological applications such as selective delignification in pulp bleaching, degradation of textile dyes and other xenobiotics generated by industrial processes, development of biosensors and biofuel cells, conversion of lignocellulosics for biofuel production and organic synthesis [3–10]. Laccases are widely distributed in nature, although enzymes from wood-decay fungi are the most studied. Fungal laccases are generally three-domain monomeric extracellular glycoproteins containing four copper atoms per molecule classified according to their spectroscopic characteristics and coordinated by 10 conserved histidine residues [11]. A type 1 (T1) mononuclear copper is responsible for the electron transfer from the substrate to a trinuclear reaction center composed by one type 2 copper atom (T2) and two type 3 copper atoms (T3) where O_2 reduction takes place [2,12]. The T1 copper is coordinated by two conserved histidines and a cysteine in a trigonal geometry and by a fourth non-coordinating axial ligand that in fungal laccases is either a leucine or a phenylalanine residue. The oxidative ability of laccases is mainly determined by the differences in redox potential between the substrate and the enzyme, being possible to extend its substrate range to non-phenolic compounds in the presence of small molecular weight molecules acting as redox mediators [13,14]. In most fungi, laccases are expressed as multiple isoforms, depending on the species, strain and culture conditions such as C:N ratio, presence of inducers, temperature, etc., although the biological implications for these redundancy is still unknown [11,15]. Although many fungal laccases have been isolated and characterized in the last years, much more effort is being done to find more accurate enzymes tailored to specific industrial processes and to high production systems. Since industrial enzymes are frequently required to be incorporated in harsh processes, characteristics such as activity in acidic or alkaline pHs, high temperature, oxidative conditions and tolerance to high salinity and/or organic solvents are highly desirable. Bioprospecting for new laccases and

* Corresponding author.
E-mail address: sonia.wirth@gmail.com (S.A. Wirth).

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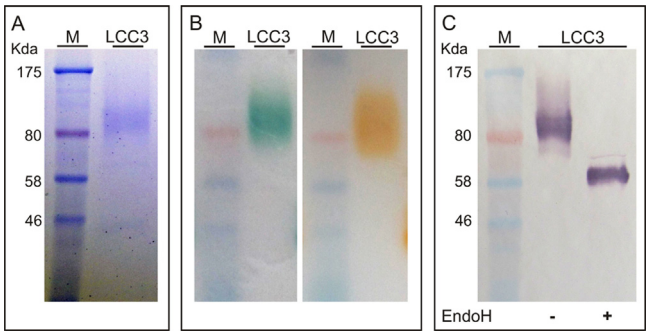

Fig. 1. Electrophoretic characterization of recombinant LCC3 expressed in *K. pastoris* (A) SDS-PAGE of purified LCC3 stained with Coomassie Brilliant Blue. (B) Native PAGE incubated with 5 mM ABTS (left) or 10 mM DMP (right) (C) western blot of crude laccase protein extracts (−) and extracts digested with EndoH endoglycosidase (+), revealed with anti-his tag antibody. M: Colorplus™ Prestained Protein Marker (New England Biolabs).

organisms that produce them is based on the fact that despite the high conservation of their amino acid sequences, these phenol oxidases belong to a diverse protein family and even isoenzymes expressed in the same species may have very different biochemical properties.

Trametes (=*Funalia*) *trogii* is a worldwide-distributed white-rot basidiomycete that has been recognized as an excellent source of ligninolytic enzymes. Production of manganese peroxidase (MnP), lignin peroxidase (LiP) and auxiliary H_2O_2-producing oxidases such as glyoxal oxidase was described in this fungus; nevertheless the more outstanding property of *T. trogii* is its ability to express high levels of laccases [16–21]. In *T. trogii*, two different laccase coding genes have been cloned from strain 201 and the presence of at least five isoenzymes has been proposed, suggesting that an extensive number of laccases is present in this species [22,23]. Studies performed in *T. trogii* BAFC 463 have shown the expression of at least two laccase isoenzymes and its ability to degrade wood lignin and organic pollutants such as nitrobenzene and anthracene as well as anthraquinonic dyes and polychlorinated biphenyls, demonstrating its potential for detoxification of xenobiotics [20,24–26]. Extracellular extracts of *T. trogii* BAFC 463 have demonstrated an extraordinary laccase activity (110 U/ml) with a half-life of more than 2 h at 60 °C [18,27]. Crude culture fluids have been assayed for the biobleaching of loblolly pine Kraft pulp and crude extracts as well as the purified laccase showed high decolorization rates of azoic, indigoid, triphenylmethane, anthraquinonic and heterocyclic dyes even at pH 7 and 70 °C and in the absence of redox mediators [18,28,29]. Additionally, a purified laccase of *T. trogii* BAFC 463 has been successfully used for the generation of biofuel cells [30,31].

In order to elucidate if high thermostable laccase activity of *T. trogii* BAFC 463 is due to the expression of a particular set of isoenzymes, we performed a PCR-based screening to clone the laccase coding genes expressed in copper induced cultures. Using this approach, we achieved to clone two novel laccase coding sequences (*lcc3* and *lcc4*) not previously reported for *T. trogii*. Here, we describe the recombinant expression in *Komagataella* (=*Pichia*) *pastoris* of the native *lcc3* cDNA sequence, demonstrating it encodes a thermostable laccase with the ability of decolorize indigoid, triarylmethane, azoic and anthraquinonic dyes in the presence of the natural redox mediator acetosyringone.

2. Material and methods

2.1. Fungal strain and culture conditions

Trametes (*Funalia*) *trogii* (Polyporaceae, Aphyllophorales, Basidiomycetes) strain BAFC 463 (*Funalia trogii* ATCC MYA2811) was obtained from the BAFC Mycological Culture Collection of the Department of Biological Sciences, Faculty of Exact and Natural Sciences, University of Buenos Aires. Stock cultures were maintained on malt extract agar slants at 4 °C.

Table 1
Kinetic constants of purified LCC3 determined at pH 4 and 30 °C.

Substrate	Km (μM)	Kcat (seg^{-1})	Kcat/Km (μM^{-1} seg^{-1})
ABTS	250 ± 9	399 ± 20	1.59
DMP	2095 ± 63	329 ± 32	0.16

Culture conditions for laccase induction were as previously described [19,27]. Four 25-mm^2 surface agar plugs from a 7-day-old culture grown on malt agar (1.3% malt extract, 1% glucose, 2% agar) were inoculated in 500 ml Erlenmeyer flasks containing 50 ml of GA medium (2% glucose, 0.5 g/l $MgSO_4{\cdot}7H_2O$, 0.5 g/l KH_2PO_4, 0.6 g/l K_2HPO_4, 0.09 mg/l $MnCl_2{\cdot}4H_2O$, 0.07 mg/l H_3BO_3, 0.02 mg/l $Na_2MoO_4{\cdot}H_2O$, 1 mg/l $FeCl_3$, 3.5 mg/l $ZnCl_2$, 0.1 mg/l thiamine hydrochloride, 3 g/l asparagine monohydrate and 1 mM $CuSO_4$). Initial pH of the medium was adjusted to 6.5 with 1 N NaOH. Incubation was carried out statically at 28 °C. Cultures were harvested at day 22 when laccase activity peaked (110 U/ml) and filtered through a filter paper using a Büchner funnel and mycelia was used for total RNA extraction.

2.2. RNA extraction and cDNA synthesis

Filtered fungal mycelium was ground into fine powder using a combination of RNAse free sea sand and liquid nitrogen. Total RNA was extracted using RNAzol RT reagent (Molecular Research Center Inc., Cincinnati, USA) according to the manufacturerís instructions. The quantity of RNA was estimated by Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies) and RNA quality was determined by formaldehyde RNA gel electrophoresis. Total RNA was treated with RNAse free DNAse I (New England Biolabs Inc., UK) and mRNAs isolated using PolyATtract® mRNA Isolation System (Promega, Madison, WI, USA). Total cDNA was synthetized using ImProm-II™ Reverse Transcriptase (Promega, Madison, WI, USA) and oligodTVN primer, according to manufacturerís instructions.

2.3. PCR and RACE PCR

For laccase cDNA amplification we designed four degenerate oligonucleotide primers recognizing the conserved sequences in copper-binding regions I to IV [32]. Combinations of forward (LacR1 5′-ACNWSNATHCAYTGGCAYGG-3′ and LacR2 5′-GGIACITTYTGGTAYCAY-3′) and reverse (LacR3 5′-CCRTGNARRTGDAWNGGRTG-3′ and LacR4 5′-RAARTCDATRTGRCARTG-3′) primers were assayed for PCR amplification. Amplified fragments of the expected sizes were gel-purified and cloned in pGEM®-T Easy Vector (Promega, Madison, WI, USA) for sequencing (Macrogen Inc). Partial coding sequences showing homologies with genes encoding fungal laccases found in NCBI Database were selected and used to design internal gene specific oligonucleotide primers for 5′-RACE and 3′-RACE PCR. For 3′-RACE PCR, template cDNA was synthesized using an oligo dT anchored primer (dTR 5′-GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTV-3′) and used for PCR amplification using forward laccase gene specific primer and reverse primer recognizing the adaptor sequence in anchored primer (5′-GACCACGCGTATCGATGTCGAC-3′). For 5′ RACE PCR, total cDNA template was subjected to incorporation of a C tract on 5′ end using Terminal Deoxynucleotidyl Transferase enzyme (Thermo Scientific) according to manufacturer's instructions, and PCR amplified using laccase gene specific primer and a 5′ poly G anchored primer (5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′),

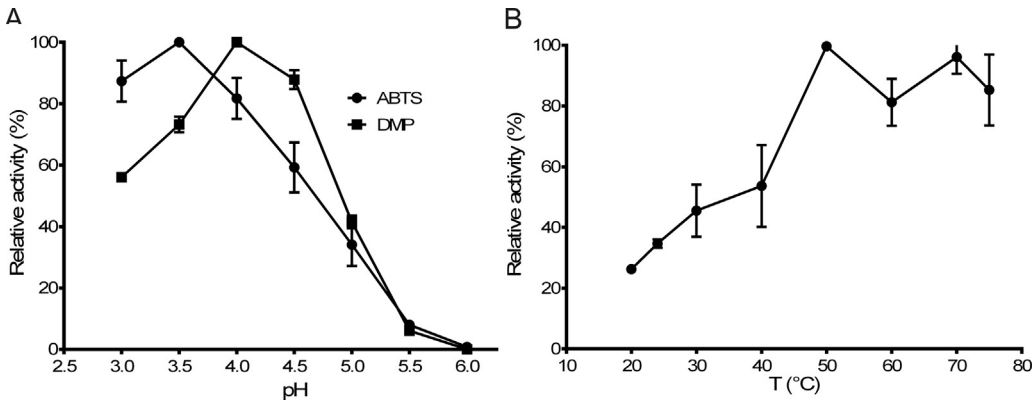

Fig. 2. Temperature and pH activity profiles for LCC3. (A) pH Profiles for ABTS (closed circles) and DMP (closed squares) determined in citrate-phosphate buffer at 30 °C (B) temperature profile using ABTS as substrate at pH 4.5. Activity is represented as % relative to the maximum, assigned as 100%. Error bars correspond to standard deviation for triplicates.

followed by a second amplification using anchored primer (5′-GGCCACGCGTCGACTAGTAC-3′). Amplified fragments of the expected sizes were gel-purified and cloned in pGEM®-T Easy Vector (Promega, Madison, WI, USA) for sequencing (Macrogen Inc.). Contigs obtained were assembled using CAP3 Sequence Assembly Program [33] for *in silico* sequence analysis and primer design for cloning.

2.4. Recombinant laccase expression in K. pastoris

Complete *lcc3* coding sequence was amplified by PCR using a 5′ primer incorporating a BamHI restriction site (L3TtPICFw 5′-GGATCCAAACGATGTTGCGCACTCGCAC 3′) and a 3′ primer incorporating a sequence coding for a 6 histidine tag and a EcoRI restriction site (L3TtPICRv 5′-GAATTCCTAATGGTGATGGTGATGGTGACTAGTCGGGTTCTCCG CGTAG-3′) for cloning in pPIC9 expression vector (Invitrogen Life Technologies Inc.), replacing the α-factor signal sequence. Recombinant vector linealized with DraI was used for transformation of *K. Pastoris* strain GS115 (Invitrogen Life Technologies) by electroporation. Recombinant clones reverting histidine auxotrophy were first selected on minimal medium MD plates (0.34% yeast nitrogen base without amino acids, 10 g/l $(NH_4)_2SO_4$, 2% dextrose and 2% agar) and then by laccase secreted activity on minimal medium MM plates (0.34% yeast nitrogen base without amino acids, 10 g/l $(NH_4)_2SO_4$, and 2% agar) supplemented with 2 mM ABTS (2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate) or 10 mM DMP (2,6-dimethoxyphenol). Induction of AOX1 (*K. pastoris* alcohol oxidase 1) promoter was achieved by adding 100 μl of 100% methanol to plate lid. Transformed clones showing green (ABTS) or orange (DMP) oxidation halos were selected and conserved on MD or YPD (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) agar slants.

For laccase production pre-inoculums were generated in 5 ml of YPD medium and used as seed to inoculate 25 ml of BMGY (1% yeast extract, 2% peptone, 0.34% yeast nitrogen base without amino acids, 10 g/l $(NH_4)_2SO_4$, 400 mg/l biotin, 1% glycerol, 100 mM potassium phosphate buffer, pH 6.0) in 250 ml shake flasks and cultivated for 48 h at 30 °C and 220 rpm. Cells were harvested by centrifugation 5 min at 1500*g* and resuspended in BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer, pH 6.0, 0.34% yeast nitrogen base without amino acids, 10 g/l $(NH_4)_2SO_4$, 400 mg/l biotin, 200 μM $CuSO_4$ and 3% sorbitol) to a final OD600 nm = 10 and cultivated in 1000 ml shake flasks at 28 °C and 220 rpm. Sterile methanol (0.5% final) was added every 24 h to maintain induction conditions.

2.5. Purification of recombinant laccase

K. pastoris cultures were harvested after 4 days of incubation in BMMY and centrifuged at 1500*g* for 10 min. The supernatant was concentrated by ultrafiltration (30 kDa MWCO, Amicon Ultra, Merck Millipore) and buffer exchanged to 50 mM sodium phosphate buffer, pH 8. Laccase was purified by gravity flow Ni-NTA affinity chromatography using His select nickel affinity gel (Sigma Chemical Co., USA). Binding was performed in equilibration buffer (300 mM NaCl, 50 mM sodium phosphate buffer, pH 8) followed by two washes with washing buffer (300 mM NaCl, 50 mM sodium phosphate buffer, pH 6.5) and eluted with washing buffer containing 250 mM imidazole.

2.6. Polyacrylamide gel electrophoresis and immunoblotting

Purified recombinant laccases were separated by 12% SDS-PAGE under reducing conditions and visually identified by Coomassie Blue staining or transferred to 0.45 μm nitrocellulose membranes (Bio-Rad Laboratories Inc., USA). The membranes were probed with 0.1 μg/ml of polyclonal rabbit anti-HIS antibody (Genescript, USA), followed by a second incubation step with alkaline phosphatase-linked goat anti-rabbit antibody diluted to 1:15000 (Sigma Chemical Co., USA) and phosphatase activity detected by a chromogenic reaction using 5 bromo-4 chloro-3 indolyl phosphate and nitroblue tetrazolium as substrates (Sigma Chemical Co., USA).

Native-PAGE was performed in 12% acrylamide gels at pH 8.8 under non-denaturing conditions. After running, gels were soaked in 100 mM sodium acetate buffer pH 4.5 for 10 min and transferred to 2 mM ABTS or 10 mM DMP solutions in 100 mM sodium acetate pH 4.5 to reveal laccase oxidative activity.

2.7. Deglycosylation assay

For *N*-glycan removal, recombinant laccases were denatured in 0.5% SDS, 40 mM DTT at 100 °C for 5 min and incubated with endoglycosidase H (Endo Hf, New England BioLabs, USA) according to manufacturer's instructions. The deglycosylated laccase was separated by SDS-PAGE and analyzed by immunoblot assay.

2.8. Enzyme activity determination assays

Laccase activity was estimated by monitoring the absorbance change at 420 nm for 1 mM ABTS oxidation (ε = 36 mM^{-1} cm^{-1}) or 469 nm for 5 mM DMP oxidation (ε = 27.5 mM^{-1} cm^{-1}) in Mc Ilvaine's buffer (citrate-phosphate), pH 4.5 at 30 °C [34,35]. One unit of enzymatic activity was defined as the amount of enzyme

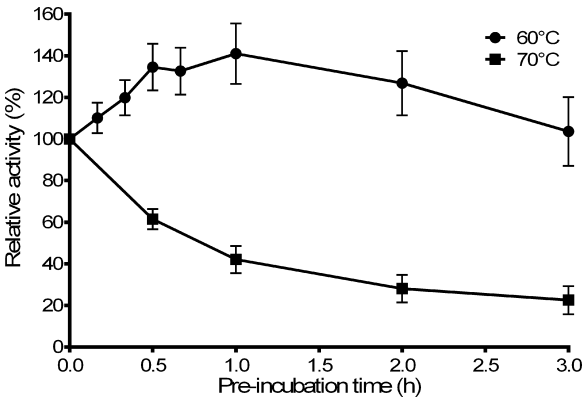

Fig. 3. Thermostability and thermal activation of LCC3. ABTS substrate oxidization activity after pre-incubation of purified LCC3 at 60 °C (closed circles) or 70 °C (closed squares) at the indicated times in citrate-phosphate buffer pH 6. Activity is represented as % of To, assigned as 100%. Error bars correspond to standard deviation for triplicates.

transforming 1 μmol of substrate per min. at the indicated pH and temperature. For temperature and pHs profiles reactions were performed either in citrate-phosphate buffer pH 6 at 24 °C, 30 °C, 40 °C, 50 °C, 60 °C, 70 °C or in citrate-phosphate pH 3, 3.5, 4, 4.5, 5, 5.5, 6, and 7 at 30 °C. Thermostability assays were performed pre-incubating purified laccase at 30 °C, 60 °C or 70 °C in citrate-phosphate buffer pH 6 for the indicated times and ABTS oxidation determined at 30 °C and pH 4.5.

The Michaelis–Menten kinetic constants were determined for laccase oxidative activity on ABTS (range of concentration 25 μM to 300 μM) and DMP (range of concentration 0.25 mM to 3 mM) at pH 4 and 30 °C by non linear regression from curves of initial velocity vs substrate concentration, using GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

2.9. Decolorization of synthetic dyes

Decolorization activity was determined by measuring the decrease in absorbance at the indicated wavelength for each dye and expressed as % of absorbance at each time point of control reactions without the addition of enzyme incubated in the same conditions of pH and temperature. Triarylmethane dyes: Malachite green, (617 nm) and Aniline blue (Acid blue 22, 590 nm); indigoid: Indigo Carmine (Acid blue 74, 610 nm); azoic dyes: Xylidine Ponceau (Acid Red 26, 497 nm) and Orange G (Acid Orange 10, 497 nm); anthraquinonic: Remazol Brilliant Blue R (RBBR, Reactive Blue 19, 590 nm); heterocyclic: Azure B (650 nm). Reactions were performed using 50 μm of dye in citrate-phosphate buffer pH 4.5 at 30 °C and laccase at final concentration of 1 U/ml or 10 U/ml. Redox mediators ρ-coumaric acid (COU), 1-hydroxybenzotriazole (HBT), and violuric acid (VA) were used at a ratio of 4:1 with dyes (200 μM). For acetosyringone (ASG) ratios of 4:1 (200 μM), 2:1 (100 μM), 1:1 (50 μM) and 0.1:1 (10 μM) with dyes were evaluated.

To test the effect of pH on dye decolorization, reactions containing 50 μm of Indigo Carmine, Malachite Green, Azure B, Remazol Brilliant Blue R or Xylidine Ponceau, 1 U/ml of LCC3 and 200 μM ASG were performed in citrate-phosphate buffer pH 3.5, 4.5, 6 or 7 and incubated at 30 °C and absorbance was measured after 1 h and 2 h. To evaluate the effect of the temperature on dye decolorization reactions were incubated in citrate-phosphate buffer pH 4.5 at 50 °C, 60 °C or 70 °C.

2.10. Data availability

Data of *T. trogii* BAFC 463 *lcc1*, *lcc2*, *lcc3* and *lcc4* nucleotide sequences was deposited at DDBJ/EMBL/GenBank under the accession numbers KU055621, KU055622, KU055623 and KU055624, respectively. Accession numbers for the corresponding encoded protein sequences are AMJ39538 (LCC1), AMJ39539 (LCC2), AMJ39540 (LCC3) and AMJ39541 (LCC4).

3. Results

3.1. Cloning of laccase coding sequences from T. trogii BAFC 463

In order to clone the coding sequences for the isoenzymes responsible for laccase activity in *T. trogii* BAFC 463, we performed a PCR-based screening in a similar approach as previously described [36,37]. Through the analysis of the reported sequences for the L1 to L4 characteristic laccase signatures for copper-binding regions found in characterized isoenzymes of *Trametes* genera, we designed four degenerate primers (two forward primers for regions I and II and two reverse primers for regions III and IV) for PCR amplification of *T. trogii* cDNA. Cloning and sequencing of PCR products followed by 5′-RACE PCR and 3′-RACE PCR techniques allowed us to complete four different cDNA sequences showing homology with other fungal laccase coding sequences reported in GenBank Database. Sequence *lcc1* (GenBank KU055621) encodes a protein with 100% amino acid identity with previously reported LCC1 of *T. trogii* strain 201 (GenBank CAC13040, [22]). Sequence *lcc2* of *T. trogii* BAFC 463 (GenBank KU055622) encodes a protein with 99% amino acid identity with the reported sequence for LCC2 of *T. trogii* 201 (GenBank CAL23367, [23]), with 4 amino acid substitutions in mature translated protein sequence: D14N, I186L, A251 V, T433A (Fig. S1). *In silico* analysis of the other two sequences, correlatively named *lcc3* and *lcc4,* showed they encode novel isoenzymes for *T. trogii*. Sequence *lcc3* (GenBank KU055623) encodes a pre-protein of 517 amino acids with 97% and 94% identity with two previously cloned laccases of the related species *Coriolopsis gallica* (TgLac3, GenBank AHM10329 [38] and cgLcc1 GenBank AAF70119 [39], respectively). Encoded LCC3 includes the eight conserved histidine residues involved in coordination of T2/T3 trinuclear copper and the two His and a Cys (H395, H456, C451) involved in coordination of T1 copper in fungal laccases [12,32]. As non-coordinating axial ligand of T1 copper, LCC3 showed a Leu (L461) instead of the Phe more frequently found in high redox potential laccases of basidiomycetes. Firsts 23 amino acids of LCC3 determine a putative signal peptide as predicted by SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/) and 4 N-X-S/T sequons (positions 54, 290, 361 and 434 of mature protein) are predicted to be N-glycosylated according to Net-glyc 1.0 program prediction (http://www.cbs.dtu.dk/services/NetNGlyc/) (Fig. S1). Sequence *lcc4* (GenBank KU055624) encodes a pre-protein of 520 amino acid length with more than 80% amino acid identity with laccases of *Trametes* sp. I-62 (GenBank AAQ12270) *Trametes versicolor* FP-101664 SS1 (GenBank XP008036898) *Trametes hirsuta* (GenBank AIZ72725) and *Dichomitus squalens LYAD-421 SS1* (GenBank XP007364547), including all the conserved residues involved in coordination of copper sites as well as a Phe (F463) as non-coordinating axial ligand of T1 copper. Firsts 21 amino acids of LCC4 determine a putative signal peptide as predicted by SignalP 4.0 and 3 N-X-S/T sequons (positions 54, 333, and 436 of mature protein) are predicted to be *N*-glycosylated according to Net-glyc 1.0 program prediction (Fig. S1). All the four laccases have the conserved Asp residue involved in stabilization of cation substrate (D205 for LCC1 and LCC3 and D207 for LCC2 and LCC4).

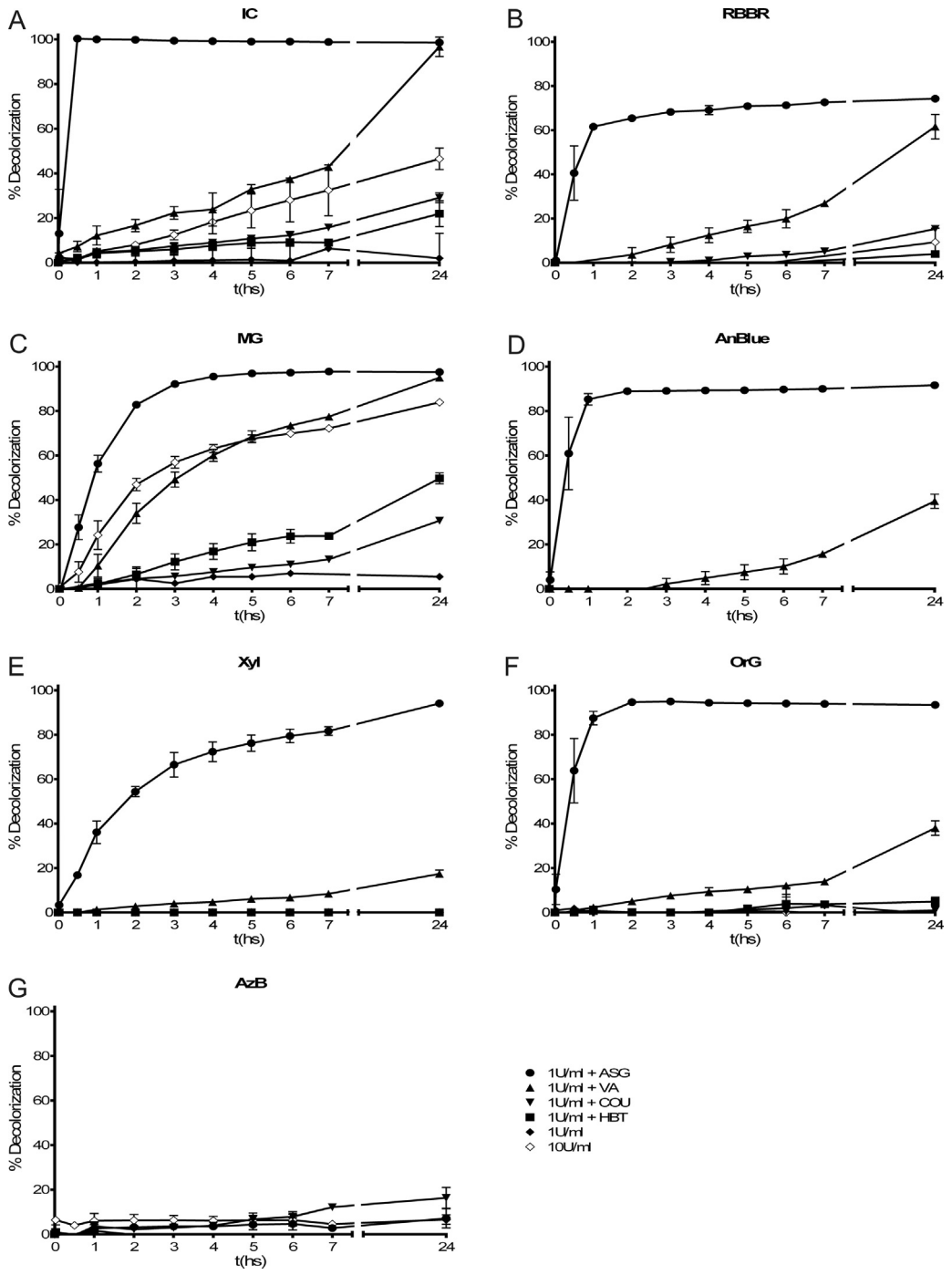

Fig. 4. Decolorization of synthetic dyes by LCC3. Decolorization of 50 μM Indigo Carmine (A), Remazol Brilliant Blue R (B), Malachite Green (C), Aniline Blue (D), Xylidine Ponceau (E), Orange G (F) and Azure B (G) was assayed in presence of 1 U/ml (closed diamonds) or 10 U/ml (open diamonds) of LCC3 or in presence of 1 U/ml LCC3 and 200 μM acetosyringone (ASG, closed circles), violuric acid (VA, closed triangles), ρ-coumaric acid (COU, closed inverted triangles) or 1-hydroxybenzotriazole (HBT, closed squares) at 30 °C in citrate-phosphate buffer pH 4.5. Decolorization is represented as % respect to To. Error bars correspond to standard deviation for triplicates.

A comparative analysis of the sequences of the predicted mature laccases encoded by *T. trogii* BAFC 463, showed that mature LCC4 shares 74% and 70% identity with LCC1 and LCC2 previously cloned and characterized in *T. trogii* 201; while mature LCC3 shares 63% and 59% identity with these two isoenzymes, respectively. As of the four laccases encoded by *T. trogii* BAFC 463, LCC3 was the more divergent and none of the proteins with which it showed highest identity were previously characterized, we selected LCC3 for recombinant expression and biochemical characterization.

3.2. *Recombinant expression in* K. pastoris

To characterize the LCC3 protein, we cloned the native coding sequence in vector pPIC9 for expression in *K. pastoris*, including a tract of 6 histidines fused in frame to C-terminal for immunodetection and Ni-NTA affinity purification (Fig. S3). Screening of transformed yeast in minimal media supplemented with ABTS or DMP showed characteristic oxidization halos surrounding colonies confirming active recombinant laccase was expressed and secreted by *K. pastoris*.

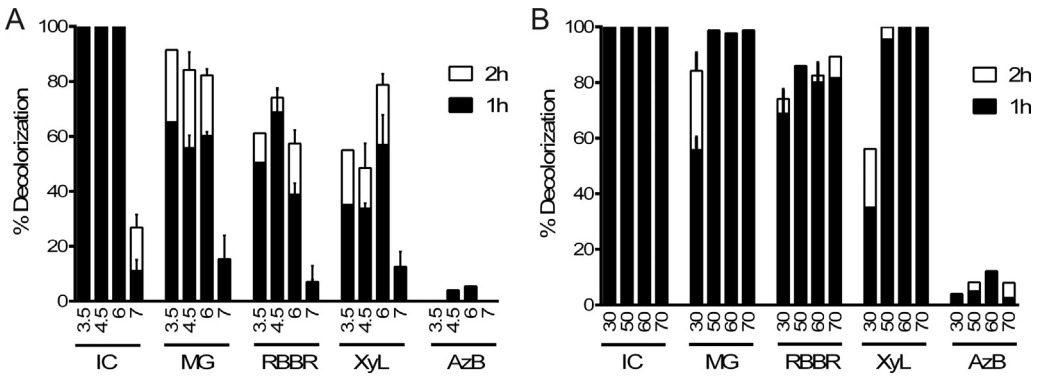

Fig. 5. Effect of pH and temperature on dye decolorization by LCC3. Decolorization of 50 μM Indigo Carmine (IC), Malachite Green (MG), Remazol Brilliant Blue R (RBBR), Xylidine Ponceau (XyL) and Azure B (AzB) was assayed in the presence of 1 U/ml of LCC3 and 200 μM acetosyringone at 30 °C in citrate-phosphate buffer pH 3.5, 4.5, 6 and 7 (A) or in citrate-phosphate buffer pH 4.5 at 30 °C, 50 °C, 60 °C and 70 °C (B). Decolorization after 1 h (solid bars) and 2 h of incubation (white bars) is represented as % respect to To. Error bars correspond to standard deviation for triplicates.

Recombinant laccase production in shake-flasks cultures gave yields of 5740 ± 500 U/l for ABTS oxidation (pH 4.5, 30 °C) in crude extracellular extracts. Further purification of recombinant enzyme by a single step Ni-NTA affinity chromatography resulted in a smear of apparent molecular mass of 85–90 kDa as visualized on SDS-PAGE stained with Coomassie Brilliant Blue (Fig. 1A). Identity of these bands was confirmed by in-gel activity assay with ABTS and DMP as substrates (Fig. 1B) and by Western blot revealed with an anti-HIS antibody (Fig. 1C, lane −), showing all the observed bands correspond to full-length active proteins. To determine if high apparent molecular weight of LCC3 was due to its glycosylation pattern, crude enzyme extract was subjected to endoglycosidase H digestion. Western blot analysis revealed a single band with a mobility of approximately 58 kDa, closer to the theoretical predicted molecular mass (54 kDa) confirming the observed bands correspond to glycosylated forms of LCC3 (Fig. 1C, lane +).

3.3. Biochemical characterization

The temperature and pH activity profiles for LCC3 were similar to those observed for other laccases of white-rot fungi [15]. LCC3 showed different pH profiles depending on the substrate, with an optimum of pH 3.5 for ABTS and pH 4 for DMP (Fig. 2A). Temperature profile showed a maximum activity at 50 °C, retaining >80% of activity at temperatures between 60 °C and 75 °C, but less than 55% of activity at temperatures below 40 °C, evidencing the thermo activity of this enzyme (Fig. 2B).

Thermal stability of purified LCC3 was assayed at 60 °C and 70 °C for ABTS oxidation. At 60 °C LCC3 retained 100% of its initial activity after 3 h of incubation, although the activity decreased to 20% when incubation was performed at 70 °C (Fig. 3). Interestingly, when LCC3 was pre-incubated at 60 °C for less than 30 min it showed an increase of up to 30% of activity in comparison with the initial and the controls incubated at 4 °C. Incubations performed at room temperature demonstrated that LCC3 did not showed any decrease of activity for at least 5 days when performed at pH 6 or pH 7, but retained only 26% and 52% of activity after 48 h when incubated at pH 3.5 and 4.5, respectively.

The kinetic constants were estimated for ABTS and DMP at pH 4 and 30 °C (Table 1). Recombinant LCC3 followed a typical Michaelis-Menten kinetics, with Km values, catalytic constants (kcat) and specificity constants (kcat/Km) in the range of those found for other laccases of white rot fungi [15].

3.4. Decolorization of synthetic dyes

Decolorization of seven synthetic dyes was assayed in presence of natural and synthetic redox mediators using concentrated crude extracts of LCC3. Observed decolorization rates were dependent on the chemical characteristics of dyes and compounds used as redox mediators. Although 1 U/ml of enzyme was not enough to observe any significant decolorization, Indigo Carmine and Malachite Green were decolorized 46% and 84%, respectively after 24 h of incubation when enzyme concentration was increased to 10 U/ml (Fig. 4A, C). A most outstanding effect was observed when 1 U/ml of enzyme was supplemented with redox mediators at a molar ratio of 4:1 with dyes. Among the redox compounds assayed, lignin-derived acetosyringone (ASG) probed to be the most efficient followed by violuric acid (VA). In the presence of ASG, LCC3 decolorized 100% of Indigo Carmine in less than 15 min and more than 80% of triarylmethane dyes Malachite Green (82.8%) and Aniline Blue (89%) and the azoic dye Orange G (94.6%) after 2 h of incubation (Fig. 4A, C, D, F). ASG also allowed high decolorization of recalcitrant anthraquinonic dye RBBR (65.4%) and azoic Xylidine Ponceau (54.4%) after 2 h of incubation (Fig. 4B, E). Supplementation with violuric acid showed a less marked effect on decolorization, with a 34% for Malachite Green and 16.6% for Indigo Carmine after 2 h of incubation and less than 30% for Orange G (13.9%), RBBR (26.9%) Aniline Blue (15.7%) and Xylidine Ponceau (8.4%) after 7 h of incubation. The other two mediators assayed, HBT and ρ-coumaric acid, showed only a slight effect on the decolorization after 24 h of incubation for Malachite Green (49.7% HBT, 30.7% COU), Indigo Carmine (21.9% HBT, 29.1% COU) and RBBR (3.9% HBT, 15.3% COU). For the highly recalcitrant heterocyclic dye Azure B, only a 16% decolorization was observed after 24 h when ρ-coumaric acid was used as redox mediator (Fig. 4G).

In order to assess whether lower concentrations of ASG were able to mediate dye decolorization by LCC3, dye solutions were supplemented with varying concentrations of ASG and incubated with 1 U/ml of enzyme. For Indigo Carmine, 100% of decolorization was reached after 1 h of incubation even with the lowest molar ratio of ASG:dye assayed (0.1:1) (Fig. S4A). For Malachite Green, a decrease from 90% to 68% of dye decolorization was observed after 4 h of incubation when the ratio of ASG:dye was reduced from 4:1 to 1:1 (Fig. S4B). A more pronounced effect was observed for Xylidine Ponceau and RBBR in which the percentage of decolorization after 4 h of incubation was reduced from 64 to 66% with a ratio of 4:1 ASG:dye to 20–25% with a ratio of 1:1 (Fig. S4C, D).

The effect of pH and temperature on dye decolorization was assayed in presence of ASG. Although pH profiles of LCC3 showed almost complete loss of activity at pH 6 for ABTS or DMP oxidation, no decrease in decolorization rates was observed for Indigo Carmine and Malachite Green after 2 h of incubation at 30 °C and pH 6 with respect to incubations performed at pH 4.5. For RBBR best results were obtained at pH 4.5 (74% after 2 h at 30 °C), in contrast

with the decolorization attained at pH 6 (57% after 2 h). Conversely, decolorization of Xylidine Ponceau increased when incubated at pH 6. Incubations performed at pH 7 showed a marked decrease in decolorization for all of the assayed dyes, with no changes in the second hour of incubation except for Indigo Carmine (Fig. 5A).

A more remarkable effect on the rate of decolorization was observed when the incubation temperature was raised above 50 °C. More than 95% of Malachite Green and Xylidine Ponceau decolorization was achieved after the first hour of incubation at 50 °C, 60 °C and 70 °C, whereas at 30 °C, decolorization was 56% and 35%, respectively (Fig. 5B). For RBBR, more than 80% of decolorization was observed after 2 h at temperatures higher than 50 °C whereas 74% was attained at 30 °C. For Azure B, although a slight increase in decolorization was observed at 50 °C and 60 °C, decolorization values remained below 15% after 2 h of incubation (Fig. 5B).

4. Discussion

In this work we used a PCR-based screening to clone four laccase coding sequences of *T. trogii* BAFC 463. Of these, sequences *lcc1* and *lcc2* encoded proteins with 100% and 99% identity with laccases LCC1 and LCC2 previously reported for *T. trogii* 201 [22,23] while *lcc3* and *lcc4* encoded two novel isoenzymes. Cloning and expression of sequence *lcc3* in *K. pastoris* showed that it encodes a laccase with a functional signal peptide, capable of oxidizing the substrates ABTS and DMP with characteristic pH activity profiles and kinetic constants commonly observed for other fungal laccases, with higher catalytic efficiency for ABTS that for the phenolic substrate DMP (Fig. S5, [15])

The most outstanding property of LCC3 was its high activity and stability at temperatures above 50 °C. Exhibiting a half-life of 45 min at 70 °C and stability higher than 3 h at 60 °C, LCC3 activity is comparable to the highly thermostable laccases described in other basidiomycetes of the genera *Trametes* [40,41], *Pycnoporus* [42,43], and *Ganoderma* [44,45] (Fig. S5). Two laccases were characterized in *C. gallica* A-241 [46] and *C. gallica* UAMH 8260 [47] showing high stability at 60 °C, but comparison of N-terminal sequences reported for the purified mature proteins showed they are different to the *C. gallica* isoenzymes TgLcc3 and cgLcc1 with which LCC3 showed high similarity (Fig. S2). None of the other reported protein sequences for *C. gallica* corresponded to TgLcc3 and cgLcc1 (Fig. S2, [48]). If these two *C. gallica* isoenzymes are also thermostable it is not known since biochemical characterization of these proteins was no reported.

None of the culture filtrates and purified laccases reported for other strains of *T. trogii* showed comparable thermostability. Both, crude preparations of *T. trogii* B6J and recombinant LCC1 of *T. trogii* 201 lost 90% of their activity after 3 h of incubation at 60 °C [49,50] and the purified laccase of *T. trogii* YDHSD showed a shorter half-life of 1.6 h at 60 °C [51]. Only a laccase purified from *T. trogii* S0301 showed a higher thermostability with a half-life of 3 h at 60 °C, although reported sequence corresponded to LCC1 [52], (Fig. S2).

Regarding *T. trogii* BAFC 463, previous assays with culture filtrates exhibited laccase activity with a half-life of more than 2 h at 60 °C and retention of 33% of activity after 1 h at 70 °C [18]. Our results suggest that thermal stability observed in *T. trogii* BAFC 463 culture filtrates could be due to the expression of thermostable LCC3. Another feature of LCC3 is the promotion of activity observed when it was pre-incubated for short periods of time at 60 °C. This characteristic has been also reported for some bacterial CotA laccase-like multicopper oxidases [53] and for a few basidiomycetes laccases, including isoenzymes from genera *Trametes* [54], *Physisporinus rivulosus* [55] and a laccase of the brown rot fungus *Postia placenta* [56].

One of the most promising industrial applications of laccases is in decolorization of synthetic textile dyes for effluent treatment. Multiple studies have shown the ability of culture filtrates of *T. trogii* for decolorization of industrial dyes, although few studies have focused on the role of individual isoenzymes. Here we demonstrated the ability of LCC3 of *T. trogii* to decolorize chemically diverse synthetic dyes. Triarylmethane type (Aniline Blue and Malachite Green), anthraquinonic (RBBR), azoic (Orange G and Xylidine Ponceau) and indigoid (Indigo Carmine) dyes were rapidly decolorized by LCC3 in the presence of the natural redox mediator acetosyringone and to a much lesser extent with violuric acid. These results are consistent with a previous study in which acetosyringone and syringaldehyde were selected as the most efficient natural mediators among 44 different compounds screened for decolorization of Reactive Black 5 by a laccase of *Pycnoporus cinnabarinus* [13]. The effectiveness of decolorization by LCC3 was dependent not only on the chemical characteristics of redox mediators and dyes, but also on the ratio between them. While decolorization of Indigo Carmine was slightly affected by the decrease of the molar ratio of ASG:dye, a decrease of 20% to 40% in the decolorization of Malachite Green, Xylidine Ponceau and RBBR was observed when molar ratio of ASG:dye was reduced from 4:1 to 1:1. Furthermore, although HBT has shown to increase decolorization of synthetic dyes by purified laccases of *T. trogii* BAFC 463 [18], *T. trogii* SYBC-LZ [57] and *T. trogii* B6J [49], it showed only a slight acceleration of decolorization of Indigo Carmine and Malachite Green by LCC3, with no effect on the other dyes assayed. A similar result was observed for cloned LCC1 that showed a better response to violuric acid than to HBT [50]. Patterns of decolorization of industrial dyes by LCC3 in the presence of acetosyringone were similar as those registered for culture filtrates of *T. trogii* BAFC 463 in absence of redox mediators, showing the same increase in observed rates when incubation was performed at temperatures above 50 °C [18]. Conversely, LCC3 was not able to decolorize dyes at pH 7 in the same extent as the culture filtrates of *T. trogii* BAFC 463 and did not show the same decolorization rates for Azure B [18]. Azure B decolorization is a characteristic of lignin peroxidase activity, and only a few laccases has been reported to oxidize it. High redox potential laccases of *P. cinnabarinus* [13] and *Trametes villosa* (*Polyporus pinsitus*) [13,58] were able to decolorize Azure B but only in the presence of natural and synthetic redox mediators, being the most efficient acetosyringone and *p*-coumaric acid (40% of decolorization after 2 h with 100 μM of mediator) [13] followed by HBT (73% of decolorization after 16 h with 2 mM of mediator) [58]. Decolorization of Azure B by *T. trogii* BAFC 463 culture filtrates and purified laccase was also highly efficient in presence of HBT. Culture filtrates attained 35% and 100% of Azure B decolorization after 24 h of incubation in absence or supplemented with 0.5 mM HBT, respectively, while purified laccase was able to decolorize more than 90% of Azure B after 1 h with 0.5 mM HBT [18]. These observations suggest the expression of more than one isoenzyme in *T. trogii* BAFC 463 and the presence of complementary activities and compounds from fungal metabolism that can act as redox mediators and stabilizing molecules.

Characteristics such as the retention of activity at pH 6, thermal stability and activity at temperatures above 50 °C, makes LCC3 an excellent candidate for incorporation in industrial processes such as dye decolorization and pulp biobleaching. High expression levels reached in *K. pastoris* even in non-optimized conditions and the availability of histidine tag for purification by a single step affinity chromatography shows good prospects for scaling up production. Also the possibility of using a natural lignin derived redox mediator as acetosyringone, has the advantage of being economic, since it can be obtained from renewable sources. Furthermore, the use of a laccase-acetosyringone system for the treatment of an effluent from the textile industry, showed a greater reduction in toxicity

that the use of a laccase-HBT system [59]. Finally, the availability of the cloned sequence of LCC3 could facilitate the improvement of its catalytic properties and contribute to the understanding of the mechanisms of thermostability in laccases through protein engineering studies.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.procbio.2016.03.015.

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