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Research Article

Removal of Phenol by Immobilization of *Trametes versicolor* in Silica–Alginate–Fungus Biocomposites and Loofa Sponge

White-rot fungi have potential in organic pollutant degradation. Immobilization of microorganisms has been successfully used for bioremediation. In this work, 25 isolates of Argentinean white-rot fungi were tested for their tolerance toward up to 10 mM phenol in agar plates. Seven isolates were further evaluated for their ability to grow on plates with 2,6-dimethoxyphenol, gallic acid, 2,4-dichlorophenol, or guaiacol (7.5 mM), or with phenol as sole carbon source. Best results were obtained with *Trametes versicolor*, *Irpex lacteus*, *Lentinus tigrinus*, and *Pleurotus lindquistii*. The ability of immobilized cultures of *T. versicolor* BAFC 2234 to remove phenol, was studied. Silica-alginate-fungus biocomposites resulted in phenol removal (10 mM) of up to 48% (mainly attributable to biosorption) within 14 days. Immobilized on *Luffa aegyptica*, it removed between 62 and 74% of phenol (15 mM) in three repeated cycles over a period of 23 days. Laccase was the main oxidative enzyme detected, and the purified enzyme oxidized 84% of phenol (0.5 mM) in vitro within 4 h, while 43% was converted by a purified Mn-peroxidase. The phenol phytotoxicity decreased noticeably. The concentrations of phenol removed are among the highest reported so far, thus this strain of *T. versicolor* may have good prospects for application in industrial wastewater treatment.

Keywords: Laccase; Mn-peroxidase; Phenolic compounds; Phytotoxicity; White-rot fungi

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1 Introduction

Phenol and its derivatives are common pollutants found in effluents from industrial operations dealing, for example, with coal conversion, pulp and paper manufacturing, wood preservation, metal casting, and production of pesticides, and they are rated as priority pollutants by the US EPA (code U188) [1]. Phenol is not readily biodegradable and was reported to be toxic or growth inhibitory to most microorganisms, even to those species that have the metabolic capacity of using it as a growth substrate [2].

Various phenol-degrading microorganisms have been extensively studied in order to develop and improve the technological processes of biodegradation. Most published studies on the degradation of phenol were carried out using bacteria or yeasts [3, 4]. The hyphal mode of growth gives a major advantage to filamentous fungi over unicellular microorganisms in the colonization of solid substrates and for the utilization of available nutrients. The basic mode of fungal growth is a combination of apical extension of hyphal tips and the generation of new hyphal tips through branching [5]. Filamentous fungi are able to grow under environmentally stressed conditions such as low nutrient availability, low water activity, and at low pH values where bacterial growth might be limited [6]. Among the phenol-degrading filamentous fungi evaluated, white-rot fungi stood out for their phenol degradation rate [4]. Basidiomycetous white-rot fungi are versatile and robust organisms having an enormous potential for biodegradation of various recalcitrant materials including toxic chemicals [7]. These fungi are capable of mineralizing even xenobiotic compounds due to the nonspecific nature of their extracellular oxidative enzymatic system that naturally completely degrades complex aromatic materials such as lignin or humic substances [8]. Many previous studies on organ pollutant degradation have focused on the lignin-degrading enzymes of the white-rot fungi *Phanerochaete chrysosporium* and *Trametes versicolor*. Over the last years, however, there has been a growing interest in studying the lignin-modifying enzymes of a wider array of white-rot fungi, not only from the standpoint of comparative biology but also with prospect of finding better lignin-degrading systems for the use in biotechnological applications.

Immobilization of microorganisms has been successfully used for bioremediation approaches in different areas including wastewater treatment and remediation of toxic chemicals. This technique generally provides several advantages over freely suspended cell systems, such as repeated use of immobilized cells, easy separation of cells from the liquid reaction medium, better protection of cells from pollutants and changes in cell permeability that allow better transfer of substrates into the cells [9–12]. Several reports

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Abbreviations: GA, glucose-asparagine; **HPLC**, high performance liquid chromatography; **TJM**, tomato juice medium

demonstrated these advantages, for example, in the degradation of chlorophenols [13], pyridine [14], coumaphos [9], and naphthalene [15]. In addition to organ pollutant removal, immobilized microbial cells can also be used to treat domestic or industrial wastewaters containing inorganic pollutants such as nitrate/nitrite or heavy metals [10]. Different types of solid matrices, such as calcium alginate, activated carbon, polyurethane foams, and *kappa*-carrageenan, were shown to be suitable for cell immobilization [16]. Fungi immobilized on silica-alginate (biocomposites) have been successfully applied for the removal of dyes [17] and complex phenolic compounds in olive oil mill wastewaters [18].

The purposes of this work were (i) to screen a selection of Argentinean white-rot fungi for their ability to tolerate and degrade phenolic compounds; (ii) to examine the ability of *T. versicolor* strain BAFC 2234 immobilized on/in two different matrices to degrade phenol; and (iii) to evaluate the possible participation of the ligninolytic oxidoreductases laccase and Mn-peroxidase (MnP) in the process.

2 Materials and methods

2.1 Fungal strains

The screening test was carried out with 25 strains of white-rot fungi isolated in Argentina, which belong to the genera *Fomes*, *Phanerochaete, Coriolus*, *Pycnoporus*, *Irpex*, *Abortiporus*, *Schizophora*, *Trametes*, *Aurantiporus*, *Ganoderma*, *Lenzites*, *Phlebia*, *Coriolopsis*, *Pleurotus*, *Flammulina*, *Lentinula*, and *Lentinus*. Full species names are listed in Table 1. The isolate subject of most of the research, *T. versicolor* BAFC 2234, was found in Rio Negro Province growing on *Nothophagus dombeyi* "Coihue." All Argentinean strains were selected from the Culture Collection of the Department of Biological Sciences, Faculty of Exact and Natural Sciences, University of Buenos Aires (BAFC). *Phanerochaete chrysosporium* strain BKM-F-1767 was used for comparison. Stock cultures were maintained on malt extract agar slants at 4°C.

2.2 Tolerance toward phenolic compounds

Phenol tolerance was evaluated using a method involving the determination of colony growth rates on agar plates containing glucose-asparagine (GA) medium [19] supplemented with different concentrations of phenol up to 10 mM (960 ppm). Phenol was dissolved in water and filter-sterilized before adding it to the agar plates with the autoclave-sterilized medium. The inoculum consisted of a 75 mm^3 agar plug of a 10-day-old culture grown on malt extract agar (MEA; 12.7 g L^{-1} malt extract, 10 g L^{-1} glucose, and 20 g L^{-1} agar) plates; a plate without phenol served as control for each fungus. Each fungal strain was tested in triplicate on plates containing 1, 5, 7.5, or 10 mM of phenol. The plates were incubated at 28 °C for 28 days. Growth was followed by measuring radial extension of the mycelium, and average growth rates (mm per day) were calculated. Additionally, from the plates containing 7.5 mM phenol, samples were taken for laccase activity measurement. For that, an agar plug with mycelium (3 cm^3) was mixed with an equal volume of distilled water, then centrifuged (10 000*g* for 8 min) and the enzymatic activity was measured using the supernatant.

Fungal strains that were capable of growing in the presence of 10 mM phenol were cultured on GA agar plates supplemented with other phenolic compounds (7.5 mM), namely 2,6-dimetoxyphenol, gallic acid (3,4,5-trihydroxybenzoic acid), 2,4-dichlorophenol or guaiacol (2-methoxyphenol). To test the ability of the fungal strains to grow on phenol as sole carbon source, growth studies were performed using a modified GA agar medium, where glucose and asparagine were replaced by phenol (5 mM) and ammonium phosphate (4 g L^{-1}). One strain of *T. versicolor*, BAFC 2234, selected, may tolerate high concentrations of phenolic compounds and is able to use phenol as sole carbon source. It was used in further tests under more stressful conditions.

2.3 Phenol transformation by *versicolor* immobilized on synthetic and natural supports

To further evaluate the degradation of phenol, *T. versicolor* was immobilized on/in two different supports, a synthetic one consisting of a silica-alginate matrix and a natural support consisting of loofa sponge (*Luffa aegyptiaca*). The encapsulation procedure used for fungal immobilization was adapted from Perullini et al. [17]. First, *T. versicolor* mycelium was encapsulated in calcium alginate by dropping a suspension of mycelium (from 15 days cultures) containing 0.5% of the fungus (dry weight) and 1.25% (w/v) sodium alginate into a 0.10 M aqueous $CaCl_2$ solution (flux velocity: 0.50 mL min^{-1}; needle diameter: 0.8 mm). The alginate beads (diameter: 4–5 mm) were collected by filtration (after stirring for 10 min). A total of four Ca-alginate beads were placed in cylindrical plastic molds (0.9 cm diameter, 4 cm height). Each mould was filled with silica nanoparticles, and a nanoporous structure was obtained by silicate polymerization. Monoliths were prepared at room temperature by adding, to each mould containing the beads, three volumes of sodium silicate (0.83 M in Si(IV)) and one volume of commercial colloidal silica Ludox HS40 (Sigma); the pH was adjusted to 4.5 with hydrochloric acid and the mixtures were stirred for 30 s. In order to distinguish between the contribution of the fungus and the monolithic structure to phenol degradation, molds containing monoliths with inactivated fungal mycelium were prepared as controls. To inactivate the fungus, the mycelium was sterilized at 121 °C for 20 min before encapsulation. Assay conditions included fungus-alginate beads either active or inactive, and monolithic structures (silica-alginate-fungi) with encapsulated active or inactive *T. versicolor* (biocomposites). Both immobilization alternatives are shown in Fig. 1.

In a second approach, mycelium of *T. versicolor* – immobilized on the endocarp of *L. aegyptiaca* as a natural sponge-like carrier material – was grown in GA-medium. Two hundred fifty milliliters flasks containing 40 mL medium and two pieces of the carrier (100 × 150 mm) were incubated under stationary conditions at 28 °C. Cultures were harvested by filtration at day 15 of incubation. Heat-inactivated cultures served as controls to determine abiotic loss and sorption of phenols to the fungal mycelium and the carrier surface. After preparation of these two variants of immobilized *T. versicolor* mycelium, fresh phenol solution (10 mM) was transferred to the systems. Phenol degradation was followed under static conditions over a period of 14 days in 250-mL flasks containing 100 mL of phenol solution; biomass was quantified in each case at the end by determination of the dry weight on pre-weighted filter papers at 80 °C. The more efficient immobilization variant (regarding phenol removal) was selected for further studies and assayed for repeated degradation of a higher phenol concentration (15 mM). This test was performed with cultures of *T. versicolor* immobilized on the

Table 1. Growth (mm per day) of 25 fungal isolates from Argentina on agar plates containing GA medium supplemented with increasing concentrations of phenol (1–10 mM). Laccase activity was measured in extracted agar plugs from plates containing 7.5 mM phenol

	Phenol					
	Growth rate (mm day^{-1})					
Strain	Control[a]	1 mM	5 mM	7.5 mM	10 mM	Laccase[b] (mU g^{-1})
Coriolopsis floccosa BAFC 2101	12.9 ± 0.28	11.6 ± 0.07	4.6 ± 0.14	2.5 ± 0.07	NG	14.7 ± 0.07
Coriolus antarcticus BAFC 266	12.9 ± 0.35	12.9 ± 0.09	6.4 ± 0.07	6.2 ± 0.09	NG	22.2 ± 0.63
Flammulina velutipes BAFC 1763	7.3 ± 0.21	9 ± 0.21	3.9 ± 0.022	NG	NG	0
Fomes fasciatus BAFC 2752	9 ± 0.07	5.4 ± 0.14	3 ± 0.08	0.5 ± 0.06	NG	10.9 ± 0.98
Ganoderma resinaceum BAFC 228	12.9 ± 0.08	11.6 ± 0.15	6.4 ± 0.06	6.4 ± 0.09	NG	34.2 ± 0.64
Abortiporus biennis BAFC 74	12.9 ± 0.07	12.9 ± 0.28	3.1 ± 0.23	NG	NG	0
Irpex lacteus BAFC 2598	12.9 ± 0.21	12.9 ± 0.14	9 ± 0.05	6.4 ± 0.05	5.6 ± 0.08	1.8 ± 0.14
Irpex lacteus BAFC 1171	12.9 ± 0.34	9 ± 0.22	5.4 ± 0.15	2.6 ± 0.07	1.8 ± 0.07	0
Pleurotus lindquistii BAFC 2102	12.9 ± 0.08	9 ± 0.28	6.4 ± 0.09	6.4 ± 0.21	6.4 ± 0.06	6.2 ± 0.15
Lentinus tigrinus BAFC 197	12.9 ± 0.14	12.9 ± 0.23	6.4 ± 0.1	6.4 ± 0.08	6.4 ± 0.10	12 ± 0.21
Lentinula edodes BAFC 3883	6.2 ± 0.13	6.1 ± 0.20	2.9 ± 0.16	NG	NG	0
Phlebia brevispora. BAFC 633	9 ± 0.21	6.4 ± 0.13	2.9 ± 0.07	NG	NG	0
Phanerochaete chrysosporium BKMF 1767	12.9 ± 0.07	9 ± 0.09	9 ± 0.06	1.7 ± 0.07	NG	1.6 ± 0.20
Phanerochaete sordida BAFC 2122	12.9 ± 0.14	12.9 ± 0.06	6.4 ± 0.07	6.4 ± 0.08	6.4 ± 0.14	12.3 ± 0.22
Pleurotus djamor BAFC 215	9 ± 0.07	6.4 ± 0.07	2.8 ± 0.08	2.1 ± 0.14	NG	34.6 ± 0.96
Pleurotus ostreatus BAFC 120	9 ± 0.13	9 ± 0.22	4.7 ± 0.15	0.7 ± 0.16	NG	11.9 ± 0.28
Pycnoporus sanguineus BAFC 98	10.3 ± 0.14	9 ± 0.06	6.4 ± 0.28	3.7 ± 0.07	NG	3.9 ± 0.13
Pycnoporus sanquineus BAFC 2126	12.9 ± 0.22	9 ± 0.23	6.4 ± 0.07	4. ± 0.12	1.3 ± 0.08	19 ± 0.07
Schizopora paradoxa BAFC 71	12.9 ± 0.13	9 ± 0.14	6.4 ± 0.08	6.4 ± 0.20	6.4 ± 0.09	0.7 ± 0.08
Aurantiporus fissilis BAFC 170	9 ± 0.23	9 ± 0.20	6 ± 0.06	3.2 ± 0.08	NG	1.1 ± 0.29
Lenzites elegans BAFC 2127	12.9 ± 0.09	12.9 ± 0.07	9 ± 0.22	6.4 ± 0.07	3.1 ± 0.05	0
Coriolopsis gallica BAFC 270	6.4 ± 0.07	4.2 ± 0.21	3.4 ± 0.08	1.8 ± 0.09	NG	34.1 ± 0.35
Trametes trogii BAFC 463	90 ± 0.18	9 ± 0.23	2.7 ± 0.14	1.1 ± 0.07	NG	23.9 ± 0.92
Trametes trogii BAFC 619	16 ± 0.14	2.3 ± 0.14	3 ± 0.07	1.7 ± 0.14	NG	14.5 ± 0.35
Trametes versicolor BAFC 2234	12.9 ± 0.12	12.9 ± 0.15	6.4 ± 0.14	6.4 ± 0.09	6.4 ± 0.08	13.3 ± 0.28
Trametes villosa BAFC 2755	12.9 ± 0.11	9 ± 0.25	6.4 ± 0.09	4.9 ± 0.15	1.4 ± 0.16	14 ± 0.84

NG, no growth.
[a] Controls (without phenol).
[b] Laccase activity was measured by the oxidation of 2,2-azino-bis 3-ethylbenzo-thiazoline-6-sulfonate at pH 4.5 after 28 days.

endocarp of *L. aegyptiaca* (Fig. 1A), as we did before, but this time the fungus was grown in a complex liquid medium: tomato juice medium (TJM), which consisted of eco-tomato juice (Albi, Germany) and distilled water (50:50 v/v). To stimulate enzyme production, $MnCl_2$ and $CuSO_4$ were added prior to autoclaving (final concentration 250 μM). Five hundred milliliters flasks containing 200 mL of TJM and three pieces of the carrier (150 × 250 mm) were incubated on a rotary shaker at 100 rpm and 24 °C. Cultures were harvested at day 15 of cultivation. Heat-inactivated cultures served as controls to determine abiotic phenol loss and its sorption to the fungal mycelium and the carrier surface. Phenol was repeatedly added (fed-batch, three times) when its initial concentration in the culture liquid had decreased by 60–70%; the whole process was followed over a period of 23 days.

2.4 Production of laccase and MnP and purification steps

MnP and laccase of *T. versicolor* were produced in TJM and purified by different steps of ion exchange chromatography using the strong anion exchangers Q Sepharose FF, Mono Q and Mono S (GE Healthcare, Freiburg, Germany) [20].

A 30-L stirred-tank bioreactor (Biostat B; Braun Biotech International, Melsungen, Germany) was used for the production of the

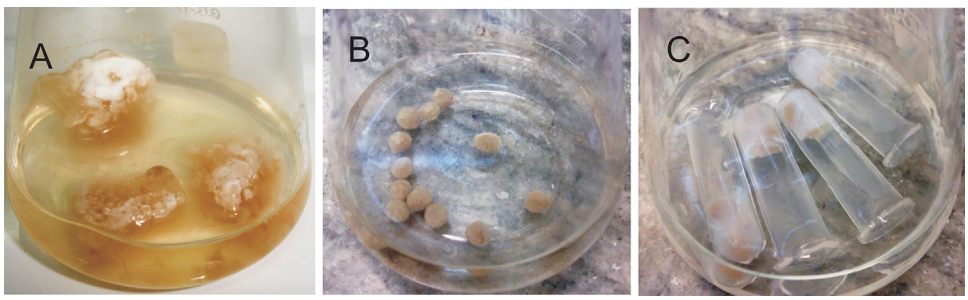

Fig. 1. *Trametes versicolor* immobilized (A) on loofa-sponge, (B) on alginate, and (C) on alginate-silica biocomposites.

enzymes. Twenty liters of the complex liquid medium TJM were used as growth medium. The inoculum consisted of 1 L of a homogenized fungal suspension pre-cultured in 500-mL Erlenmeyer flasks containing 200 mL of TJM on a rotary shaker at 100 rpm and 24 °C for 10 days. Fermentation was carried out under following conditions: 120 rpm, 4 L min^{-1} aeration rate and 28 °C; the pH was not adjusted. Whole cultures were harvested after 7 days.

To obtain pure laccase and MnP, the major part of the crude extract produced in TJM was further purified by up to three steps of fast protein liquid chromatography. In the first step, extracellular proteins were separated on Q-sepharose® (strong anion exchanger, column: 16 × 100 mm, GE Healthcare, Freiburg, Germany) and eluted with a linear gradient of 0–0.8 M NaCl in 10 mM sodium acetate buffer (pH 5.5) at a flow rate of 5 mL min^{-1}. Fractions containing laccase/MnP activities were pooled, concentrated, dialyzed against 10 mM sodium acetate (pH 5.5–7.0) with 10 kDa Vivaspin concentrators (Sartorius Stedim Biotech; Göttingen, Germany) and loaded onto a Mono-Q® column (10 × 100 mm, GE Healthcare). Bound proteins were eluted with 10 mM sodium acetate buffer containing 2 M NaCl at a flow rate of 6 mL min^{-1} (these two steps were sufficient for laccase separation). Fractions containing MnP activity were further purified by loading them onto a Mono-S column (10 × 100 mm, GE Healthcare) that was eluted with 10 mM sodium acetate buffer containing 2 M NaCl at pH 4.5. After the respectively last purification step, the final fractions of laccase and MnP were pooled, concentrated and stored at −20 °C. The elution of proteins was monitored at 280 nm (total protein) and 407 nm (heme). SDS–PAGE was performed to verify the purity of the enzyme preparations (12% NuPAGE Bis–Tris gel; Invitrogen, Karlsruhe, Germany; Fig. 2). Analytical isoelectric focusing was carried out using isoelectric focusing precast gels (pH 3–10; Invitrogen). After electrophoretical separation, the protein bands were visualized with the Colloidal Blue Staining Kit (Invitrogen). Protein concentration was determined by the method of Bradford using the Roti®-Nanoquant Protein Assay Kit (Roth, Karlsruhe, Germany) with bovine serum albumin as the standard.

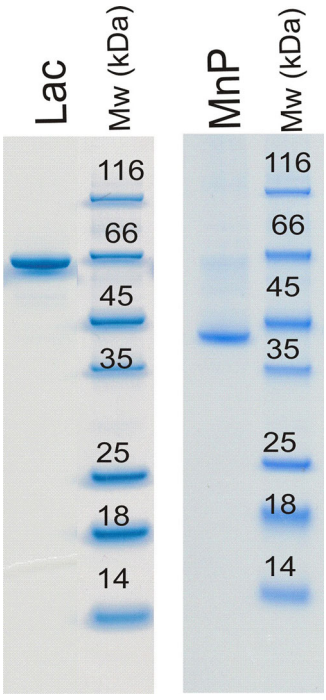

Fig. 2. SDS–PAGE of purified laccase and MnP produced by *T. versicolor*: lane 1, laccase; 3, MnP; 2 and 4, molecular weight markers.

2.5 Phenol removal in vitro

To evaluate the possible participation of the lignin-modifying oxidoreductases (laccase and MnP) in phenol conversion, a number of in vitro experiments were performed. Enzymatic reactions were directly carried out in 1.5-mL high performance LC (HPLC) vials and small fractions (20 μL) were repeatedly injected into the HPLC system by an autosampler. The reaction mixture (500 μL) contained dissolved phenol (0.5 mM) and sodium citrate (50 mM, pH 4.5) in case of laccase or sodium malonate (50 mM; pH 4.5) for MnP. Laccase reaction was started by the addition of the purified *T. versicolor* laccase (1 U mL^{-1} final concentration) and the MnP reaction by adding the purified enzyme (1 U mL^{-1} final concentration) and H_2O_2 (0.5 mM final concentration). Controls did not contain enzyme. Reaction progress was analyzed by HPLC using the Agilent Series 1200 system described above under the same conditions.

2.6 Enzymatic assays

Laccase activity was determined by following the oxidation of 2,2-azino-bis 3-ethylbenzo-thiazoline-6-sulfonate by 50 μL enzyme sample (culture liquid) in 50 mM citrate-phosphate buffer (pH 4.5, 25 °C) at 420 nm ($\varepsilon_{420} = 36\,000\ M^{-1}\,cm^{-1}$) [21]. The activity of MnP was measured at 270 nm ($\varepsilon_{270} = 11\,590\ M^{-1}\,cm^{-1}$) by the formation of Mn^{3+} malonate complexes in 50 mM sodium malonate buffer (pH 4.5) containing 0.5 mM $MnCl_2$, 0.2 mM H_2O_2, and 50 μL enzyme sample (culture liquid); the reaction was initiated by adding H_2O_2 [22]. Lignin peroxidase activity was measured with veratryl alcohol [23] by following the formation of veratraldehyde at 310 nm ($\varepsilon_{310} = 9300\ M^{-1}\,cm^{-1}$) in 50 mM sodium tartrate buffer (pH 3) containing 5 mM veratryl alcohol, 0.2 mM H_2O_2, and 100 μL enzyme sample; the reaction was started with H_2O_2. Versatile peroxidase activity was measured at 598 nm ($\varepsilon_{598} = 47\,600\ M^{-1}\,cm^{-1}$) by monitoring the conversion of the azo dye Reactive Black 5 in 50 mM sodium tartrate buffer (pH 3) containing 0.1 mM Reactive Black 5 and 0.2 mM H_2O_2 [24]. Enzymatic activities were expressed in units (U) defined as the amount of enzyme required to produce 1 μmol of product or, in case of versatile peroxidase, to cleave 1 μmol min^{-1}.

2.7 Phenol quantification

Phenol concentration was first estimated measuring the phenolic content according to Vazquez Roncero et al. [25], using gallic acid as standard. Phenol removal in later experiments was followed by HPLC. An Agilent HPLC system (Agilent, 1200 series, Waldbronn, Germany) equipped with a diode array detector and a diphenyl column (100 × 2.0 mm, 2.4 μm particle size, Varian, Darmstadt, Germany) was used for analyses. Samples (1–1.5 mL) were centrifuged and transferred to 2-mL HPLC vials and then injected into the HPLC system by an autosampler. Analyzes were performed at a column temperature of 50 °C using a mixture of phosphoric acid (20 mM) and acetonitrile as eluent (nitrophenol/phenol, 82:18 and 90:10 v/v, respectively), at a flow rate of 0.4 mL min^{-1} and under

isocratic conditions. Eluted substances including phenol were detected at 270 nm.

2.8 Phytotoxicity bioassay

The phytotoxicity of phenol and its derivates were determined according to the method described by Zucconi et al. [26] using *Lepidium sativum* seeds incubated for 48 h at 25 °C with either a fresh phenol solution (10 mM), or the phenol solution treated for 23 days with *T. versicolor* immobilized on sponge carrier. The germination index (GI) was calculated according to the expression:

$$GI = ((G/G_o)/(L/L_o)) \times 100$$

where G_o and L_o are, respectively, the percent germination and radicle growth of the control. All chemicals used were obtained from Sigma–Aldrich (Weinheim, Germany) or Merck (Darmstadt, Germany) in highest purity available.

2.9 Statistical analysis

All experiments were carried out in triplicate. The results are expressed as mean values with standard deviation; variance analysis was done out using the ANOVA program followed by Tukey's test using the Statistica 7.0 program. Differences at $p < 0.05$ were considered to be significant.

3 Results and discussion

3.1 Fungal screening

The first steps in developing an efficient fungal bioremediation process are the identification of suitable strains and the optimization of the culture conditions necessary for their well growth and the production of relevant enzymes [27]. With this aim, 25 Argentinean white-rot fungi belonging to four different orders were tested for phenol tolerance during growth on MEA plates in comparison with the model organism *P. chrysosporium* (BKM-F-1767), a widely studied ligninolytic fungus. Most fungal strains tested showed already a notably reduced growth (up to 85% in case of *T. trogii*) at the lowest phenol concentration (1 mM) tested, which is indicative of a strong inhibitory effect of phenol. Ten of the fungal strains were able to grow at a phenol concentration of 10 mM – interestingly *P. chrysosporium* was not among them. Six strains, among others *T. versicolor*, still reached 45–50% of the growth rate observed in the absence of phenol (Table 1). Extracellular laccase activities were measured in agar extracts from the plates containing 7.5 mM phenol. Six fungal species known to cause a strong white-rot did not exhibit any laccase activity under these conditions (*Flammulina velutipes*, *Irpex lacteus*, *Lentinula edodes*, *Lenzites elegans*, *Abortiporus biennis*, and *Phlebia brevispora*). The highest laccase activities were detected for *Pleurotus djamor*, *Ganoderma resinaceum*, and *Coriolopsis gallica* (around 35 mU g^{-1}), but none of these strains was capable of growing in the presence of 10 mM phenol.

Among the 26 isolates, seven strains were selected, based on their phenol tolerance, for a second screening step using GA agar plates supplemented with 7.5 mM of different phenolic compounds (2,6-dimethoxyphenol, gallic acid, 2,4-dichlorophenol or guaiacol; Table 2). Furthermore, fungal growth was tested on agar plates containing phenol as sole carbon source. None of the fungal strains was able to grow in the presence of dichlorophenol. 2,4-dichlorophenol is one of the most recalcitrant chlorophenols, known to be toxic for fungal growth. 10 to 50 ppm was equal to one-half the lethal dose for fungi such as *Trichoderma viride*, *Mortierella isabellina*, and *Saprolegnia parasitica* [28]; in vitro and in situ assays conducted with 2,4-dichlorophenol and fungal isolates from soil demonstrated that this compound was toxic to fungal propagules at similar concentrations [29]. In this work, also guaiacol had a strong inhibitory effect, and only *I. lacteus*, *T. versicolor*, *Lentinus tigrinus*, and *Pleurotus lindquistii* could grow to some extent in the presence of this compound (5–50% growth inhibition). Interestingly, all fungal strains selected were seemingly capable of growing with phenol as sole carbon source. Lignin degradation by white-rot fungi is known as a co-metabolic process (with sugars from hemicelluloses or cellulose as easily assimilable carbon sources), and growth of white-rot fungi on monoaromatics such as phenol has only scarcely been described [3]. On the other hand, a number of basidiomycetous yeasts such as *Trichosporum cutaneum* [30] and *Rhodotorula mucilaginosa* [31] as well as ascomycetous molds like *Penicillium frequentans* [32] or *Aspergillus niger* [33] were shown to utilize various aromatic compounds including lignin-related aromatic alcohols, aldehydes and acids as sole sources of carbon and energy. Intracellular key enzymes of aromatics degradation from *T. cutaneum*, for example, catechol 1,2-dioxygenase and phenol hydroxylase, were characterized in detail [34–36]. These activities were recently found in *T. versicolor* [3, 37]. Among the fungi tested here, *T. versicolor* (strain BAFC 2234) showed the highest growth rate with phenol as carbon source and was therefore chosen for further studies in liquid culture.

Table 2. Growth of fungal isolates, selected according to the results of Table 1, on agar plates containing GA medium supplemented with different phenol derivatives (7.5 mM), or on plates containing phenol (2.5 mM) and ammonium phosphate (4 g L^{-1}) as sole sources of carbon and nitrogen, respectively

	Growth rate (mm day^{-1})				
Strain	Gallic acid	2,6-Dimethoxyphenol	Guaiacol	2,4-Dichlorophenol	Phenol
Irpex lacteus	12.2 ± 0.35^{a}	12.4 ± 0.15^{a}	0.6 ± 0.05^{c}	NG	$1.2 \pm 0.11^{a,b}$
Lentinus tigrinus	12.6 ± 0.28^{a}	12.3 ± 0.13^{a}	2.7 ± 0.12^{b}	NG	$0.8 \pm 0.05^{b,c}$
Pleurotus lindquistii	12.5 ± 0.22^{a}	12.4 ± 0.14^{a}	2.4 ± 0.10^{b}	NG	$0.8 \pm 0.10^{b,c}$
Phanerochaete sordida	12.4 ± 0.15^{a}	12.1 ± 0.25^{a}	NG	NG	$0.9 \pm 0.08^{b,c}$
Schizopora paradoxa	12.4 ± 0.12^{a}	12.2 ± 0.15^{a}	NG	NG	1.1 ± 0.09^{b}
Lenzites elegans	12.5 ± 0.25^{a}	12.4 ± 0.21^{a}	NG	NG	$0.9 \pm 0.07^{b,c}$
Trametes versicolor	12.8 ± 0.22^{a}	12.6 ± 0.25^{a}	6.4 ± 0.15^{a}	NG	1.4 ± 0.11^{a}

NG, no growth. Mean values superscript with different letters are significantly different ($p < 0.05$).

3.2 Phenol removal by *versicolor* immobilized on synthetic and natural supports

Processes using growing, immobilized cells are more suitable for biodegradation studies than free cells in suspension, since immobilization allows us to use the fungal cells repeatedly or even continuously and it facilitates liquid–solid separation. Furthermore, immobilized cells tend to accomplish higher levels of activity and they are more resilient to environmental perturbations such as pH or temperature changes, or exposure to toxic chemicals [38]. *T. versicolor* BAFC 2234 immobilized on both supports assayed here was able to remove 10 mM phenol in aqueous solution. The best result was achieved when the fungus had been immobilized on the sponge-like carrier prepared from fruits of *L. aegyptica*. It could remove up to $87 \pm 1.2\%$ of phenol, although it is recognized that about $30 \pm 1.1\%$ of this removal may be attributed to adsorption to the mycelium and material support. Active fungi-alginate beads removed $41 \pm 0.5\%$ and the inactivated system $33 \pm 0.9\%$ of phenol; the inactive biocomposites (silica-alginate-inactivated fungus) removed $41 \pm 1.7\%$ (attributed to adsorption) and the active biocomposites $48 \pm 0.5\%$. In consequence, the sponge carrier was chosen as the support of choice for the immobilization of *T. versicolor* and further tested using a 15 mM phenol solution. 71 ± 2.4, 62 ± 0.5, 74 ± 0.4, and $73 \pm 0.3\%$ of the phenol added was depleted in three repeated cycles over a total period of 23 days (phenol was added after three, ten and 16 days, respectively; Fig. 3). In the control, a phenol decrease was only observed within the first cycle and amounted to $39 \pm 0.5\%$ compared with the active fungus (again attributable to adsorption to the mycelium, while fungal/enzymatic conversion made up $32 \pm 1.2\%$). In subsequent cycles, adsorption was not further observed and significant variation in the phenol content of the controls was not detected (*i.e.*, the possible binding positions for adsorbing phenol molecules were occupied). Although fungal biomass when using vegetable sponge as carrier cannot be determined accurately since the fungus may use as C source the polymers of loofa sponge, in order to compare both immobilization systems, we used similar biomass (around 16 ± 0.1 mg).

Santos and Linardi [39] screened 30 filamentous fungi isolated from industrial effluents for their ability to degrade phenol. The extent and time required for phenol degradation varied as a function of strain and the initial phenol concentration. Phenol concentrations of >6 mM lowered the percentage of phenol degradation to levels below 5%, a behavior that is characteristic for the metabolism of toxic compounds [40]. In this context, it has to be noted that the strain of *T. versicolor* used here removed 10 mM phenol. The successful bioremediation of a phenolic wastewater by *T. versicolor* was reported to be dependent on a range of factors including fungal growth, culture age and laccase production. The phenolic effluent boosted laccase production [27]. In our study, the only relevant activity of a lignin-modifying enzyme in cultures of *T. versicolor* supplemented with phenol was related to laccase (up to $160\,U\,L^{-1}$; Fig. 3), MnP, lignin-peroxidase, or versatile peroxidase activities were not detectable (data not shown). Laccase activity at first decreased right after addition of the pollutant but increased again in coincidence with phenol disappearance. This effect was observable after each phenol supplementation. In fact, during the oxidative transformation of phenolic compounds, permanent inactivation through interactions of the enzyme with radical products and through entrapment in polymer precipitates can take place [41]. One of the proposed natural functions of laccases is the detoxification of phenolic compounds by initial oxidation followed by radical coupling and polymerization. Strong indication for the latter reaction was the formation of black precipitates concomitantly to the increase in laccase activity [27, 42]. These precipitates were observed from the 3rd day of cultivation on, and may represent humic-like substances as observed during the oxidation of phenols by the laccase of the fungus *Chaetomium thermophilum* [43].

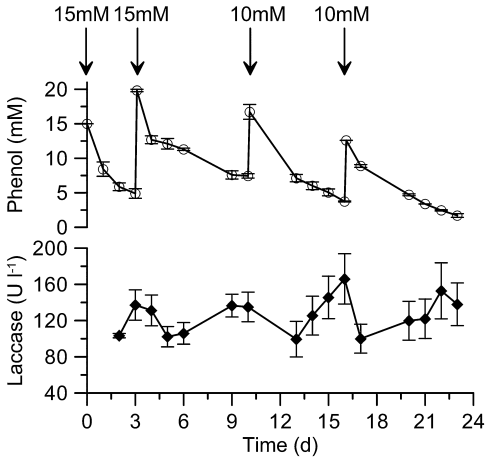

Fig. 3. In vivo removal of phenol by mycelium of *T. versicolor* immobilized on loofa-sponge under fed-batch conditions. Diamonds: laccase activity, circles: phenol concentration.

Selection of the immobilization technique as well as of the immobilization material is essential to design an effective system for each particular purpose [44]. We tested here two different materials for immobilization of *T. versicolor* and the subsequent transformation of phenol. Both supports proved to be effective for phenol removal by *T. versicolor*, but immobilization on loofa sponge gave better results. A main factor involved in the removal of phenol by the biocomposites was the adsorption to the matrix. Thus, the results observed with the matrix-fungus system are the consequence of the adsorption and the regulated transport of phenol as well as of the retention of the degrading enzymes inside the alginate beads. Massalha et al. [45] reported that a physical adsorption to Ca-alginate beads immediately started after adding phenol to the medium. In our case, adsorption was also observed for the sponge system, but the action of enzymes was obviously the key factor for phenol removal. Rodríguez Couto et al. [44] reported similar results when comparing different materials for *T. hirsuta* immobilization. They found that the fungus immobilized on stainless steel sponge established superior efficiency in the removal of industrial dyes, while other carriers such as alginate-silica gave only moderate results. However, there is an important point to consider regarding the Ca-alginate beads: this immobilization procedure allows the separation of the microorganisms from the polluted stream and the whole environment. Thus, it was found in experiments on dye degradation by white-rot fungi that the transport of the dye through the matrix facilitates constant biodegradation by maintaining a low dye concentration near the fungus [17]. In particular, when high pollutant concentrations are treated, this system will keep the microorganism viable for a longer period of time.

The ability of *T. versicolor* BAFC 2234 to reduce phenol concentrations as high as 15 mM is a further indication of its bioremediation potential. For the respective test, an alternative complex

culture medium based on TJM was chosen, which resulted in higher biomass (25 instead of 16 mg). In a previous study, *T. versicolor* secreted several ligninolytic enzymes during cultivation in TJM supplemented with copper and manganese [20]. TJM was also found to stimulate the production of extracellular oxidoreductases (MnP, laccase) by several other agaric fungi [46, 47].

3.3 Removal of phytotoxicity

The germination index was used to test the phytotoxicity of phenol with *L. sativum* seeds. This index was almost zero for 15 mM phenol solution and controls containing phenol and heat-inactivated mycelium. After treatment with *T. versicolor*, the phytotoxicity decreased noticeably and led to a germination index of $63 \pm 5.7\%$. Aranda et al. [48] recently demonstrated that the treatment of solid olive-mill residues with laccase from white-rot fungi remarkably reduced the phytotoxicity caused by the phenolic ingredients.

3.4 Phenol degradation in vitro

Finally, we examined the ability of purified laccase and MnP from *T. versicolor* strain BAFC 2234 to oxidize phenol in vitro. Both enzymes were purified from TJM cultures of the fungus and diminished the concentration of phenol. Laccase converted 84% of the phenol within 4 h, while 43% was degraded by MnP. Control experiments were carried out without enzymes and resulted in negligible removal of phenol (Fig. 4). *T. versicolor* is known to produce high levels of different lignin-modifying enzymes, but each one may differently contribute to the final degradation of lignin. So it produces several laccases, lignin-peroxidases, MnP, and aryl alcohol oxidase, but probably utilizes primarily laccase and MnP in lignin degradation [49]. Our tests with the latter enzymes confirmed their high degradative potential and interestingly laccase showed the better performance in phenol removal. Ullah and Evans [50] reported that purified laccase of *T. versicolor* removed pentachlorophenol also more efficiently than MnP did. Treatment with laccases of *T. versicolor* and *Pleurotus sajor caju* reduced from 4 up to 70% of phenol in olive oil mill wastewater [51]. Laccases are copper-containing oxidases that catalyze the oxidation of aromatic compounds with phenolic and/or amino groups [49, 52]. The typical catalytic action of laccase involves oxidation of the phenolic substrates by H-abstraction transferring the electrons to dioxygen (one-electron oxidation). This results in the formation phenoxy radicals that in turn may undergo disproportionation to quinones [53] or coupling to humic substance-like polymerization products [42, 54]. However, in our study, *p*-benzoquinone was not observed in HPLC elution profiles of phenol samples treated with laccase; only dark colored products that partly precipitated were found. This result led us assume that oxidative coupling of phenoxy radicals is the major pathway of phenol conversion by *T. versicolor* laccase. This is in agreement with findings of Ryan et al. [27] who reported that the increase of laccase activity in fungal cultures coincided with the formation of dark precipitates, which were proposed to originate from the coupling of phenolic inducers used to stimulate enzyme production. Font et al. [55] observed substantial polymerization and the formation of oligomeric and high-molecular weight compounds during the treatment of black liquor with *T. versicolor*. We noticed also formation of dark precipitates and the lacking of *p*-benzoquinone during in vitro treatment of phenol with MnP. MnP of another white-rot fungus, *Bjerkandera adusta*, was found to polymerize, among others, guaiacol, *o*-cresol, 2,6-dimethoxyphenol and aromatic amines [56]. The incubation of dry olive mill residue with peroxidase-producing agaricomycetes also led to a decrease in its phytotoxicity and phenol depletion caused by polymerization [57].

4 Concluding remarks

An Argentinean strain of *T. versicolor*, BAFC 2234, immobilized on natural plant sponge was found to remove up to 15 mM phenol and decreased the phytotoxicity of respectively treated samples. The purified ligninolytic enzymes laccase and MnP were able to remove phenol, thus contributing to the process. Immobilization on sponge-like carriers allows combining a high rate of phenol degradation with the re-utilization of the biomass over several cycles and the development of continuous bioprocesses. To our best knowledge, the concentrations of phenol removed by *T. versicolor* here are among the highest concentrations reported so far. On the basis of these results,

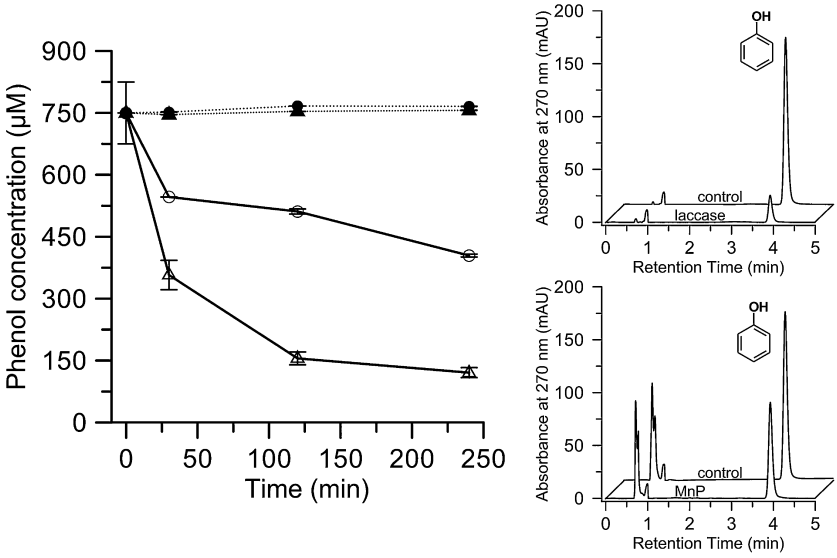

Fig. 4. (Left) In vitro oxidation of phenol by purified *T. versicolor* laccase (triangles) and MnP (circles). Black symbols correspond to control experiments and empty symbols correspond to assays performed with enzymes. (Right) HPLC elution profiles (recorded at 270 nm) of corresponding phenol samples treated with MnP bottom and laccase top, respectively.

the investigated strain may have good prospects for application in industrial wastewater treatment.

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