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Preliminary studies of new strains of *Trametes* sp. from Argentina for laccase production ability

*María Isabel Fonseca**, *Marcos Raúl Tejerina*, *Silvana Soledad Sawostjanik-Afanasiuk*, *Ernesto Martin Giorgio*, *Mónica Lucrecia Barchuk*, *Pedro Darío Zapata*, *Laura Lidia Villalba*

Laboratorio de Biotecnología Molecular, Módulo de Bioquímica y Farmacia, Facultad de Ciencias Exactas Químicas y Naturales, UNaM, Posadas, Misiones, Argentina

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ABSTRACT

Oxidative enzymes secreted by white rot fungi can be applied in several technological processes within the paper industry, biofuel production and bioremediation. The discovery of native strains from the biodiverse Misiones (Argentina) forest can provide useful enzymes for biotechnological purposes. In this work, we evaluated the laccase and manganese peroxidase secretion abilities of four newly discovered strains of *Trametes* sp. that are native to Misiones. In addition, the copper response and optimal pH and temperature for laccase activity in culture supernatants were determined.

The selected strains produced variable amounts of laccase and MnP; when Cu^{2+} was added, both enzymes were significantly increased. Zymograms showed that two isoenzymes were increased in all strains in the presence of Cu^{2+}. Strain B showed the greatest response to Cu^{2+} addition, whereas strain A was more stable at the optimal temperature and pH. Strain A showed interesting potential for future biotechnological approaches due to the superior thermo-stability of its secreted enzymes.

Introduction

In recent years, many innovative technologies have been developed to reduce pollution and generate ecologically friendly energy. Searching for new promising microorganisms with significant enzymatic secretion systems has provided innovative biotechnological resources. The province of Misiones has one of the highest biodiversities in Argentina, and together with the Yungas region, these regions are the biologically richest natural areas of Argentina. However, the study of fungi, especially microfungi, has been neglected, and many fungi await extensive studies. This wonderful natural area is a mycologist's paradise.[1] Many white rot fungi (WRF) species grow in forest areas of Misiones and produce a complex system of ligninolytic enzymes with biotechnological potential.

Lignin peroxidase (Lip), manganese peroxidase (MnP) and laccase (Lac) are the most widely distributed ligninolytic

* *Corresponding author.*
E-mail: biotecmol2010@gmail.com (M.I. Fonseca).
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enzymes.[2–4] The synthesis of these enzymes is regulated by various factors, and heavy metals significantly affect enzyme production.[5–7] Temperature and pH also greatly influence the activity and thermostability of the secreted enzymes.[8]

Principally due to the polymorphism of Basidiomycetes, diverse taxonomical criteria have been used to classify these fungi; therefore, the correct name for many taxa used in various studies have remained unclear.[9,10] However, the phylogenetic diversity of strains is related to the environmental, ecologic, geographic and genetic conditions in which they live.[11] Molecular techniques are useful tools to study the phylogeny and intraspecific genetic variations, and provide essential information for understanding enzyme variation among strains of the same fungal species.[9,12] The internal transcribed space (ITS) regions of ribosomal DNA have also been used to analyze various genera and species of fungi.[13–15] The sequences of these regions are more diverse among species than within species.[16] The aim of this work was to evaluate the laccase and manganese peroxidase secretion abilities of new strains of *Trametes* sp. isolated from Misiones, Argentina. The effect of the addition of Cu^{2+}, the thermostability, and the optimal pH and temperature of laccase activity in the culture supernatants were also analyzed.

Materials and methods

Microorganisms and culture conditions

White rot fungi strains A, B, C, and E were provided by the Culture Collection of the Faculty of Forestry, National University of Misiones, Argentina; the strains were subjected to molecular phylogenetic classification as described below. Stock cultures were maintained on malt agar at 4 °C.

To prepare liquid inocula, each fungus was grown for 5–7 days on malt agar plates (MEA: 20 g L^{-1} agar, 12.7 g L^{-1} malt extract), and 36-mm^2 agar plugs were then cut from the agar and transferred to 100-mL Erlenmeyer flasks containing 20 mL of medium (ME: 12.7 g L^{-1} malt extract and 5 g L^{-1} corn steep liquor) and incubated at 29 °C in steady-state conditions.

Biomass and protein determination

Biomass growth was determined by measuring the mycelium dry weight, and proteins in the conditioned medium were measured using the Bradford method.

Liquid medium was separated from the supernatant mycelium by filtering through fiberglass filters (GF/C) in a Büchner funnel and frozen at −20 °C until use. Biomass dry weight was determined by measuring the difference between the fiberglass filter (GF/C) weight before filtration and after filtration and subsequent drying at 80 °C until a constant weight was attained.

Protein was determined using a micro-test based on the Bradford technique (BioRad) following the manufacturer's instructions; bovine serum albumin was used as the standard. Secreted protein concentrations are expressed in units of μg/mL.

Enzyme assays

Laccase (EC 1.10.3.2) activity was measured at 30 °C using 1 mL of 5 mM 2,6-dimethoxyphenol (DMP) in 0.1 M sodium acetate buffer pH 3.6 and 50 μL of filtered supernatant mycelium. Absorbance was monitored at 469 nm (E_{469} = 27.5 mM^{-1} cm^{-1}) using a Shimadzu UV-3600 spectrophotometer. One laccase activity unit was defined as the amount of enzyme required to oxidize 1 μmol of DMP per min at 30 °C and is expressed in units of U mL^{-1}.[7]

Manganese peroxidase (EC 1.11.1.13) activity was measured at 30 °C using 2.5 mL of 0.1 M phenol red in sodium dimethylsuccinate buffer pH 4.5 and 50 μL of filtered supernatant mycelium. The reaction was initiated using 20 μL of 0.2 mM H_2O_2. Absorbance was monitored at 610 nm (E_{610} = 22 mM^{-1} cm^{-1}) using a Shimadzu UV-3600 spectrophotometer. One MnP activity unit was defined as the amount of enzyme required to oxidize 1 μmol of phenol red per min at 30 °C and is expressed in units of U mL^{-1}.[17]

Polyacrylamide gel electrophoresis

To identify the number of isoenzymes involved in Cu^{2+} induction, the crude enzyme was subjected to native polyacrylamide gel electrophoresis (ND-PAGE) and denaturing polyacrylamide gel electrophoresis (SDS-PAGE) in 7.5% gels. After resolving the proteins using ND-PAGE, the gel was incubated in 0.1 M sodium acetate buffer containing 5 mM DMP before detecting laccase activity.[18] After incubating the gel for 5 min, the dye solution was discarded; the gel was immediately scanned using a scanner (HP Deskjet F300 All-in-One series).

The molecular weight of the isoenzymes was evaluated using 7.5% SDS-PAGE including a molecular weight marker (Kaleidoscope, BioRad). SDS was removed by incubating the gel in 50 mM sodium acetate containing 0.2% Triton X-100 and then staining with 5 mM DMP to detect laccase activity.[19]

Determination of the thermostability and optimal temperature and pH of laccase activity in culture supernatants

The laccase activity in the culture supernatants was tested at pH 3.6–5.6 in 50 mM sodium–acetate buffer using DMP as substrate. After determining the optimum pH, laccase activity was measured in the range 10–80 °C.

The thermostability was evaluated at the optimal values of pH and T using culture supernatants that had been incubated for 7 h.

DNA extraction

Fungi mycelia were filtered and washed with 0.1 M Tris–HCl pH 8 and 0.02 M EDTA. DNA was extracted in buffer solution (100 mM Tris–HCl pH 8, 1.5 M NaCl, 50 mM EDTA pH 8) at 60 °C containing 0.1 mg mL^{-1} proteinase K, 10 mM β-mercaptoethanol and 2% SDS. The DNA was purified with chloroform, isoamyl alcohol (24:1) and 3 M potassium acetate and then precipitated using isopropyl alcohol.

ITS amplification and sequencing analysis

ITS-1 (partial sequence), the 5.8S rRNA gene, ITS 2, and the 28S rRNA gene (partial sequence) region, (ITS1-5.8S-ITS2-28S), were amplified and sequenced; the sequences were then deposited in GenBank.

The genes were amplified using the primer sequences described by White et al.[16] PCR was carried out in 20-μL reactions containing 1× KCl buffer, 2.5 mM $MgCl_2$, 200 μM dNTPs, 10 pmol of each primer, 0.5 U Taq polymerase and 25 ng of DNA. The PCR program was as follows: 4 min at 94 °C; 35 rounds of 40 s 94 °C, 40 s 50 °C, and 40 s 72 °C; and final extension for 10 min at 72 °C. All fragments were then submitted to a sequencing service (Macrogen, Korea).

All sequences were analyzed using Chromas Lite 2.01, BLASTn, BioEdit and CLUSTAL W before phylogenetic tree construction. Phylogenetic analysis was carried out using the T.N.T. program.[20] Gaps (indels) were treated as a 5th state because they represented insertion–deletion events. The analysis included 42 sequences, and *Daedaleopsis tricolor*, *Hexagonia nitida* and *H. mimetes* were used as outgroups.[4,21,22] Because the data set was reduced, the heuristic searches were implemented using 1000 RAS, saving one tree per TBR. To assess the support for the identified groups, bootstrap and parsimony jack-knifing analyses were performed.[23] Both the bootstrap and jack-knife analyses included 1000 resampled matrices. For each resampled matrix, we performed 100 RAS + TBR cycles.

Statistical analysis

Two-way ANOVA and Bonferroni's post test were performed using GraphPad Prism 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Cultural characteristics and phylogenetic studies of new Trametes strains

In this work, we worked with four strains of white rot fungi that were characterized as members of the *Trametes* genus. The four strains showed white mycelia development on MEA but exhibited different cultural characteristics: strains A, C and E showed cotton-like colonies, whereas strain B showed colonies with smoother surfaces (Fig. 1).

To conduct phylogenetic studies, we determined the nucleotide sequence of the ITS1-5.8S-ITS2-28S region of strain A (538 bp), strain B (548 bp), strain C (547 bp), and strain E (475 bp); each fungus exhibited 97% sequence identity with

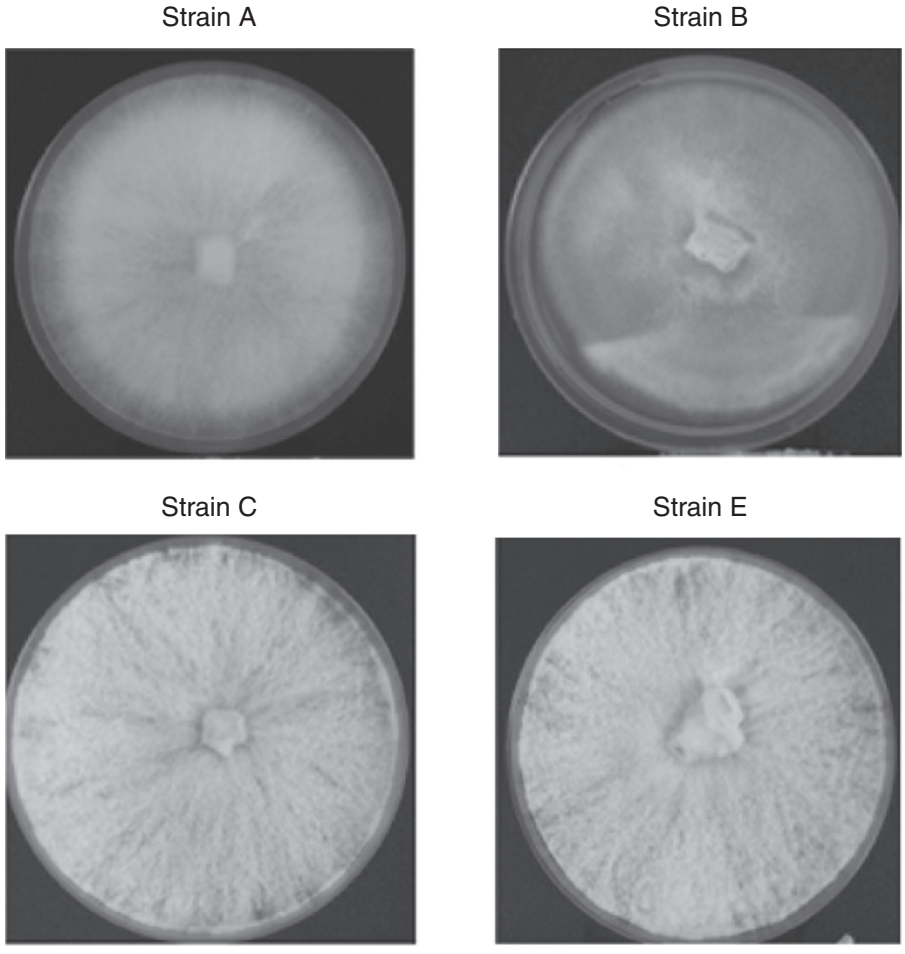

Fig. 1 – Cultural characteristics of *Trametes* sp. strains on MEA. Fungi were grown for 7 days on malt agar plates (20 g L^{-1} agar, 12.7 g L^{-1} malt extract). Letters indicate strain codes.

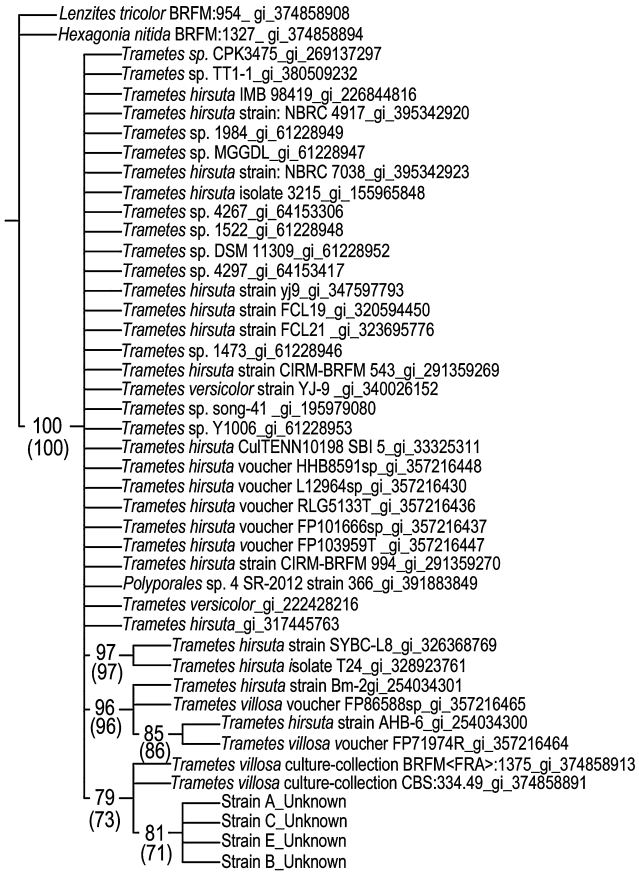

Fig. 2 – ITS region of 5.8S rDNA phylogenetic relationships between strains. Group support was assessed using 1000 bootstrapping and parsimony jack-knifing replicates. Numbers above branches correspond to jack-knife support. Bootstrap supports are given in parentheses.

Trametes hirsuta, *Trametes versicolor* and *Trametes villosa*. Blast and Fungi Barcode (http://www.fungalbarcoding.org) searches showed that the fungi were closely related to the genus *Trametes* among the examined basidiomycetes. Based on the results of the Blast search, subsequent phylogenic analysis was performed using mainly species from the genus *Trametes*. Sequences obtained from all strains were deposited in GenBank under the following access numbers: HM222419.2 (strain A); HM622150.1 (strain B); HM622151.1 (strain C) and HM622153.1 (strain E).

A phylogenic tree was obtained from overlapping data sets of the ITS region (Fig. 2). Informative sites (defined by the presence of any gaps, ambiguity symbols, and nonconsensus sequence at each site) included those at 88/612. After removing poorly aligned positions and divergent regions of DNA, the ITS sequences were aligned over 612 characters, 88 of which were parsimoniously informative. Parsimony analysis resulted in 80 equally parsimonious trees that were 185 steps long. Both bootstrap and jack-knife processes yielded nearly the same topology, and their support values were very similar. The resulting trees showed that all strains studied in this work formed a monophyletic clade that was closely related to *T. villosa*.

Biomass, and protein and oxidative enzyme secretion

The biomass and secreted protein in the liquid medium were significantly different among the strains (Fig. 3). Strain A showed a significant increase in biomass with days of culture ($p < 0.001$). All strains secreted similar quantities of protein, and the highest contents were found on culture day 10.

To verify the laccase and MnP secretion patterns of the analyzed strains, all fungi were cultured for 7 days in liquid medium containing malt extract. The strains secreted laccase to differing extents (Table 1). Strains A and C showed the highest amounts of total laccase, and strain C showed the highest

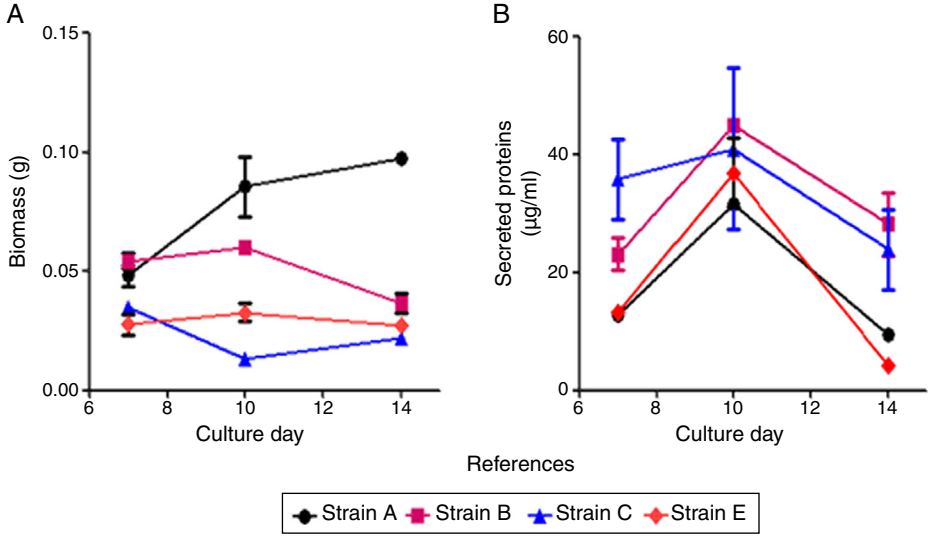

Fig. 3 – Biomass (a) and secreted proteins (b) of *Trametes* sp. strains. All strains were cultured on ME medium for 14 days at 29 °C and pH 4.8. Biomass accumulation and amount of secreted proteins were determined for all sub-strains at 7, 10 and 14 culture days. All experiments were carried out in triplicate.

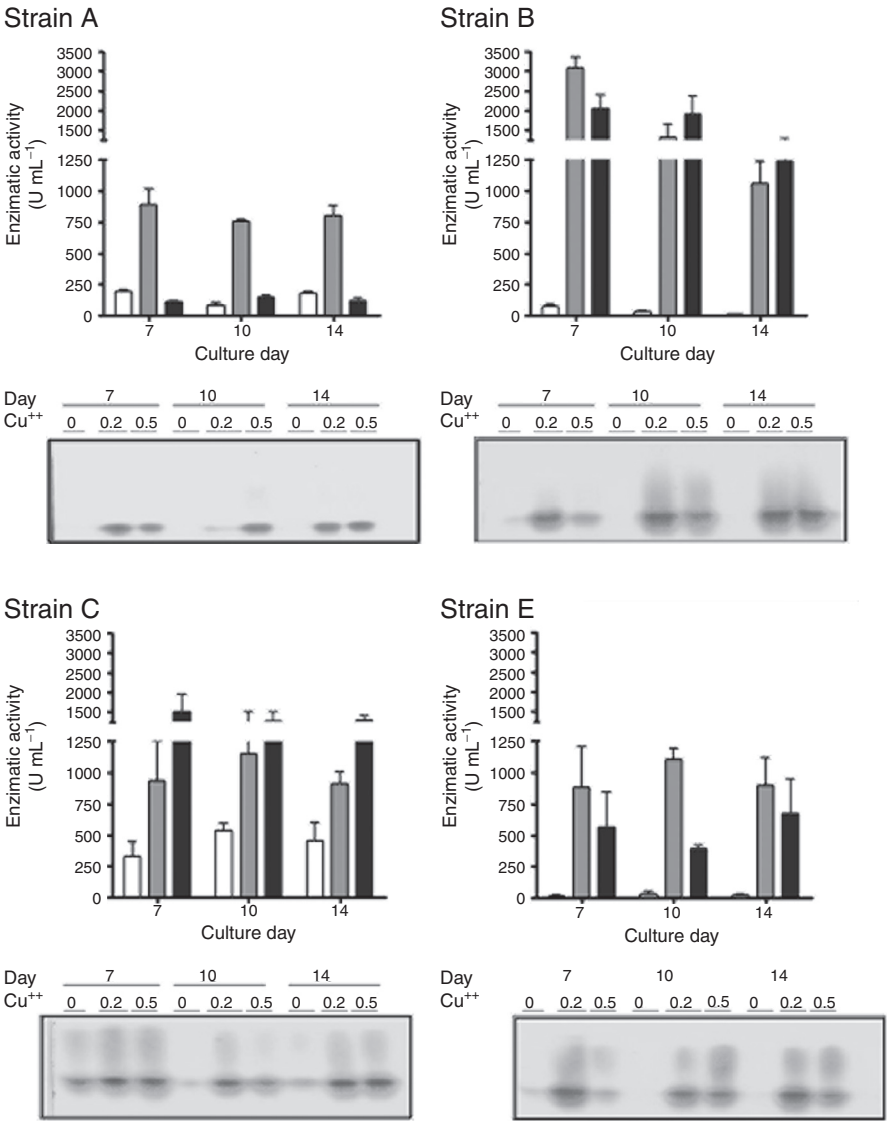

Fig. 4 – Effect of Cu^{2+} on laccase activity in the studied *Trametes* sp. strains. All strains were cultured on ME for 14 days in the presence or absence (□) of 0.2 mM (■) and 0.5 mM Cu^{2+}(■). The experiments were carried out in ME medium, and enzyme activity was measured on 7, 10, and 14 days of culture (upper graphs). All determinations were carried out in triplicate, and the results are expressed as U mL^{-1}. Laccase isoenzymes were detected using ND-PAGE analyses with DMP (lower graphs).

Table 1 – Laccase and MnP secretion by *Trametes* sp. strains.

	Laccase Enzymatic activity U mL^{-1}	MnP Enzymatic activity U mL^{-1}
Strain A	190.74 ± 15.33	60.42 ± 29.32
Strain B	78.76 ± 12.74	37.44 ± 19.74
Strain C	325.00 ± 19.05	Undetectable
Strain E	19.65 ± 4.21	Undetectable

Strains were grown on ME under steady-state conditions at 29 °C and pH 4.5.
Enzymatic activities were measured on day 7 of culture in triplicate. Data are shown as medians ± SD. Determinations were carried out in triplicate.

production of laccase per unit of biomass production (U g^{-1} of mycelia). Only strains A and B showed detectable quantities of MnP on the day 7 of culture.

Effect of the addition of Cu^{2+} on laccase and manganese peroxidase activity

Previously published data showed that the addition of Cu^{2+} increases laccase activity in Basidiomycetes. To verify that this also occurs in the newly discovered strains, laccase activity was measured in the absence and presence of 0.2 mM and 0.5 mM Cu^{2+}. The experiments were carried out in ME medium, and enzyme activity was measured on days 7, 10 and 14 of culture (Fig. 4).

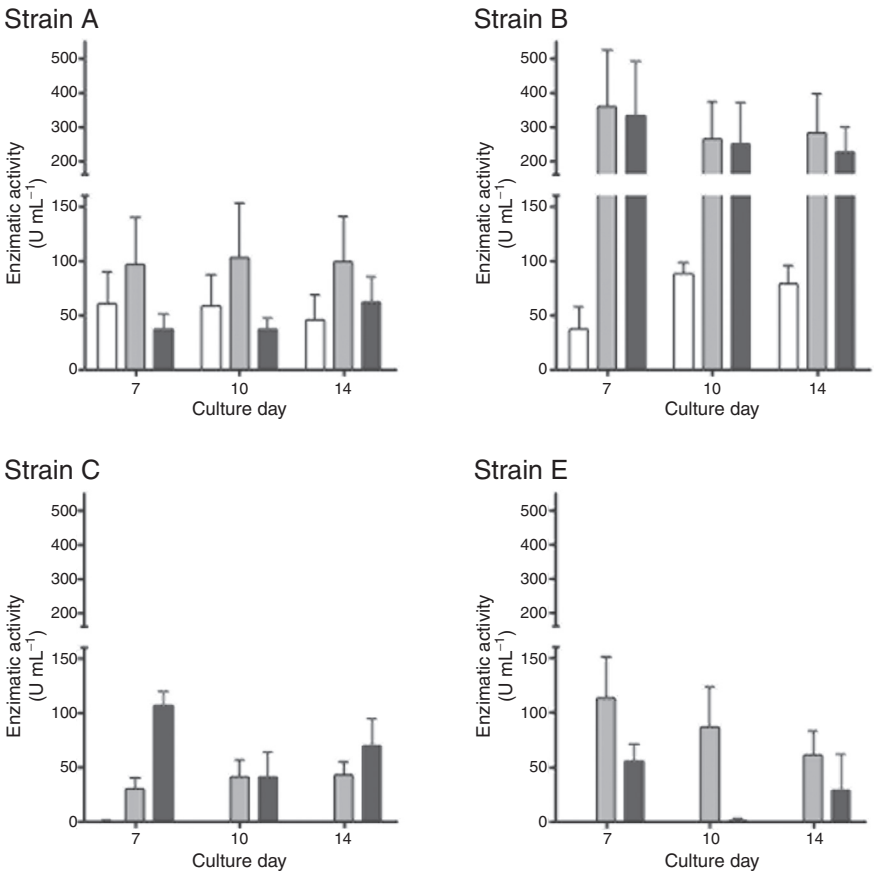

Fig. 5 – Effect of Cu^{2+} on MnP activity in *Trametes* sp. strains. All strains were cultured on MEA for 14 days in the presence or absence (□) of 0.2 mM (■) and 0.5 mM Cu^{2+}(■). The experiments were carried out in ME medium, and enzyme measurements were made on days 7, 10 and 14 of culture (upper graphs). All determinations were carried out in triplicate, and the results are expressed as U mL^{-1}.

The addition of Cu^{2+} led to a significant increase in laccase secretion in all strains ($p < 0.05$). Maximum secretion occurred upon the addition of 0.2 mM Cu^{2+} to strains A, B and E. Strain C exhibited maximal laccase secretion upon the addition of 0.5 mM Cu^{2+}. Strain B responded most strongly to Cu^{2+} induction on day 7 of culture (3085.5 U mL^{-1}; i.e., 39.5-fold greater than the secretion without Cu^{2+} (78.7 U mL^{-1})). Strain E responded most strongly to Cu^{2+} induction on day 10 of culture (1104.5 U mL^{-1}); i.e., 33.2-fold greater than the secretion without Cu^{2+} (33.2 U mL^{-1}); $p < 0.001$). However, strains A and C exhibited only 4.67-fold induction by Cu^{2+}. Strain A secreted 891 U mL^{-1} of laccase in the presence of Cu^{2+} and 191 U mL^{-1} in its absence. Strain C secreted 1519 U mL^{-1} of laccase in the presence of Cu^{2+} and 325 U mL^{-1} in its absence.

A previous report described that the number of laccase isoenzymes varies among Basidiomycetes.[7] To examine the presence of laccase isoenzymes and their differential response to Cu^{2+} induction, ND-PAGE analyses with DMP were carried out (Fig. 4). Zymogram analysis showed a clear increase in the density of two laccase bands for all strains in the presence of Cu^{2+}. The upper bands appeared for strains A, B and E only in the presence of Cu^{2+}, whereas strain C showed an upper band in the absence of Cu^{2+}. Using SDS-PAGE with DMP detection, we established the molecular weight of the detected laccase bands: 70 kDa for the lower band and 140 for the upper band.

We also determined MnP activities under basal conditions without the addition of exogenous Mn^{2+} in the presence of 0.2 or 0.5 mM Cu^{2+}. All strains showed a clear increase in MnP secretion in the presence of Cu^{2+} ($p < 0.05$). Strain B showed the highest response to the addition of Cu^{2+} on day 7 of culture day; similar amounts of MnP were secreted using both Cu^{2+} concentrations (357.4 U mL^{-1}; 9.5-fold higher than that in the absence of Cu^{2+} (37.4 U mL^{-1})). Strain A showed high MnP secretion in the presence of 0.2 mM of Cu^{2+} on day 10 of culture day (102.7 U mL^{-1}; 1.7-fold higher than that in the absence of Cu^{2+} (58.6 U mL^{-1})). Strains C and E only secreted MnP (in variable amounts) in the presence of Cu^{2+} (Fig. 5).

Thermostability and temperature and pH optima for laccase activity

We determined the optimal T and pH for laccase activity in culture supernatants in the presence and absence of Cu^{2+}. Culture supernatants of all strains exhibited maximal activity at 55 °C in the presence of Cu^{2+}. In the absence of Cu^{2+}, strains A and E exhibited maximal activity at 55 °C, strain B exhibited maximal activity at 50 °C, and strain C exhibited maximal activity at 45 °C (Fig. 6).

Culture supernatants lacking Cu^{2+} did not show any difference in laccase activity between pH 3.6 and 5.6. All culture

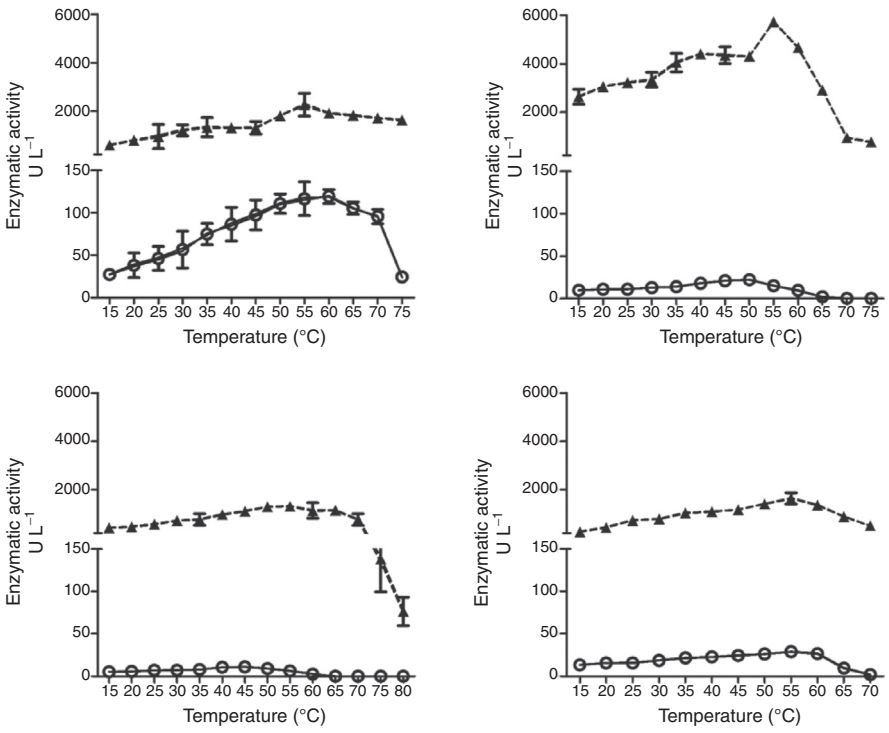

Fig. 6 – Effect of temperature on laccase activity in culture supernatants of *Trametes* sp. strains. All strains were cultured on ME for 14 days in the absence (triangle) or presence of 0.5 mM Cu^{2+} (◯). The experiments were conducted between 10° and 80 °C. All determinations were carried out in triplicate using DMP as substrate, and the results are expressed as U mL^{-1}.

supernatants including Cu^{2+} exhibited maximal laccase activity at pH 3.6; the maximal activity in the presence of Cu^{2+} was greater than that in the absence of copper (13-fold for strain A ($p < 0.001$), 300-fold for strain B ($p < 0.001$), 140-fold for strain C ($p < 0.001$), and 100-fold for strain E ($p < 0.001$); Fig. 7).

To measure the thermostability of the enzyme, culture supernatants were incubated for 7 h at the optimal pH and T. Residual laccase activity was estimated based on the highest value obtained (100%). Strain A had a half-life of 300 min in culture supernatants including Cu^{2+} and a half-life of 120 min in supernatants including Cu^{2+}. Strains B, C and E had a half-life of 40 min in the presence and in the absence of Cu^{2+} (Table 2).

Discussion

In this work, we evaluated oxidative enzymes secreted by four newly discovered *Trametes* strains and focused on laccase activity. These strains showed differences in biomass

Table 2 – Residual laccase activity in culture supernatants incubated at optimal temperature and pH.

Time (min)	Residual enzymatic activity							
	Strain A		Strain B		Strain C		Strain E	
	With 0.5 mM of Cu^{2+}	Without Cu^{2+}	With 0.5 mM of Cu^{2+}	Without Cu^{2+}	With 0.5 mM of Cu^{2+}	Without Cu^{2+}	With 0.5 mM of Cu^{2+}	Without Cu^{2+}
0	100	100	100	100	100	100	100	100
20	91	113	82	92	63	65	81	92
40	55	97	58	61	52	47	69	32
60	59	96	31	58	43	54	36	7
120	56	51	36	13	31	12	23	8
180	52	49	32	14	30	–	18	–
240	48	36	27	–	17	–	10	–
300	56	32	29	–	13	–	10	–
360	40	31	26	–	13	–	6	–
420	45	–	24	–	–	–	6	–

Residual enzymatic activity is expressed as a % of the initial activity. Data are shown as medians ± SD.

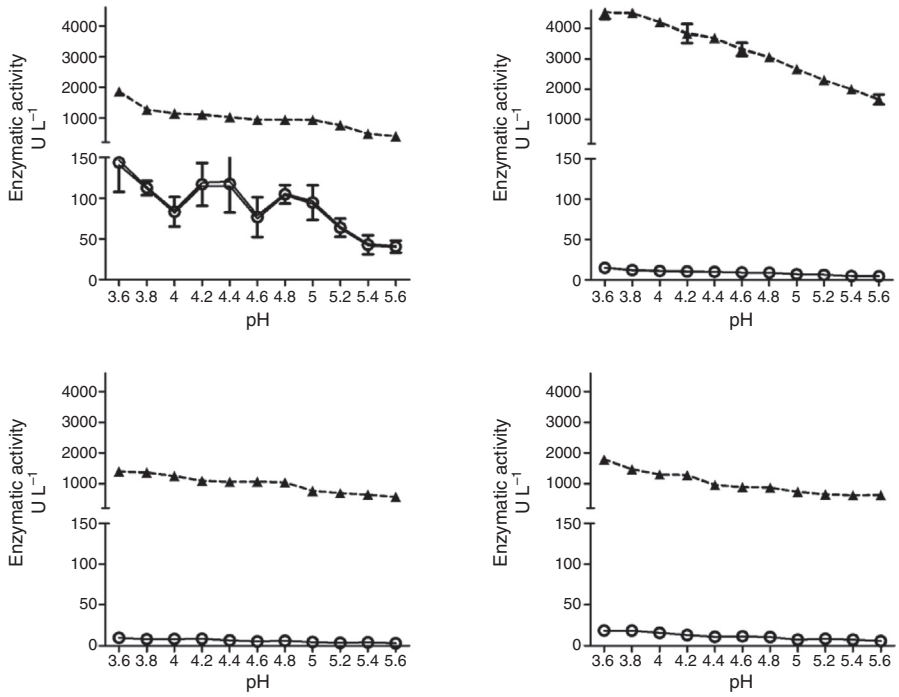

Fig. 7 – Effect of pH on laccase activity in culture supernatants of *Trametes* sp. strains. All strains were cultured on ME for 14 days in the absence (triangles) or presence of 0.5 mM Cu^{2+} (○). The experiments were conducted in the pH range 3.6–5.6 using 0.1 M sodium acetate buffer. All determinations were carried in triplicate out using DMP as substrate, and the results are expressed as U mL^{-1}.

production, levels of secreted proteins and levels of the main oxidative enzyme laccase.

The ITS1 and ITS2 regions, which are separated by the conserved short 5.8S rRNA, may exhibit nucleotide variations because their transcripts are excised from the final rRNA fragments. Consequently, we decided to study these sequences. Sequences are commonly used to infer phylogenetic relationships of closely related species and to assess the variability present within a population; e.g., among geographically distant isolates (ecotypes).[10] Although a phylogenetic analysis of the ITS1-5.8S-ITS2-28S regions revealed that the studied strains are closely related to the *T. villosa* clade branch, we were only able to identify the strains at the genus level. *T. villosa* was recently isolated in Guadeloupe and Argentina.[4] More extensive ITS trees were constructed using all the available ITS1-5.8S-ITS2 sequences of good quality that were 520–560 nt in length from *Trametes* spp. and members of closely related genera (data not shown); these trees indicate that these strains might represent divergent isolates of the species *T. villosa*. Finally, to clarify the taxonomic position of the strains, it will be necessary to complement our molecular studies with classical genetic analysis; e.g., based on mating experiments.[24] The *Trametes* species identified and studied in this work are related to the third clade obtained by Welti et al.,[4] comprising a group of specimens from *Trametes pubescens* to *T. hirsute*. Three distinct sub-clades have been identified within this clade, and the sub-clade of interest here comprises genuine *Trametes* species (i.e., with strictly poroid hymenophores): *T. versicolor*, *T. hirsuta*, *T. ochracea*, *T. suaveolens* and *T. chinese* close to *T. junipericola*, *T. socotrana*, *T. pubescens* and *T. villosa*. Most of these species, except for *T. socotrana* and *T. villosa*, are found in temperate areas.

In other fungal species, such as *Trametes trogii*, previous data indicated that concentrations of Cu^{2+} up to 1 mM are associated with increased mycelial growth.[25] However, the response to Cu^{2+} is affected by concentration because, at extremely high doses, decreased fungal growth leads to decreased laccase activity. Ramsay et al.[26] found that in *Trichoderma virens* and *Rosea clonostachys*, high concentrations of toxic metals, such as Cu^{2+}, decrease the carbon utilization rate.[26] This effect might occur because the presence of Cu^{2+} decreases the ability of fungi to use resources such as carbon that can therefore not be used for fungal nutrition.[27] Some authors have suggested that fungi develop mechanisms to protect them against the toxic effect of ions; such mechanisms include the immobilization of these ions through the intracellular and extracellular production of organic acid-chelating compounds and siderophores.[28]

Some authors have demonstrated a close association between protein secretion and enzyme activity.[29] Our data showed an average correlation between secreted proteins and laccase activity, indicating that protein concentrations are not a good indicator of enzyme activity; thus, laccase activity measurements are warranted. It is useful to report enzyme activity in relation to biomass levels (expressed as U g^{-1} of mycelium)[30] because this parameter can reveal the true increase in enzyme activity accounting for fungal growth. However, in previous work, we found that biomass levels and

laccase secretion did not always follow a common pattern; thus, generalizations used to predict the behavior of a given fungus are impeded.[31]

In terms of the effect of Cu^{2+} on laccase production, our results are comparable to those obtained in other studies on *T. trogii* (Levin et al., 2001),[25] *Pleurotus ostreatus*[5,32] and *T. pubescens*[33]; in these species, enzyme activity was increased in the presence of Cu^{2+}. Enzyme activity was affected by Cu^{2+} concentrations, and activity was higher at lower concentrations, as for strains A, B and E. Some authors have described how Cu^{2+} interacts with the ACE transcription factor-1 and thereby induce *lac* gene expression.[34,35] However, not all *lac* genes respond equally to this transcription factor and some genes might be regulated by alternative routes. These data could explain the presence of Cu^{2+}-inducible and non-inducible isoenzymes. Our study found two Cu^{2+}-inducible laccase isoenzymes with different responses to Cu^{2+}, as previously described.[7,11,36] All strains showed an increase in the activity of both isoenzymes in the presence of Cu^{2+}. This is in agreement with the clustering results observed in the phylogenetic study.

MnP activity was affected in all strains by the presence of Cu^{2+}. This could be due to a metal response element-binding transcription factor, consistent with previous data indicating the possible regulation of MnP by extracellular concentrations of metal ions, such as Cu^{2+} and Mn^{2+}.[37–39] In this context, Alvarez et al.[35] observed an effect of Cu^{2+} on the expression of genes encoding ligninolytic laccase (*lcs*) and manganese peroxidase (*mnp*) in *Ceriporiopsis subvermispora*.

Enzymatic activity is affected by proteins, minerals and chemicals that are present in culture supernatants.[40,41] The thermostability and the pH and temperature optima of isoenzymes should be determined under specific conditions.[41–45] Our data showed that laccase activity was maximal at pH 3.6, consistent with reported data.[46] This value is somewhat lower than the usual pH range for enzyme activity (between 4 and 6).[2,47]

Strain B did not show significant variation in enzyme activity across the studied pH range, indicating the biotechnological potential of this strain to perform under acidic pH bioprocess conditions. Similar results have been reported by Sathishkumar et al.[48]

The optimal temperature for laccase activity in the presence of Cu^{2+} was 55 °C, consistent with published data.[49] However, we found some differences in culture supernatants lacking Cu^{2+} (strains B and C); these differences might be due to differences in glycosylation among the isoenzymes.[46]

Thermostability is a desired property for industrial enzyme application because it reduces the demand for fresh enzyme.[50,51] Our data indicated extended laccase stability in strain A at the optimal temperature and pH; 50% laccase activity remained at 4 h, and 40% activity remained at 7 h.

In conclusion, all strains were affected by the presence of Cu^{2+}, which increased laccase and MnP activity. Laccase increases occurred in the same strain due to the presence of two isoenzymes that respond to Cu^{2+}. Strain A was very responsive to Cu^{2+} and presented good thermostability, demonstrating its potential for future biotechnological applications. This work constitutes a biochemical study of the potential of newly discovered strains of WRF that secrete laccase and their response to the presence of Cu^{2+}. This and previous studies[6,7,52] aimed to evaluate the capacity of newly discovered native fungi to produce enzymes for biotechnological applications.

Conflicts of interest

The authors declare no conflicts of interest.

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