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Research article

Simple screening protocol for identification of potential mycoremediation tools for the elimination of polycyclic aromatic hydrocarbons and phenols from hyperalkalophile industrial effluents

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A B S T R A C T

A number of fungal strains belonging to the ascomycota, basidiomycota and zygomycota genera were subjected to an *in vitro* screening regime to assess their ligninolytic activity potential, with a view to their potential use in mycoremediation-based strategies to remove phenolic compounds and polycyclic aromatic hydrocarbons (PAHs) from industrial wastewaters. All six basidiomycetes completely decolorized remazol brilliant blue R (RBBR), while also testing positive in both the guaiacol and gallic acid tests indicating good levels of lignolytic activity. All the fungi were capable of tolerating phenanthrene, benzo-α- pyrene, phenol and *p*-chlorophenol in agar medium at levels of 10 ppm. Six of the fungal strains, *Pseudogymnoascus* sp., *Aspergillus caesiellus*, *Trametes hirsuta* IBB 450, *Phanerochate chrysosporium* ATCC 787, *Pleurotus ostreatus* MTCC 1804 and *Cadophora* sp. produced both laccase and Mn peroxidase activity in the ranges of 200–560 U/L and 6–152 U/L, respectively, in liquid media under nitrogen limiting conditions. The levels of adsorption of the phenolic and PAHs were negligible with 99% biodegradation being observed in the case of benzo-α-pyrene, phenol and *p*-chlorophenol. The aforementioned six fungal strains were also found to be able to effectively treat highly alkaline industrial wastewater (pH 12.4). When this wastewater was supplemented with 0.1 mM glucose, all of the tested fungi, apart from *A. caesiellus*, displayed the capacity to remove both the phenolic and PAH compounds. Based on their biodegradative capacity we found *T. hirsuta* IBB 450 and *Pseudogymnoascus* sp., to have the greatest potential for further use in mycoremediation based strategies to treat wastestreams containing phenolics and PAHs.

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1. Introduction

Wastes generated by industrial processes are mainly composed of ubiquitous organic compounds. In general, aromatic compounds are extensively distributed in nature, showing a rich structural diversity and are considered as strong pollutants (Fuchs et al., 2011). Polycyclic aromatic hydrocarbons (PAHs) and phenolic compounds are well established as being among the most toxic environmental

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priority pollutants known (Gupta et al., 2015; Tisa et al., 2015). Since aromatic compounds have a poor reactivity, they can stably remain in the environment for long periods of time (Fuchs et al., 2011). However, when activated, they are extremely dangerous because they can react with biological molecules, such as DNA, as well as lipids present in cellular membranes and with proteins (Bragin et al., 2016; Long et al., 2016).

PAHs are a wide and heterogeneous group of compounds (more than 100 compounds) with two or more condensed benzene rings in linear, angular or cluster arrangements (Scott, 2015). They can be either naturally produced or derived from anthropogenic sources. PAHs can be found in oil and carbon deposits, and their adverse effects which include toxicity, teratogenicity, mutagenicity and carcinogenicity have been extensively demonstrated (Bragin et al., 2016; Long et al., 2016). PAHs are semi-volatile, thermodynamically stable, and show low aqueous solubility (Keyte et al., 2013). Consequently, they are highly recalcitrant compounds that persist over long periods of time in ecosystems (Kuśmierz et al., 2016). PAHs immobilization in nature mainly occurs through adsorption into soil or sediment particles due to their hydrophobic characteristics and by bioaccumulation processes in trophic chains (Petit et al., 2013; Shuttleworth and Cerniglia, 1995).

Phenols are widely used in numerous industrial processes. Consequently, several toxic phenols are generated from industrial operations (Pontes et al., 2010). They are highly toxic even at low concentrations; are one of the most prevalent forms of chemical contaminants, and the major pollutants found in industrial wastewaters (Gayathri and Vasudevan, 2010). Since 1989, phenolic compounds have been considered as the second major class of pollutants in the environment (Guang, 1998). Particularly, phenols can be found in liquid effluents such as petrochemical, papermaking, oil refining, resin manufacturing, coking, iron-smelting, pharmaceutical industries, among others (Tisa et al., 2015). Phenolic compounds present in wastewaters represent a potential risk for the aquatic biota (Gayathri and Vasudevan, 2010).

PAHs and phenolic compounds in the environment can be transformed by different mechanisms such as photolysis and chemical or biological oxidation (Biache et al., 2015; Riva et al., 2015). Microbial transformation is recognized as the main process in their mineralization in nature (Haritash and Kaushik, 2009; Tisa et al., 2015). Different microbial catabolic pathways are involved in the PAHs and phenolic biodegradation or mineralization. PAHs and phenol catabolic genes have been studied in both bacteria and fungi, and several microbial treatment strategies have been described in response to these pollutants (Aranda, 2016; Haritash and Kaushik, 2009). The resonance energy that stabilizes the aromatic ring systems is the main base of the aromatic substance recalcitrance, which confers a high redox potential for electron transfer reactions. For instance, only a small group of enzymes are able to cleave the aromatic rings of PAHs or phenol-based compounds (Fuchs et al., 2011). While dioxygenases, dihydroxygenases, monooxygenases, phenol hydroxylases, serine hydrolases and aldolases are involved in the bacterial degradation of PAHs and phenols via either metabolism or cometabolism (Moody et al., 2004; Rentz et al., 2008), only two major fungal mechanisms have been described. They are *(i)* the cytochrome P-450 monooxygenase pathway and *(ii)* extracellular enzymes of lignin catabolism such as lignin peroxidases (LiP), manganese peroxidase (MnP) and laccases (Lac) (Aranda, 2016; Cerniglia, 1997; Krastanov et al., 2013; Mhuantong et al., 2015). These three ligninolytic enzymes oxidize a wide range of organic compounds (including several oil fractions, phenols and PAHs) by virtue of their low specificity (Andriani et al., 2016; Pang et al., 2015; Zafra and Cortes-Espinosa, 2015).

The bacterial metabolism of PAHs and phenolic compounds have been extensively studied and their molecular mechanisms extensively elucidated. However, some fungi have shown the ability to remove and mineralize PAHs and phenolic compounds in a more competent way than bacteria (Fernández-Luqueño et al., 2010; Fuentes et al., 2014; Juhasz and Naidu, 2000). Moreover, microbial-based approaches to remove phenols or PAHs under extreme conditions (e.g. high pH and salinity) provide an opportunity for the screening of microorganisms with extremophile characteristics. The use of fungi to remove aromatic compounds from industrial wastewaters enriched with phenols or PAHs derived from chemical industry, provides an opportunity for downstream biotechnological applications on hypersaline or alkaline liquid wastes (Acikgoz and Ozcan, 2016; Christen et al., 2011). Fungi growing under extremophile conditions (e.g. alkaline pH) in real wastewaters provide an attractive resource for the development of bioprocesses and ecological restoration.

There is an increasing interest in exploring as yet unstudied or understudied fungal species/strains in order to degrade PAHs and phenol-based compounds, and to select ideal candidates for bioremediation of industrial wastewaters contaminated with organic pollutants. The environmental problems caused by the anthropogenic activities demand new screening regimes to uncover fungal phylotypes with robust potencies for downstream biotechnological applications.

This work aimed to study the removal of phenols and PAHs *in vitro* by the screening of a fungal collection and to identify relevant strains capable of eliminating phenolic compounds and PAHs from biorefinery industrial wastewaters. To this end, twelve fungal strains were screened based on their ability to secrete ligninolytic enzymes to degrade PAHs and phenols. Additionally, we tested the tolerance of these strains at a variety of different PAHs and phenol concentrations, and their efficiency in the removal of these compounds from wastewaters.

2. Materials and methods

2.1. Microorganisms and chemicals

Zygomycetes, ascomycetes and basidiomycetes species isolated from different environments or deposited in different microbial collections were analyzed in this work (Table 1). Spores and mycelia

Table 1
Zygomycetes, ascomycetes and basidiomycetes utilised in this study.

Species	Remarks
Zygomycete	
Cunninghamella elegans ATCC 36112	
Ascomycetes	
Cadophora sp. TS2	Isolated from the deep sea
Emericellopsis sp. TS11	sponge *Stelleta normani*
Pseudogymnoascus sp. TS12	
Aspergillus caesiellus H1	Halophilic fungus
Trichoderma atroviride CEIB 206040	
Trichoderma atroviride + Lac of	Recombinant clone
Trametes sanguineus CeIB MD01	of *T. atroviride*
Basidiomycetes	
Pleurotus dryinus IBB 903	
Trametes hirsuta IBB 450	
Phanerochaete chrysosporium ATCC 787	
Trametes hirsuta MTCC 1171	
Pleurotus ostreatus MTCC 1804	

ATCC: American Type Culture Collection.
TS: Reference in School of Microbiology, University College Cork. Cork, Ireland.
CEIB: Biotechnology Research Center of the Autonomous University of the Morelos State.
IBB: Institute of Biochemistry and Biotechnology, Tbilisi, Georgia.
MTCC: Indian Microbial Type Culture Collection.

obtained on Malt extract agar (MEA) from fresh cultures of each fungal species assessed were used in all experiments.

All chemicals were of analytical grade or higher purity. MEA, gallic acid, remazol brilliant blue R (RBBR), guaiacol, thiamine, 2,6-dimethoxylphenol (DMP), 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 4-aminoantipyrine (4-AAP), $K_3[Fe(CN)6]$, veratryl alcohol, benzo-α-pyrene, phenanthrene, acetone, hexane, and all inorganic salts were obtained from Sigma-Aldrich (Saint-Louis, MO, USA).

2.2. Optimal growth temperature

Growth rates of each fungus in the mesophile temperature range (25, 30, 35 °C) were determined. A 7 mm diameter plug was inoculated on solidified MEA agar plates and incubated for 10 days. The radial growth (diameter) of the colony was measured in triplicate each 24 h. The temperature that supported the best growth rate (expressed in mm/d) was selected as the optimal growth temperature.

2.3. Fungal screening for detection of ligninolytic activities

2.3.1. Dye decoloration test

MEA supplemented with 50 mg/L of RBBR was used as a culture medium during the screening regime to determine the laccase (Lac) production of the tested fungi. RBBR is known to be strongly decolorized by Lac-producing fungi (Pasti and Crawford, 1991). A plug of 7 mm diameter of each fungus was inoculated on the above medium, and cultures were incubated for 20 days at their optimal growth temperature. Petri dishes were visually examined daily, to determine the decoloration in each case.

2.3.2. Gallic acid test

MEA supplemented with 5 g/L of gallic acid was used as a culture medium to determine the ligninolytic enzyme production ability of the tested fungi. Ligninolytic enzyme activities in fungi oxidize gallic acid oxidation to form brown-colored quinonic form in solid media (Leonard, 1971; Shleev et al., 2004). A plug of 7 mm diameter of each fungus was inoculated in the above medium, and cultures were incubated for 20 days at their optimal growth temperatures. Petri dishes were visually examined daily in each case to monitor the production of brown color due to the gallic acid oxidation.

2.3.3. Guaiacol test

MEA supplemented with 0.2% (*v*/*v*) of guaiacol was also used as a screening method to determine the Lac and peroxidases (Per) production in the fungi. The oxidation of guaiacol to its reddish brown-colored form in agar is indicative of Lac and Per activity in fungi (Kiiskinen et al., 2004; Kumar and Rapheal, 2011). A plug of 7 mm diameter of each fungus was inoculated in the above medium, and cultures were incubated for 20 days at their optimal growth temperature. Petri dishes were visually examined daily in each case to monitor the production of a reddish brown-color due to the oxidation of guaiacol.

Fungal cultures in MEA without the addition of RBBR, gallic acid and guaiacol were inoculated and incubated under the same conditions were used as controls in all above tests. Three replicates of each culture were analyzed in each experiment.

2.4. Production of Lac and Per in liquid medium

The enzymatic screening was performed in a 250 mL Erlenmeyer flasks containing 50 mL of the basal mineral medium as previously reported by Tien and Krik (1988). This saline medium was selected to stimulate ligninolytic enzyme (Lac and Per) production, and its composition per liter was as follows: 0.2 g ammonium tartrate, 3.28 g sodium acetate, 0.002 g thiamine, 2 g KH_2PO_4, 0.53 g $MgSO_4·7H_2O$, 0.1 g $CaCl_2$, 0.001 g $CuSO_4$, 0.005 $MnSO_4$, 0.0001 g H_3BO_3, 0.0001 g $NaMoO_4·2H_2O$, 0.001 g $ZnSO_4·7H_2O$, 0.001 g $CoCl_2$, 0.01 g NaCl and 0.001 g $FeSO_4·7H_2O$. Four grams of cylindrical wood chips (dimensions: 6 mm diameter and 10 mm height) from *Pinus arizonica* were added as the carbon source to boost the ligninolytic enzyme production. Each fungal species was inoculated in Erlenmeyer flasks ($\cong 10^8$ spores), and cultures were incubated at their optimal growth temperature shaking at 150 rpm for 12 days. Lac, MnP and LiP activities were determined every 2 days. Three replicates of each culture were analyzed and three independent measurements were performed.

2.5. Enzyme activity measurements

For Lac activity, a solution containing 1.0 mM ABTS, 0.1 M sodium acetate buffer (pH 4.0) and supernatant from fungal cultures was used. For MnP activity a mixture containing fungal supernatants, 0.5 M sodium malonate (pH 4.5), 5 mM $MnSO_4$, 1 mM DMP and 1 mM H_2O_2 was used. For LiP activity fungal supernatants, 0.25 M sodium tartrate (pH 2.5), 10 mM veratryl alcohol and 5 mM H_2O_2 were added at the reaction mixture. Supernatants in all cases were obtained by centrifugation of fungal cultures at 10,300 g for 10 min at 4 °C.

The Lac and Per activities were monitored by UV–Vis spectrophotometer (SpectraMax Plus 384, Molecular Devices Corporation, Sunnyvale, CA) according to previously reported methods (Haroune et al., 2014; Touahar et al., 2014; Wang et al., 2008). Experiments were performed in triplicate, involving three independent measurements in each case. Lac and Per activities were expressed as U/L. One unit (U) of enzymatic activity is defined as the amount of enzyme catalyzing the oxidation of 1 μmol of substrate per minute.

2.6. Fungal screening for PAHs and phenolic compounds degradation

2.6.1. PAHs and phenol tolerance

Phenanthrene and benzo-α-pyrene at 1, 5 and 10 ppm (final concentration) were added to the MEA medium to evaluate the fungi's tolerance to the PAHs. The tolerance of the fungi to phenol and *p*-chlorophenol was also evaluated using these compounds at 5 and 10 ppm (final concentration).

PAHs were previously dissolved in acetone before addition to the MEA medium. Triplicate plugs of 7 mm diameter of each fungus were inoculated in plates containing PAHs, phenol or *p*-chlorophenol, and cultures were incubated at their optimal growth temperature for 7 days. All the fungi were also inoculated into the MEA plates in the absence of the aromatic compounds (control cultures also included culture with acetone). The tolerance rate (TR) expressed as percentage of mycelial growth inhibition (% MGI) was calculated in triplicate [see Equation (1)] (Lee et al., 2014):

$$TR = FGR/GRC \times 100, \quad (1)$$

where FGR is the fungal growth rate and GRC is the growth rate of a control culture.

2.6.2. PAHs and phenol removal

The best fungal strains were selected, based on their tolerance to aromatic compounds (PAHs and phenols), for the PAHs/phenols removal study. Two hundred and fifty (250) mL Erlenmeyer flasks containing 50 mL of Malt Extract Broth (MEB) with 10 ppm of phenanthrene, benzo-α-pyrene, phenol or *p*-clorophenol were

aseptically inoculated with 10^8 spores of the selected fungi, incubated shaking at 150 rpm for 10 days at their optimal growth temperatures. Flasks were removed every 24 h in order to determine Lac and Per activities together with PAHs and phenols concentration. Both, supernatants and mycelium were collected for analysis.

For PAH determinations, liquid-liquid (*v*/*v*) (from supernatants) and solid-liquid (from mycelium) extractions were performed using 2 mL of hexane. Two mL of the supernatant and 1 mg of wet mycelium were used. Extractions were repeated three times for each sample (supernatant and mycelium). The recovered hexane (final volume 6 mL) was concentrated 3 times using a vacuum rotatory evaporator. Gas chromatograph-mass spectrometer (GC-MS) was used to determine the PAH concentration according to previously published methods (Lee et al., 2010, 2014). GC-MS analysis was performed on a G1800A gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with an electron ionization detector and an HP-5 MS fused-silica column (30 m × 0.25 mm i.d., 0.25 mm film thickness) (Hewlett-Packard, Palo Alto, CA).

Total phenolic compound removal percentages were calculated for each treatment using the total phenol estimation in accordance with the previously reported method (Scott, 1931). The phenol content of the solution was monitored using the conventional Aminoantipyrine method (Guang, 1998; Wei, 1990). Briefly, 50 μL of ammonium hydroxide solution (0.5 mol/L) was added to 2 mL of the sample, whose pH was adjusted to 7.9 using phosphate buffer of pH 7.0. Twenty μL of each, 2% 4-AAP and 8% $K_3[Fe(CN)_6]$ solutions were added, mixed well and the absorbance was measured at 500 nm. Phenol and *p*-clorophenol levels in the test samples were determined from their standard calibration curves in the range of 1.0–70.0 mg/L. This method is restricted to phenolic molecules unsubstituted at *para* - position, as the coupling reaction between 4-AAP and phenol provides the colored product whose absorption is measured spectrophotometrically, essentially takes place at the *para* position. Thus, this method is robust for estimation of generic phenol and 2-chlorophenol (substitution at *ortho* - position), since both are unsubstituted at the *para* − position.

Uninoculated flasks were used as controls. All experiments and analysis were performed in triplicates.

2.7. Removal of PAHs and phenolic compounds in industrial wastewater

A non-sterile wastewater containing both PAHs and phenolic compounds (Balcázar-López et al., 2016) collected from a biofuel production facility (Quebec, Canada) was used to study the potentiality of these fungi to remove PAHs and phenolic compounds from industrial wastewaters. Each fungus ($\cong 10^8$ spores) was inoculated in 50 mL of wastewater contained in 250 mL Erlenmeyer flasks. Two different scenarios were investigated: (*a*) wastewater supplemented with 0.1 mM of glucose, and (*b*) wastewater without the addition of glucose. The cultures were incubated for 12 days at their optimal growth temperature shaking at 150 rpm.

PAHs and phenols determinations were performed as previously described in section 2.6. Lac and Per activities were also determined as previously described. Triplicates of each culture were analyzed and three independent measurements were performed in each case.

2.8. Data analysis

Analysis of variance (ANOVA) tests were applied to determine significant differences between the different cases. Firstly, the assumptions of ANOVA were verified: analysis of homogeneity of variance (Hartley-Cochran-Bartlett test) and normal distribution (Kolmogorov-Smirnov and Lilliefors tests) were performed. Subsequently ANOVAs were conducted to demonstrate similarities or differences between measurements of the data. Finally, a Tukey HSD (honest significance difference) *post hoc* analysis indicating the nature of the differences found in the ANOVAs was performed. All statistical calculations were performed using StatSoft, Inc. (2004) STATISTICA software (data analysis software system), version 7. Levels of significance are always expressed as a p value < 0.05. All experiments were performed in triplicate ($n = 3$).

3. Results and discussion

3.1. Optimal growth temperature

The optimal growth temperature of the 12 tested fungi was determined in MEA medium in the mesophilic temperature range (25, 30 and 35 °C) (Table 2). All the basidiomycetes (41.7% of the fungi total tested) had an optimal growth temperature of 30 °C, while the ascomycetes and *Cunninghamella elegans* (a Mucoral fungus) grew better at 25 °C. *Pleurotus dryinus* IBB 903 displayed the highest growth rate (14.3 mm/d, Tukey HSD, total degrees of freedom (df) = 71, $p = 0.016$, $t = 4.761$), while *Emericellopsis* sp. and *Cadophora* sp., both isolated from the deep marine sponge *Steletta normani*, had the lowest growth rates showing no statistically significant differences between their growth rates (0.51 and 0.54 mm/d, respectively). Given that these fungi were recovered from *S. normani* at a depth of 751 m, where water temperatures in the deep-ocean are typically around 0-3 °C, these results are perhaps not surprising.

The growth rates at 25 °C for both wild type *Trichoderma atroviride* IMI 206040 and the recombinant *T. atroviride* strain, showed no significant differences (Tukey HSD, total df = 71, $p = 0.931$, $t = 4.761$). These two strains had the best growth rates among all the ascomycetes tested. Thus the heterologous expression of the *Trametes sanguineus'* Lac in *T. atroviride* did not appear to affect the growth rate of *T. atroviride*. *C. elegans* (a zygomycete) had a growth rate of 10.5 mm/d which was comparable with that of the basidiomycetes, being even slightly better than *P. ostreatus* MTCC 1804 and *T. hirsuta* MTCC 1171 (9.2 and 9.7 mm/d, respectively) (Table 2).

The determination of the optimal growth temperature is very useful to select different fungal species as potential candidates for bioremediation strategies. Mesophilic temperatures were chosen here, given that the intention was to subsequently employ these fungi in PAHs degradation and phenol removal approaches, under mesophilic conditions.

3.2. Screening for ligninolytic enzymes in solid medium: RBBR discoloration, gallic acid and guaiacol reactions

Although some of the species considered in our study have been previously studied with respect to their ligninolytic potential, we focused on characterized the ligninolytic properties of a number of the new fungal strains. A large fungal diversity has been reported in studies conducted on the screening of ligninolytic enzymes (Casieri et al., 2010). Furthermore, it has been observed that huge variations in both Lac and Per activities can occur within the same fungal species (Casieri et al., 2010). For this reason, we characterized phylogenetically related and non-related fungal species belonging to different genera and phyla. Basidiomycetes have been extensively studied for their ability to produce Lac and Per (mainly LiP and MnP) over a wide range of environmental conditions and substrates of different chemical natures such as PAHs, pesticides and asphaltenes, amongst others (Barrasa et al., 2009; Jeon et al., 2012; Liu et al., 2014; Mikolasch and Schauer, 2009; Si et al., 2013). However, studies on both zygomycetes and ascomycetes

Table 2
Fungal growth rates at optimal temperature, tolerance tests to PAHs, phenols, RBBR test, guaiacol and gallic acid reaction for each isolate.

Fungal species	Optimal temperature (°C)	Growth rate (mm/d)	Growth in different ligninolytic media			Tolerance test (10 ppm for each compound)			
						PAHs		Phenols	
			RBBR[a]	Gallic acid reaction[b]	Guaiacol[c]	PHE[d]	BAP[d]	P[e]	CP5
Zygomycetes:									
Cunninghamella elegans ATCC 36112	25	10.5 ± 0.1[c]	C	–	–	+++++	+++++	+++++	+++++
Ascomycetes:									
Cadophora sp. TS2	25	0.5 ± 0.1[f]	D	–	–	+++	+++	++++	++++
Emericellopsis sp. TS11	25	0.5 ± 0.3[f]	E	–	–	++	++	+++	+++
Pseudogymnoascus sp. TS12	25	2.1 ± 0.1[e]	E	DB	–	++	++	+++++	+++++
Aspergillus caesiellus H1	25	1.9 ± 0.1[e]	E	–	–	+++	+++	++++	++++
Trichoderma atroviride IMI 206040 (wild type)	25	6.1 ± 0.2[d]	E	–	–	++	++	+++	+++
Trichoderma atroviride IMI 206040 (Lac+)	25	6.1 ± 0.3[d]	D	DB	–	+++	+++	++++	++++
Basidiomycetes:									
Pleurotus dryinus IBB 903	30	14.3 ± 0.3[a]	A	DB	A	+++++	+++++	+++++	+++++
Tramates hirsuta IBB 450	30	11.3 ± 0.2[b]	A	YB	A	+++++	+++++	+++++	++++
Phanerochaete chrysosporium ATCC 787	30	11.9 ± 0.2[b]	A	YE	–	+++	++	+++++	++++
Tramates hirsuta MTCC 1171	30	9.7 ± 0.4[c]	D	YE	–	++	+	+++++	+++++
Pleurotus ostreatus MTCC 1804	30	9.2 ± 0.4[c]	B	DB	A	++	++	+++++	++++

Superscripts letters indicate levels of significance statistically differences.
[a] Time to decolorize the full dish within: A, 3 days; B, 4 days; C, 5–7 days; D, 8–15 days; and E over 15 days. RBBR, remazol brilliant blue R.
[b] Gallic acid reaction columns indicate; DB, dark brown; YB, yellowish brown; and YE, yellow of brown-colored quinonic form.
[c] Guaiacol to form reddish brown color; A 3–5 days; B 5–10 days; C 11–15 days.
[d] PAHs tolerance was determined by the percentage of mycelial growth inhibition (% MGI) on MEA media amended with phenanthrene (PHE) or benzo(a)pyrene (BAP), and was expressed as +++++ ($0 \le$ % MGI < 10), ++++ ($10 \le$ % MGI < 30), +++ ($30 \le$ % MGI < 50), ++ ($50 \le$ % MGI < 70), + ($70 \le$ % MGI < 100).
[e] Phenol tolerance was determined by the percentage of mycelial growth inhibition (% MGI) on MEA media amended with phenol (P) or *p*-chlorophenol (CP), and was expressed as +++++ ($0 \le$ % MGI < 10), ++++ ($10 \le$ % MGI < 30), +++ ($30 \le$ % MGI < 50), ++ ($50 \le$ % MGI < 70), + ($70 \le$ % MGI < 100).

are much less common, and for that reason we included some of these in this ligninolytic enzyme characterization.

RBBR and gallic acid tests were used to determine the fungus' potential to produce Lac. All the tested strains were able to decolorize RBBR (Table 2). Only 4 (41.7% of the tested strains) basidiomycetes strains decolorized the entire dish within 7 days, 2 ascomycetes (25%) within 15 days and the remaining strains (33.3%) in more than 15 days. In general, the ascomycetes took longer to achieve discoloration of the entire dish than the basidiomycetes. The highest growth rates observed for the basidiomycetes (Table 2) may explain this as other studies have reported a significant positive correlation between growth rates and *in vitro* discoloration of RBBR (Lee et al., 2010, 2014).

Regarding the RBBR discoloration by the Lac recombinant clone in *T. atroviride*, both the recombinant and wild type strain showed the ability to decolorize the dish completely. However, the recombinant clone achieved this within 10 days, while the wild type took longer achieving discoloration after 15 days. This result reveals that the RBBR discoloration only suggests the presence of Lac but it is not a confirmative test to determine its enzymatic activity. The selection of fungi for bioremediation purposes based on time (days) to achieve extensive discoloration of RBBR has also been used by other groups (Lee et al., 2010). RBBR, also known as Reactive Blue 19, is one of the most important synthetic dyes in the textile industry and represents an emergent class of toxic organopollutants (Palmieri et al., 2005). Studies conducted by Vyas and Molitoris (1995) showed the involvement of ligninolytic enzymes in RBBR discoloration (Vyas and Molitoris, 1995). Additionally, because RBBR is an anthracene derivative and structurally resembles certain PAHs, it has been proposed as an efficient screening method for fungi with the ability to degrade aromatic compounds (such as PAHs and phenols) (Hammel, 1992). It has been suggested that RBBR decolorizing enzymatic activity may play an important role in the degradation of aromatic rings-based compounds, such as lignin and other xenobiotics (Vyas and Molitoris, 1995). However, there is not always a strong correlation between RBBR discoloration and PAH tolerance/degradation (Lee et al., 2014).

The gallic acid test was performed to confirm the secretion of phenol oxidases (Lac, LiP and MnP) by these fungi (Alexopoulus, 2007; Gigi et al., 1981; Scharf, 2015), with seven strains (58.3%), including two ascomycetes and all five basidiomycetes being positive. Different oxidation products resulting in the generation of a variety of different colours (brown, dark brown, yellowish brown) occurred during the analysis (Table 2). The production of a brown shaded color produced in the agar has previously been strongly correlated with the ability of fungi to oxidize gallic acid by ligninolytic enzymes (Shleev et al., 2004). The *T. atroviride* strain containing the Lac recombinant clone oxidized the gallic acid into a brown-colored quinonic form, while the *T. atroviride* wild type was negative for this reaction. The Lac heterologous expression in *T. atroviride* supports the above result. This screening is consistent with results previously published by other authors, which concluded that the positive RBBR and gallic acid reactions do not always correlate (Lee et al., 2014).

During screening of the fungi on guaiacol only 3 (25%) of the basidiomycete strains (*P. dryinus* IBB 903, *T. hirsuta* IBB 450 and *P. ostreatus* MTCC 1804) generated the reddish brown-colored form in agar, which is indicative of guaiacol oxidation (Table 2). Guaiacol is a phenolic natural product that was first isolated from the oxidation of lignin and it is routinely used in screening regimes for the identification of fungi with phenol oxidase activity, because upon oxidation it turns yellowish brown or reddish brown.

The ligninolytic qualitative prospection has been performed with a non-inducing solid medium. Frequently, ligninolytic screening regimes are performed under nitrogen-limited conditions and use inducers of Lac and Per activities (Casas et al., 2013; Janusz et al., 2006). It is known that nitrogen sources in the culture can inhibit the expression and extracellular secretion of these enzymes (Elisashvili and Kachlishvili, 2009; Janusz et al., 2013). However, it is often useful when screening for Lac and Per activity to use nitrogen-enriched conditions. Thus it is really very convenient to characterize fungi with the capability to efficiently produce Lac and Per in the presence of sugars and under nitrogen-enriched conditions in different pollutant aqueous matrices derived from

industrial complexes. Sometimes, mixtures of wastewaters are enriched with nitrogen, sugars and polluted with xenobiotic compounds (e.g. PAHs and phenolic compounds), all derived from industrial and domestic activities. In this case, phenol oxidases will be required to be active under these conditions to effect biological based treatments.

3.3. Laccase and peroxidase production in liquid medium

Lac together with both LiP and MnP activities were tested in the twelve fungal strains cultured under nitrogen-limited conditions in the presence of wood chips as a carbon source. Wood chips from *Pinus arizonica* were added given that it is well established that lignocellulose in this form, induces the expression of fungal ligninolytic enzymes (Aranda, 2016). Lac production at varying levels was observed in all the fungi studied, except in the non-recombinant clone (wild type) of *T. atroviride* (Fig. 1). *P. chrysosporium* ATCC 787 and *T. hirsuta* IBB 450 displayed the highest Lac activities (541.1 and 421.3 U/L, respectively), while *Pseudogymnoascus* sp. and *Cadophora* sp. produced the lowest levels of Lac activity (23.1 and 32.2 U/L, respectively) under the test conditions (Fig. 1).

T. hirsuta MTCC 1171 had a lower Lac activity (Tukey HSD, total df = 65, $p = 0.028$, $t = 4.686$) than *T. hirsuta* IBB 450 (346.2 and 421.1 U/L, respectively). However, *P. dryinus* IBB 903 had a much higher Lac activity (Tukey HSD, total df = 65, $p = 0.009$, $t = 4.686$) than *P. ostreatus* MTCC 1804 (317.5 and 48.7 U/L, respectively). These data clearly demonstrate that different strains of the same species and different species of the same genus, can display a large variability in ligninolytic enzyme levels when screening for ligninolytic activity (Casieri et al., 2010).

High levels of Lac activity were observed in the *T. atroviride* recombinant Lac strain (173.2 U/L as the maximum activity at the eighth day). This evidence supports *T. atroviride* as a good heterologous expression system for fungal proteins (Balcázar-López et al., 2016). The deep sea sponge fungal isolate *Emericellopsis* sp. also displayed quite good levels of Lac activity (161.4 U/L) after two days of growth. There were no differences between Lac activity from *Emericellopsis* sp. and the recombinant Lac clone of *T. atroviride*. This result highlights the potential of the *Emericellopsis* sp. as producer of Lac which is comparable with a heterologous expression system.

The moderate halophilic fungus *A. caesiellus* (Batista-García et al., 2014) had a laccase activity of 65.5 U/L; this is the first report of Lac activity in this fungus. Although basidiomycetes have previously been reported as the best Lac-producing organisms (Arakaki et al., 2013; Ntougias et al., 2015), *A. caesiellus* and *P. ostreatus* MTCC 1804 showed no significant statistically difference between them in relation to levels of Lac production (65.5 and 48.7 U/L, respectively).

It should be noted that there is no reported information in the literature regarding quantitative Lac activity production from *Emericellopsis* sp., *Cadophora* sp. and *Pseudogymnoascus* sp. This study also supports the widely held belief that fungi isolated from extremophile environments, such as the deep sea, can exhibit enzymatic activities with potential biotechnological applications in different fields (Burgaud et al., 2009; Bonugli-Santos et al., 2015). At the same time, marine sponges are also well established as an interesting source of microbes with new enzymes (Höller et al., 2000; Suryanarayanan, 2012). These results may also ultimately also provide new insights into the ecological niches of different fungus genera and species such as *A. caesiellus*, *Emericellopsis* sp., *Cadophora* sp. and *Pseudogymnoascus* sp.

Regarding the Per activity, in liquid cultures only 6 of the strains tested (50%) displayed MnP activity (Fig. 2), and it was not possible to detect LiP in any of the supernatants derived from the fungal cultures. *Cadophora* sp. produced high levels of MnP reaching 150.3 U/L by day ten, which was much higher than the other marine fungus *Emericellopsis* sp. where MnP levels were much lower at 6.81 U/L by day ten. However, *Emericellopsis* sp. did produce Per activity earlier than the other fungi, with levels of 3.28 U/L being observed on day four. We also found Per activity in *A. caesiellus* (12.8 U/L in the eighth day) as previously reported (Batista-García et al., 2014). *P. chrysosporium* ATCC 787 and *P. ostreatus* MTCC 1804 also displayed good MnP activity by day eight (54.1 and 20.2 U/L, respectively), while lower levels of MnP activity being observed in *P. dryinus* IBB 903 of 16.7 U/L by day ten.

These results also suggest potential new roles for these enzymes in fungal physiology, particularly in the genera derived from the deep marine sponge (*Emericellopsis*, *Cadophora* and *Pseudogymnoascus*) given that this is the first study where MnP has been reported in these genera. This work highlights the importance of

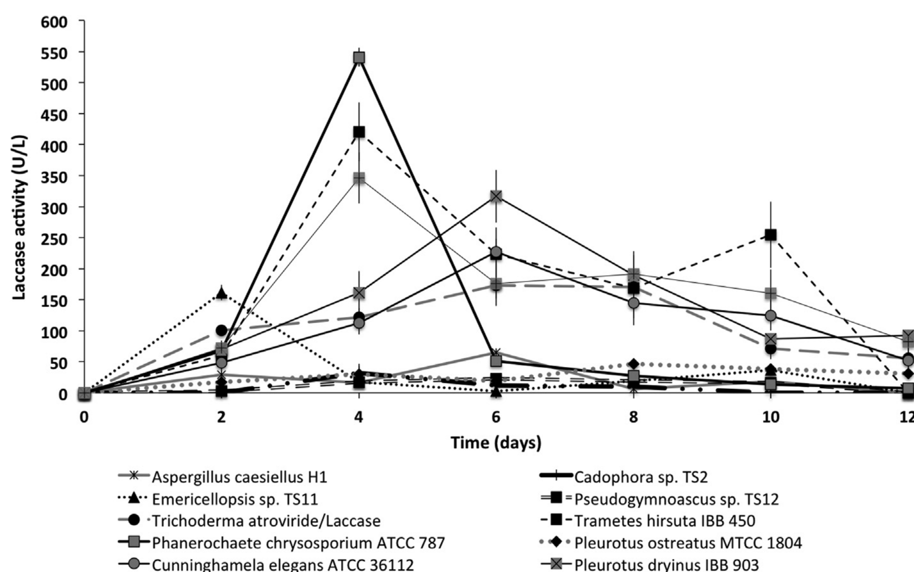

Fig. 1. Laccase activity (U/L) during 12 days.

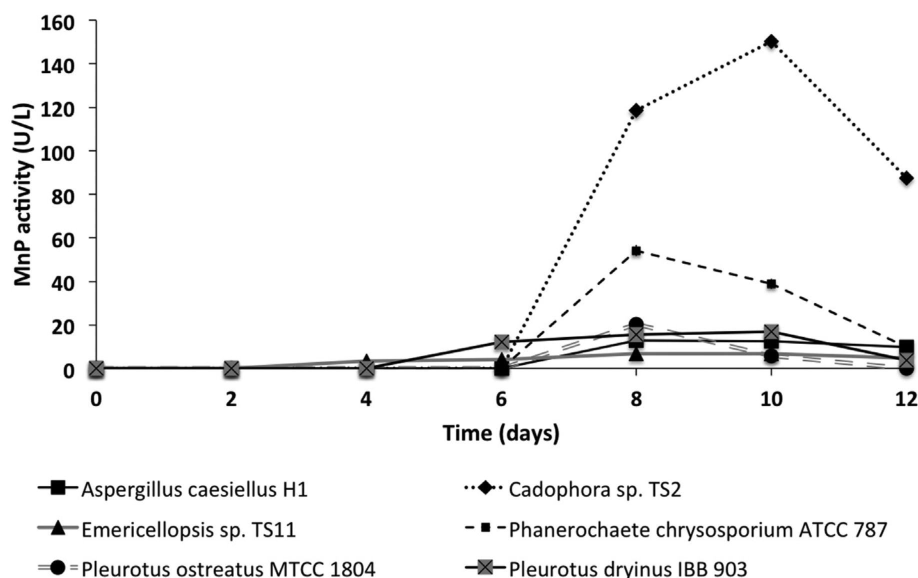

Fig. 2. Peroxidase activity (U/L) during 12 days.

continuing to extend the spectrum of fungi which are screened for ligninolytic enzymes, to as-yet-unconsidered strains and species that could ultimately lead to better yields of ligninolytic enzymes and possibly Lac or MnP activities with desirable characteristics such as higher redox potential, broader substrate range, robust thermo stability, lower Kms, etc.

3.4. PAHs and phenols: tolerance, biodegradation and adsorption

The tolerance to phenantrene and benzo-α-pyrene (1, 5 and 10 ppm) and phenol and *p*-chlorophenol (5 and 10 ppm) were evaluated (Table 2). No growth inhibition was observed for any fungal strain grown on media containing up to 10 ppm of either of the PAHs or of the phenolic compounds. For this reason, Table 2 shows the PAHs and phenol tolerance results for 10 ppm. Species *P. dryinus* IBB 903, *T. hirsuta* IBB 450 and *C. elegans* displayed better PAH tolerance than any of the tested ascomycetes. Species *A. caesiellus*, *Cadophora* sp. and *T. atroviride*-Lac were the ascomycetes with the highest growth in presence of both phenantrene and benzo-α-pyrene. The Lac activity in both the recombinant and wild type *T. atroviride* confirmed the expression of a laccase in the recombinant *Trichoderma* clone and its increase tolerance to PAHs versus the wild type.

Regarding the phenol and *p*-chorophenol tolerance, *C. elegans*, *Pseudogymnoascus* sp. and all basidiomycetes; in general revealed the lowest percentage MGI (Table 2). For the fungi collection, *p*-chorophenol was more toxic than phenol. This result is congruent with previous reports (Michałowicz and Duda, 2007) where *Aspergillus* spp. grew less well in the presence of *p*-chorophenol. Most of the strains (83%) showed MGI less than 30%. Therefore, the fungi screened here represent good candidates for downstream biotechnological application such as the removal of phenols.

Based on the high lignin modifying enzyme secretion potential obtained in the ligninolytic screening in both solid and liquid media as well as the high PAHs and phenol tolerances observed, we focused on *Pseudogymnoascus* sp., *A. caesiellus*, *T. hirsuta* IBB 450, *P. chrysosporium* ATCC 787, *P. ostreatus* MTCC1804, and *Cadophora* sp. to assess PAH and phenol biodegradation and removal abilities.

With respect to PAHs removal in liquid media, *A. caesiellus* was the best strain removing 80.5% of the phenantrene in 10 days, while the lowest level of removal was observed for *Pseudogymnoascus* sp. Notwithstanding this the *Pseudogymnoascus* sp. did perform reasonably well by removing 53.2% of the PAH (Table 3a). All of the six strains exhibited very high levels of benzo-α-pyrene removal in liquid media within 10 days (Table 3a), with removal levels higher than 92% being observed for all the tested fungi. It is possible that the differences observed between the phenantrene and benzo-α-pyrene removal could be related to the spore germination inhibition effect that phenantrene is known to exert over some fungal spores (Cerniglia and Sutherland, 2010). Additionally, the results confirmed that the benzo-α-pyrene adsorption is an insignificant mechanism, while the phenantrene adsorption occurred to a greater extent (Table 3a). *Pseudogymnoascus* sp. adsorbed 33.5% of the phenantrene, which it is the largest adsorption percentage for both hydrocarbons. In all cases, the biodegradation percentage was higher than the bioadsorption percentage, and all the species evaluated were capable of biodegrading more than 50% of each xenobiotic compound. In general, the tested fungi showed a better potential to remove benzo-α-pyrene than phenantrene. Consequently, our results are quite interesting since *(a)* higher molecular weight PAHs, such as benzo-α-pyrene, are typically more recalcitrant to degradation than lower molecular weight PAHs (Gupta et al., 2015; Labana et al., 2007), and *(b)* benzo-α-pyrene is more toxic in aqueous environments than phenantrene according to the US Environmental Protection Agency (EPA) (see Table 3b).

In relation to overall elimination, considering mycelium adsorption and total removal of both phenol and *p*-chlorophenol, all the fungal strains tested showed total removal percentages higher than 91%, except for *Cadophora* sp. (77%) when grown in the presence of *p*-chlorophenol. In general, phenols were less adsorbed than PAHs, *Cadophora* sp. being the strain that revealed the highest adsorption percentages: 4 and 7.2% for *p*-chlorophenol and phenol, respectively. Thus these *in vitro* studies, clearly demonstrate that *Pseudogymnoascus* sp., *A. caesiellus*, *T. hirsuta* IBB 450, *P. chrysosporium* ATCC 787, *P. ostreatus* MTCC1804 and *Cadophora* sp. are promising strains for bioremediation purposes in wastewater polluted with aromatic compounds such as PAHs and phenols.

Temperature and pH are well known to be crucial factors potentially affecting the biological removal of aromatic compounds (Laurent et al., 2012; Tran et al., 2013). In the current study, the PAHs and phenols removal experiments were carried out at

Table 3a
PAHs: Degradation, mycelium adsorption and total removal.

Culture	Phenanthrene (PHE)					Benzo-a-pyrene (BAP)				
	Biodegradation		Adsorption		Total	Biodegradation		Adsorption		Total Removal (%)
	Res. Conc. (ppm)	Biod. (%)	Ads. PHE (ppm)	Adsp. (%)	Removal (%)	Res. Conc. (ppm)	Biod. (%)	Ads. BAP (ppm)	Adsp. (%)	
Abiotic Control	10	0	0	0	–	10	0	0	0	–
P. ostreatus MTCC1804	3.5	64.7	2.0	20.0	84.7	0.14	98.6	0.03	0.3	98.9
T. hirsuta IBB 450	3.2	68.3	1.6	16.2	81.5	0.08	99.2	0.08	0.8	100
A. caesiellus H1	2.0	80.5	1.8	18.1	98.6	0.004	99.8	0.02	0.2	100
Cadophora sp. TS2	2.6	74.3	0.6	5.7	80.0	0.07	99.4	0.006	0.06	99.5
P. chrysosporium ATCC787	2.7	73.4	1.8	18.4	91.8	0.05	99.5	0.003	0.03	99.5
Pseudogymnoascus sp. TS12	4.7	53.2	3.4	33.5	86.7	0.71	92.9	0.05	0.5	93.4

Res. Conc.: Residual concentration; Biod. Biodegradation; Ads. BAP.: Adsorbed benzo-a-pyrene; Ads. PHE.: Adsorbed phenanthrene. Adsp.: Adsorption.

Table 3b
Phenol: Degradation, mycelium adsorption and total removal.

Culture	*p*-chlorophenol (CP)					Phenol (P)				
	Biodegradation		Adsorption		Total	Biodegradation		Adsorption		Total Removal (%)
	Res. Conc. (ppm)	Biod. (%)	Ads. PCP (ppm)	Adsp. (%)	Removal (%)	Res. Conc. (ppm)	Biod. (%)	Ads. CP (ppm)	Adsp. (%)	
Abiotic Control	10	0	0	0	–	10	0	0	0	–
P. ostreatus MTCC1804	0.08	99.2	0.006	0.06	99.3	0.2	98	0.07	0.7	98.7
T. hirsuta IBB 450	0.1	99	0.006	0.06	99.1	0.06	99.4	0.003	0.3	99.7
A. caesiellus H1	1.1	89	0.2	2	91	0.9	91	0.12	1.2	92.2
Cadophora sp. TS2	2.7	73	0.4	4	77	1.6	84	0.72	7.2	91.2
P. chrysosporium ATCC787	0.08	99.2	0.03	0.3	99.5	0.09	99.1	0.006	0.06	99.2
Pseudogymnoascus sp. TS12	0.9	91	0.03	0.3	91.3	0.3	97	0.005	0.05	97.1

Res. Conc.: Residual concentration; Biod. Biodegradation; Ads. P. Adsorbed phenol; Ads. CP.: Adsorbed p-chlorophenol. Adsp.: Adsorption.

25–30 °C and pH 6, and have shown promising results. However, a full characterization of operational conditions would be required in future studies, in order to reduce the time needed for PAHs and phenols biodegradation/removal and increasing the biodegradability of phenantrene.

3.5. Phenols and PAHs biodegradation in industrial wastewaters

The presence of phenols and PAHs in industrial wastewaters is a major concern worldwide. For this reason, we decided to test the phenols and PAHs removal in a non-sterilized industrial wastewater containing these aromatic compounds (Balcázar-López et al., 2016). For this experiment we used the six most promising strains: *Pseudogymnoascus* sp., *A. caesiellus*, *T. hirsuta* IBB 450, *P. chrysosporium* ATCC 787, *P. ostreatus* MTCC1804 and *Cadophora* sp. The tested wastewater, with an initial pH of 12, was obtained from a biofuel industry plant containing a range of aromatic compounds (eg. cresols, nitrophenols, chlorophenols, pyrene, naphthalene and phenantrene, amongst others (see (Balcázar-López et al., 2016)) and was used to explore the potential of these fungi to remove phenolic compounds and PAHs in an actual industrial liquid waste stream. Among the six strains tested, only one fungus (*Cadophora* sp.) was able to remove phenolic compounds in wastewater treatments in the absence of glucose (Table 4). On the other hand, when the wastewater was supplemented with 0.1 mM glucose, all of the tested fungi, except *A. caesiellus*, displayed the capacity to remove phenolic compounds (Table 4). In presence of glucose, *Pseudogymnoascus* sp. removed 15.5% of the phenolic compounds present in the wastewater after 12 days of cultivation, being the best of the fungi tested for that purpose. In general, the addition of 0.1 mM of glucose improved the phenol removal from the wastewater. It would be interesting to evaluate if higher glucose concentrations enhance the phenol removing capacity (Table 4). Intriguingly, Lac activity was not detected in fungal cultures where phenol concentration decreased, for example *Pseudogymnoascus* sp. TS12, which was able to eliminate 15% of the phenol content. MnP and LiP activities were also not detected in the wastewater treatments under the tested conditions, even under supplemented or non-supplemented wastewater with 0.1 mM glucose. In this respect some reports suggest that there is no clear relationship between the ligninolytic enzyme activities and some phenolic compounds degradation such as dichlorophenols, and even in some

Table 4
Phenol and PAHs removal (%) from wastewater after 12 days of culture.

Fungal species	Phenols		PAHs	
	Removal with glucose (%)	Removal with no glucose (%)	Removal with glucose (%)	Removal with no glucose (%)
Ascomycetes:				
Cadophora sp. TS2	1.1	5	85.6	87.6
Pseudogymnoascus sp. TS12	15.5	0	0	87.9
Aspergillus caesiellus H1	0	0	28.1	87.5
Basidiomycetes:				
Tramates hirsuta IBB 450	6.9	0	84.9	62.8
Phanerochaete chrysosporium ATCC 787	8.4	0	70.3	65.7
Pleurotus ostreatus MTCC 1804	3.8	0	78.6	80.4

Removal mains degradation + adsorption.

cases (eg. *Coprinopsis* species) laccases and some peroxidases are not necessarily involved in phenolic compounds biotransformation (Singh, 2006). Some reports also suggest that intracellular enzymes, such as cytochrome P450, may be involved in fungal-mediated xeniobiotic transformation (Jauregui et al., 2003; Marco-Urrea et al., 2010).

Regarding the PAHs removal in wastewater, the ascomycetes showed better removal percentages than the basidiomycetes. *Cadophora* sp., *Emericellopsis* sp. and *A. caesiellus* removed 87% of the PAHs present in the liquid waste. The addition of glucose negatively affected the ability of *Pseudogymnoascus* sp. and *A. caesiellus* to remove the PAHs, but this effect was not seen in the *Cadophora*'s treatments (Table 4). In contrast, PAHs removal by the basidiomycetes was favored by the addition of glucose. It is important to note that the wastewater collected from the industry in Quebec has an initial pH of 12. So, these fungi exhibited an excellent capacity to remove PAHs under hyper alkaline conditions. In contrast, their phenol removing abilities were much lower. To our knowledge, this is the first report describing phenol and PAHs removal under harsh high alkaline conditions (pH 12) using *Cadophora* sp., *A. caesiellus* and *Pseudogymnoascus* sp. At the same time, we demonstrated for the first time the alkalotolerance of these fungi given ability to grow in a real pollutant matrix enriched with phenols and PAHs. At the end of the experiments all fungi tested significantly decreased the pH to between ($\cong$ 7–9), a factor which is desirable in biological based environmental treatments. This would appear to suggest that: *(i)* extracellular acid based fungal metabolism could be involved in the removal of the xenobiotics or *(ii)* aromatic compounds (wastewater composition) stimulate fungal metabolism and acid production. Further work will be necessary to understand in depth the molecular mechanisms involved in the xenobiotic removal by these fungi.

Finally, it is important to note that phenols and PAHs were removed from a non-sterile wastewater. This is a relevant point in terms of cost effectiveness because the use of a previously sterilized wastewater increases the cost of any environmental treatment. Thus, it is very desirable to find candidates such as the fungi described here, which have utility in mycoremediation on non-sterile industrial effluents. Non-sterile effluents (especially from older wastewaters or sediments) may contain groups of microorganisms that could potentially inhibit fungal growth.

4. Conclusions

Mycoremediation has many advantages such as the high potency of fungi to degrade contaminants of emerging concern through inducible enzymes such laccases and peroxidase and being able to tolerate high concentrations of polyaromatic hydrocarbons and phenols. The fungi studied here possess the capability to grow well in ambient conditions of 25–30 °C and mineralize xenobiotics under highly alkaline conditions (pH 12) with great efficacy. The strains demonstrated excellent abilities to remove both benzo-α-pyrene and phenantrene from liquid media within a short period. In addition they displayed a better potential to remove benzo-α-pyrene than phenanthrene, which is quite interesting as higher molecular weight PAHs, such as benzo-α-pyrene, are typically more recalcitrant to biodegradation than lower molecular weight PAHs. This approach could provide an excellent treatment strategy for industrial effluents containing such contaminants.

Even though there are reports on the use of basidiomycetes in mycoremediation-based strategies, based primarily on for their ability to produce Lac and Per, studies on the use of both zygomycetes and ascomycetes are much rarer. This report shows good levels of contaminant removal potentially mediated through the production of ligninolytic enzymes, in addition to tolerance of PAHs present in hyperalkalophile industrial effluents. Overall, the results reported here demonstrate a clear biotechnological potential for the use of these fungal strains in the treatment of PAH and phenol contaminated waste streams, the optimization of which will necessitate additional work on the mechanisms involved. Following this, it is likely that these fungi can emerge as highly efficient and trustworthy tools for wastewater engineers to use in the treatment of various waste streams containing chemical pollutants such as PAHs and phenols.

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