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Full Length Research Paper

New laccase-producing fungi isolates with biotechnological potential in dye decolorization

Raúl Tapia-Tussell[1]*, Daisy Pérez-Brito[1], Rafael Rojas-Herrera[2], Alberto Cortes-Velazquez[1], Gerardo Rivera-Muñoz[3] and Sara Solis-Pereira[3]

[1]Laboratorio GeMBio, Centro de Investigación Científica de Yucatán A.C., Calle 43 # 130, Chuburná de Hidalgo, C.P 97200, Mérida, Yucatán, México.
[2]Facultad de Ingeniería Química, Campus de Ingenierías y Ciencias Exactas, UADY, Periférico Norte Kilometro 33.5, Tablaje Catastral 13615, Col. Chuburna de Hidalgo Inn, C.P. 97203. Mérida, Yucatán, México.
[3]Instituto Tecnológico de Mérida, Av. Tecnológico km. 4.5, Mérida Yucatán, México.

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Thirty six (36) native fungal strains from the state of Yucatan were first screened for ligninolytic activity on solid media containing ABTS. Molecular identification based on ITS rDNA region and PCR fingerprinting of seven selected fungi isolates were carried out. Molecular characterization based on genetic fingerprinting was helpful in determining unequivocally the differences between isolates at genera and species levels. The seven isolates showed ABTS oxidation zones in plates but only five strains produced extracellular laccase. The strains identified as *Trametes hirsuta* (GenBank accession numbers GQ280372 and GQ280373) showed the highest laccase production. The strain Bm-*2* displayed the greatest laccase activity and dye decolourization ability in 72 h without the addition of mediators. Both the high laccase activity shown by Bm-2 and its ability to decolorize dyes are a good indication of its possible use in the treatment of textile effluents.

Key words: Laccase, *Trametes hirsuta*, dye decolorization, PCR fingerprinting.

INTRODUCTION

Biological processes represent a good alternative for remediation and decontamination of environmental pollutants given the ability of some microorganisms to mineralize a wide variety of toxic xenobiotics and to oxidise substrates with low solubility, such as chlorinated phenolics, synthetic dyes, pesticides and polycyclic aromatic hydrocarbons (Baldrian, 2006; Reddy, 1995; Rodriguez et al., 1999; Torres et al., 2003). White-rot fungi including *Trametes, Pleurotus, Coriolopsis* and other genera, degrade a variety of dyes without generating toxic by-products. Studies on these organisms have identified laccase as a significant component of their enzyme system (Levin et al., 2004; Peláez et al., 1995). The effectiveness of the process using laccase has been proven and colour removal from 40 to 95% in common textile dyes has been demonstrated (Cristovão et al., 2008; Rodriguez et al., 2005; Tavares et al., 2009).

Identification and characterization of fungi species and strains is based on morphological, physiological, biochemical and genetic characteristics. The whole procedure is time-consuming and technical skills are required for proper interpretation of results (Fleet, 1992). On the other hand, DNA-based identification is faster and more reliable than phenotypic characterization (Kurtzman et al., 2003). By using PCR-based methods, fungi can be identified to the level of genus, although, the identification to species level is dependent on the choice of a specific method. Appropriate molecular methods for identification of fungi species and strains are PCR-fingerprinting, RAPDs and restriction analysis of non-coding ribosomal DNA (rDNA) (PCR-RFLP of internal transcribed spacer [ITS]), cleaved amplified polymorphic sequence and simple sequence repeats (SSRs). The efficacy of identification can be greatly increased when combinations of these methods are used (Leaw et al.,

*Corresponding author. E-mail: rtapia@cicy.mx. Tel: (0052) 999-942-8330. Fax: (0052) 999-981-3900.

2006; Perez-Brito et al., 2007; Tapia-Tussell et al., 2008).

In developing countries, many textile-manufacturing industries release dyes into the environment, leading to the accumulation of highly undesirable pollution load in water bodies. As a result of their recalcitrance to biological degradation, these pass into the environment on a large scale. Adding to this problem is the fact that the dyes are aromatic in nature and contain a variety of linkages. Biodegradation, using enzymes such as laccases has been suggested as one of the most attractive alternatives for treatment of dyes (Robinson et al., 2001). However, in most fungi the yield of laccases is too low for commercial purposes even when their genes are expressed in various heterologous hosts (Hong et al., 2006). Therefore, it is of great benefit to explore new strategies for overproduction of lacasses or to screen new microorganisms with high yields of the enzyme. In Yucatan, Mexico, in particular, many of textile industries are posing an environmental problem mainly because of the peculiar characteristics of soil structure and shallow underground water. In the search for fungi strains well adapted to the environmental conditions of the region and with high laccase activity to be used in effluent decontamination, the aim of this study was to screen selected Yucatan native fungi for their ability to decolourize effluent and textile dyes and to carry out the molecular characterization of these fungi.

MATERIALS AND METHODS

Fungi isolation

Fruiting bodies fungi were collected from henequen by-products in a processing factory (12 isolates) and wood decay (24 isolates) in different municipalities of the state of Yucatan (Hunucma, Baca, Dzemul and Merida). Isolation was carried out on plates of ME medium containing 2% malt extract, 250 µg/l amikacin, 6 mg/l Benlate® (in order to prevent the growth of filamentous fungi) and 2% agar. Plates were incubated at 35°C for 3 to 5 days. Fungi were subcultured three times to obtain stable, pure cultures.

Primary screening for ligninolytic activity

As an initial screening method for detecting the ability of fungal strains to produce lignin modifying enzymes, an ABTS (2,2'-Azino-bis(3-ethylbonzotiazoline-6-sulfonic acid) diammonium salt) oxidation based method was used (Matsumura et al., 1987). A disk of 1 cm diameter of mycelium from each strain was inoculated on ME plates containing 5 mM ABTS and then incubated for four days at 35°C. The formation of a dark-green halo in ME plates indicated a positive extracellular laccase secretion. The diameter and intensity of the halo were used as an indicator of the level of ligninolytic enzyme production. Strains with a dark-green colouration appearing on the second day of incubation and with a ratio above 1 cm were classified as highly ligninolytic and were chosen for further molecular identification and characterization.

PCR-fingerprinting

Arbitrarily, primed (AP)-PCR was carried out using seven 15 bp primers according to previous reports: $(GTG)_5$, $(GAC)_5$ and M13 (5´-GAGGGTGGCGGTTCT–3´) were used at 50°C of annealing temperature (Perez-Brito et al., 2007; Cadez et al., 2002); $(TCC)_5$ and $(GACAC)_3$, were used at 42°C (Bridge et al., 1997) and $(CAG)_5$ was used at 60°C (Talhinhas et al., 2002). PCR reactions were performed in 25 µl reaction volumes containing PCR buffer (20 mM Tris-HCl, 50 mM KCL, pH 8.4), 0.25 mM of each of dNTPs (Invitrogen), 2 mM $MgCl_2$, 0.8 µM of each primer, 10 ng of template DNA and 1 U Taq polymerase (Invitrogen). DNA amplification was performed in a GeneAmp 9700 DNA Thermal Cycler (Perkin-Elmer), with an initial denaturing step of 5 min at 95°C, followed by 40 cycles of 40 s at 95°C, 60 s at the appropriate annealing temperature for each primer and 60 s at 72°C; and a final extension step of 5 min at 72°C. Amplified DNA fragments were separated by electrophoresis in 1.5% (w/w) agarose gels (Invitrogen), in 1X TBE (0.9 M Tris-Borate-20 mM EDTA) buffer at 100 V for 40 min and stained with ethidium bromide. DNA banding patterns were visualized on a UV transilluminator and images were acquired with UVP Biolmaging systems.

Production of laccase

Cultures of the seven selected fungi were maintained on liquid basal medium, pH 6 (Tien and Kirk, 1984). Erlenmeyer flasks containing 50 ml of media were inoculated with 1 ml of homogenized mycelia, from inoculums previously produced in liquid ME medium, and incubated for 96 h at 35°C on a rotary shaker (150 rpm). Cultures were harvested, filtered and centrifuged at 7 800 x *g* for 20 min to remove mycelia and the supernatant was used to measure the laccase activity by oxidation of ABTS (Pal et al., 1995). Reaction mixture contained 1 M acetate buffer pH 5 (100 ul), 5 mM ABTS (100 µl), deionized water (700 µl) and enzyme extract (100 µl). Oxidation of ABTS was determined at 40°C by increasing in A 420 nm (ε_{max}= 36,000 M^{-1} cm^{-1}). One unit of enzyme activity is defined as 1 µmol of product formed per min.

PCR amplification and sequencing of 5.8S-ITS of rDNA

Fungal DNA was extracted from lyophilized mycelia of selected strains according to a protocol previously reported (Tapia-Tussell et al., 2006). PCR amplification of the internal transcribed spacer (ITS) region of the ribosomal DNA was performed using primers ITS1 and ITS4 (White et al., 1990) and PCR products (650 bp) were purified and sequenced by Macrogen Inc, Korea.

Sequence analysis and phylogenetic inference

Sequence alignment was done with ClustalW (Thompson et al., 1994) and taxonomic classification and nearest neighbors were evaluated with GenBank database using BLAST (Altschul et al., 1997). Phylogenetic inferences were carried out following the maximum parsimony method by using bootstrap values based on 1000 replications with program MEGA 4 (Tamura et al., 2007). Only parsimony informative sites were considered for analysis.

Microbial treatment of textile dyes

The strain with the highest laccase activity (Bm-2) was used for the evaluation of textile dye decolourization. Erlenmeyer flasks containing 200 ml of liquid ME supplemented with 0.01% of each textile dye (acid blue 74, reactive green 19 and reactive red 195 dyes) were inoculated with 4 ml of homogenized inoculum from selected fungi and incubated at 35°C in a shaker (150 rpm) for three days. Samples were withdrawn at regular intervals (0, 24, 48

Table 1. Screening of strains for laccase production by ABTS oxidation.

Strain	Size of halo (cm)
Bm-2	5.8
Bm-4	-*
Bm-5	-*
AHB-1	4.6
AHB-3	3.3
AHB-6	6.5
VIC-3	4.4

*No halo formation.

and 72 h) and analysed for laccase activity (as previously described) and colour removal. Non-inoculated controls were run under the same conditions. Decolouration was determined as a percentage of the absorbance reduction at the wavelength of maximum absorbance for each eye. Rate colour removal (P) was calculated as follows: $P= (A_1-A_2)/A_1 \times 100\%$, where A_1 represents the absorbency of the control without inoculums and A_2 represents the absorbency of samples.

RESULTS

Primary screening for ligninolytic activity

From a total of 36 fungal strains autochthonous to Yucatan tested by the ABTS oxidation-based method, three isolates from henequen by-products and four from wood decay showed high ABTS oxidation capacity in agar plates (Table 1), as demonstrated by the dark green colour observed in the plates.

In the case of Bm-4 and Bm-5 strains, no extracellular laccase activity was produced since no halo formation was observed, but at the end of mycelia growing, ABTS oxidation was observed below the fungal colony what might suggest that laccase activity in these isolates would be associated to cell wall.

In Figure 1, strains with the highest oxidative activity isolated from henequen by-products (AHB-6) and wood decay (Bm-2) are shown. These results indicated that the plate assay can be used as a simple rapid assay for visual demonstration of presence of laccase.

PCR-fingerprinting

AP-PCR analysis performed with six arbitrary primers to characterize seven fungi isolation is shown in Table 2. As can be seen, the resulting patterns for each strain are different to all primers; a different letter (A to F) was given to each combination of pattern type, except for strains Bm-4 and Bm-5 which were identical in all cases. Band patterns obtained from single primers varied from simple patterns consisting of 1 to 4 bands to complex consisting of up to 12 bands (Figure 2). In spite of the fact that the detected level of polymorphism was generally high with all primers tested, CAG_5 (Figure 2a) and $GACAC_3$ (Figure 2c) revealed the highest differences among isolates and gave the highest levels of resolution for all primers.

Production of laccase

The different levels of laccase production in liquid medium for the seven strains selected are shown in Figure 3. The highest levels of laccase production were obtained for strains Bm-2 and AHB-6, which produce high titres of laccase activity without the addition of mediators coinciding with results obtained for plaque activity (Figure 1), while strains Bm-4 and Bm-5 did not produce extracellular laccase and were indistinguishable at molecular level suggesting that these two isolates are redundant. In the case of strains AHB-1, AHB-3 and VIC-3, the levels of laccase production were low, they were in the range of 2.5 to 5 U/ml.

PCR amplification, sequencing of 5.8S-ITS of rDNA and phylogenetic inference

The amplified fragment from the 5.8S-ITS region for seven selected isolates was 650 bp long. Sequence analyses showed a similarity level of 97 to 99% to other sequences from different species belong to *Basidiomycota* and *Ascomycota*. Bm-2 and AHB-6 had a 98% of homology with *Trametes hirsuta*, meanwhile Bm-4 and Bm-5 had 99% of similarity with *Phanerochaete chrysosporium*. This result supports the hypothesis that Bm-4 and Bm-5 are the same strain.

The other three isolates AHB-1, AHB-3 and VIC-3 were identified as *Cochliobolus lunatus*, *Athelia rolfsii* and *Bipolaris* sp with 99, 97 and 99% of homology, respectively. These species are plant pathogens.

As can be observed in the consensus tree depicted in Figure 4, all strains grouped in different clades indicating that these isolates belong to different taxonomic ranks with high support (100 and 95%) in the branches. All strains identified in this study grouped were with other from the same species. In the case of *T. hirsuta*, strains Bm-2 (GQ280373) and AHB-6 (GQ280372), although, they are the same species grouped in different clades.

Microbial treatment of textile dyes

The ability of Bm-2 identify as *T. hirsuta* to decolourize three synthetic (one indigoid and two azo) dyes in a liquid medium was evaluated. Although, the decolourization rates of all dyes were different, in all cases a high rate of decolourization was observed (Figure 5a). Bm-2 started to decolourize acid blue 74 immediately after growth commenced, with a colour removal of 50% at 24 h and reaching 90% after 48 h (Figure 5a, b). In the medium

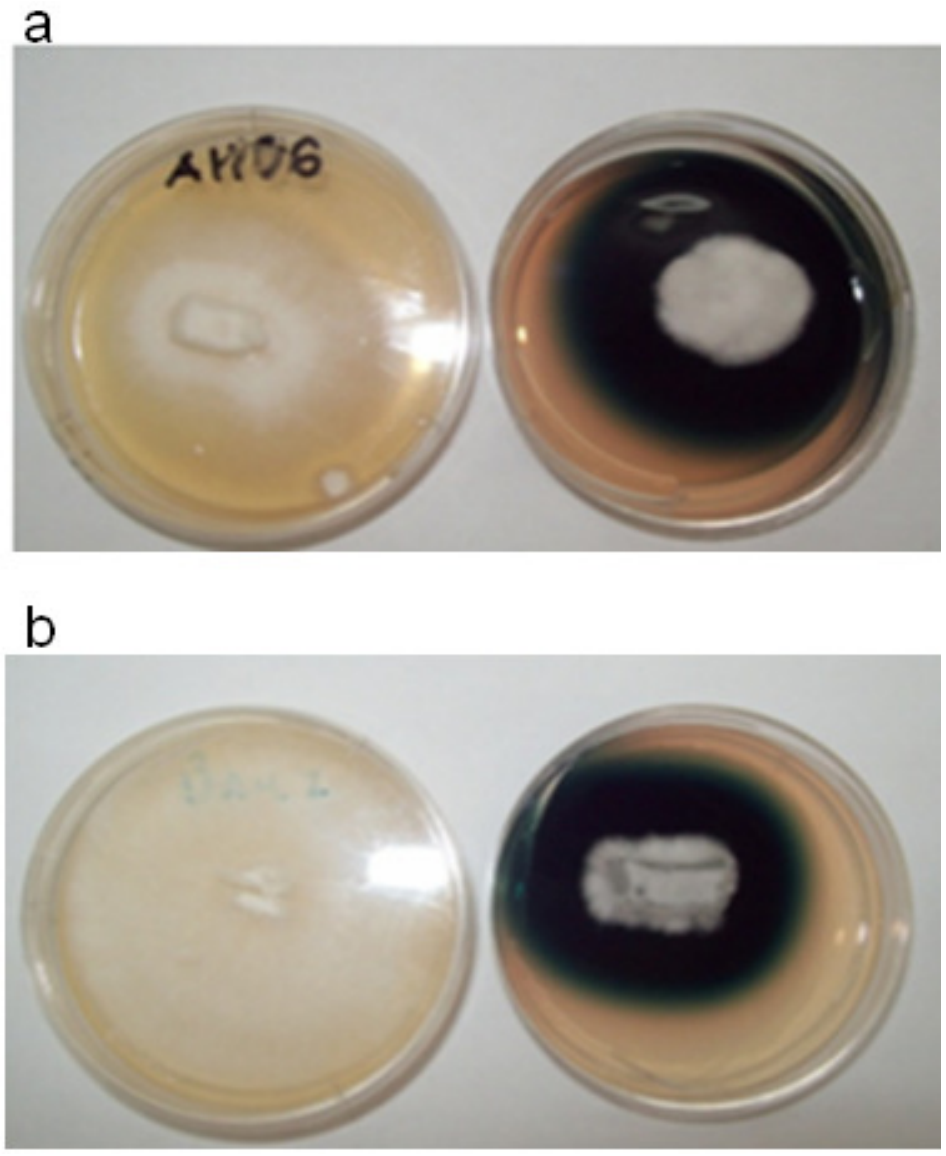

Figure 1. Primary screening for ligninolytic activity in plates. (a) *T. hirsuta,* AHB-6 strain isolated from henequen by-products, in the left media without ABTS (control), in the right media with ABTS; (b) *T. hirsuta* Bm-2 strain isolated from wood decay, in the left media without ABTS (control), in the right media with ABTS.

Table 2. AP-PCR pattern of ligninolytic fungi strains. The last column, overall pattern, was obtained from the combination of the results from the different primers. A different letter was given to each combination of pattern types.

Strain	AP-PCR with primer						Overall pattern
	$(GTG)_5$	M13	$(GAC)_5$	$(CAG)_5$	$(TTC)_5$	$(GACAC)_3$	
Bm-2	A	A	A	A	A	A	A
Bm-4	B	B	B	B	B	B	B
Bm-5	B	B	B	B	B	B	B
AHB-1	C	C	C	C	C	C	C
AHB-3	D	D	D	D	D	D	D
AHB-6	E	E	E	E	E	E	E
VIC-3	F	F	F	F	F	F	F

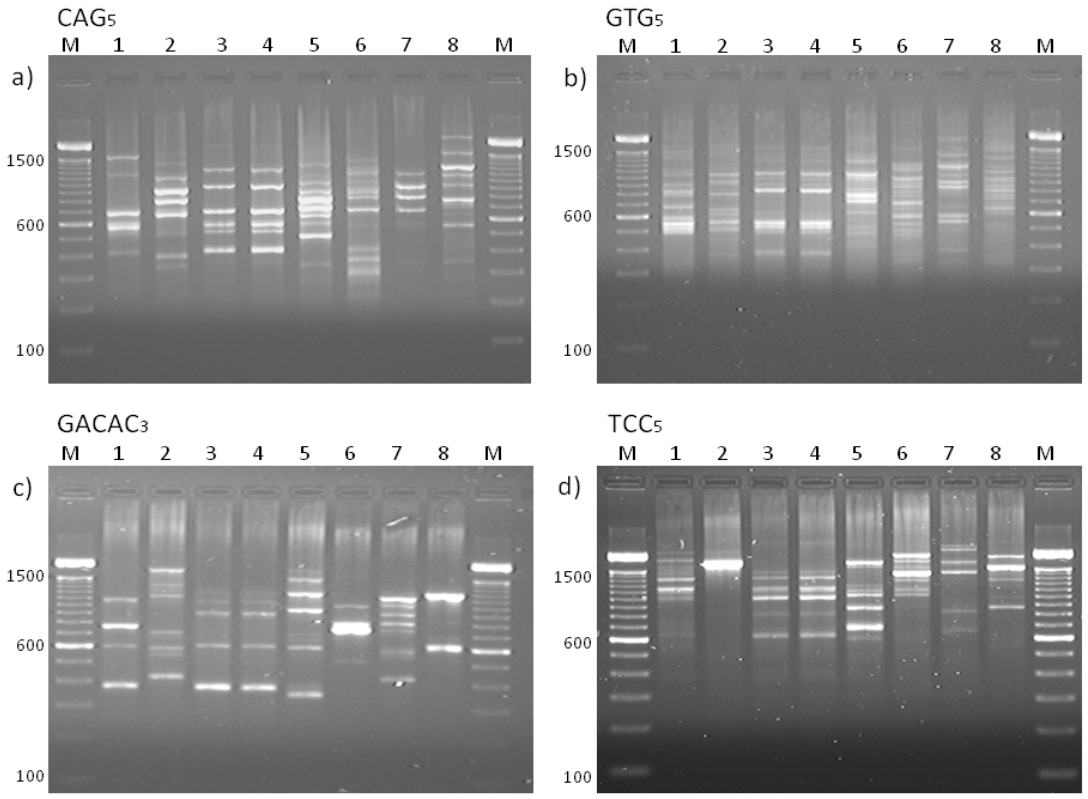

Figure 2. PCR-fingerprinting of fungal strains with different primers. M: molecular marker 100 bp ladder; Lane 1: *P. chrysosporium* (control); lane 2: Bm-2; lane 3: Bm-4, lane 4: Bm-5, lane 5: AHB-1, lane 6: AHB-3, lane 7: AHB-6 and lane 8: VIC-3.

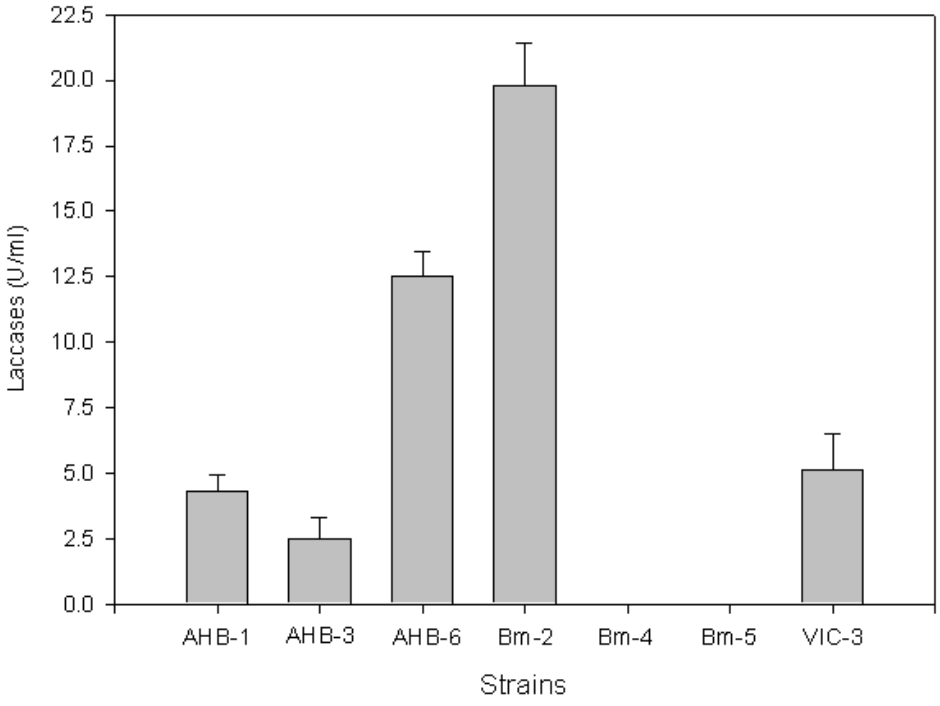

Figure 3. Laccase production by seven white rot fungi strains, AHB-1:*C. lunatus,* AHB-3 *A. rolfsii,* AHB-6 and Bm-2:*T. hirsuta,* Bm-4 and Bm-5: *P. chrysosporium,* VIC-3: *Bipolaris* sp.

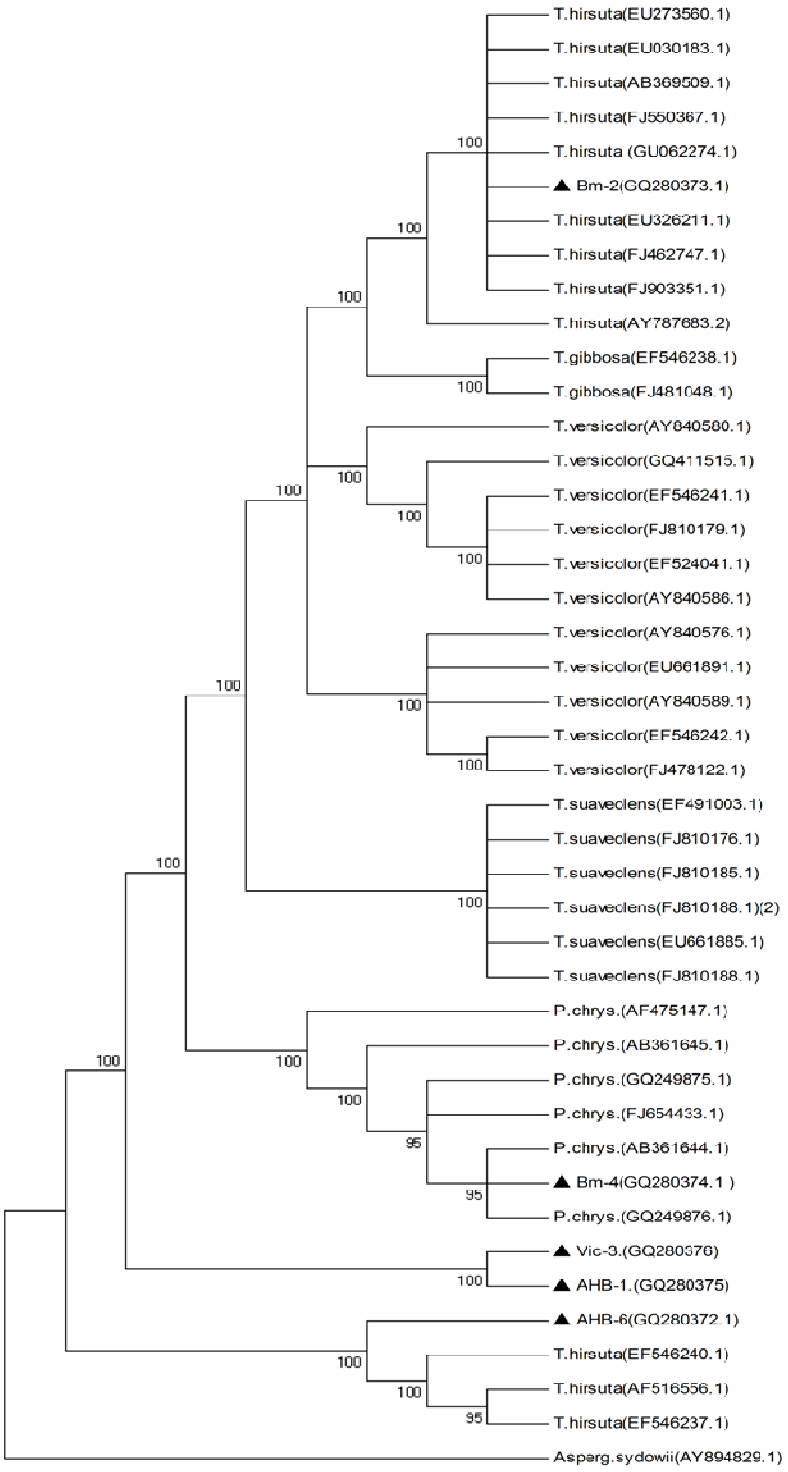

Figure 4. The evolutionary history was inferred using the maximum parsimony method (Eck and Dayhoff, 1966). The consensus tree inferred from 86 most parsimonious trees is shown. Branches corresponding to partitions reproduced in less than 50% trees are collapsed. The consistency index is (0.867277), the retention index is (0.949345) and the composite index is 0.826712 (0.823345) for all sites and parsimony-informative sites (in parentheses). The percentage of parsimonious trees in which the associated taxa clustered together is shown next to the branches. There were a total of 240 positions in the final dataset, out of which 217 were parsimony informative. *Aspergillus sydowii* (AY 894829.1) was employed as the out-group to root the tree. Strains used in this study.

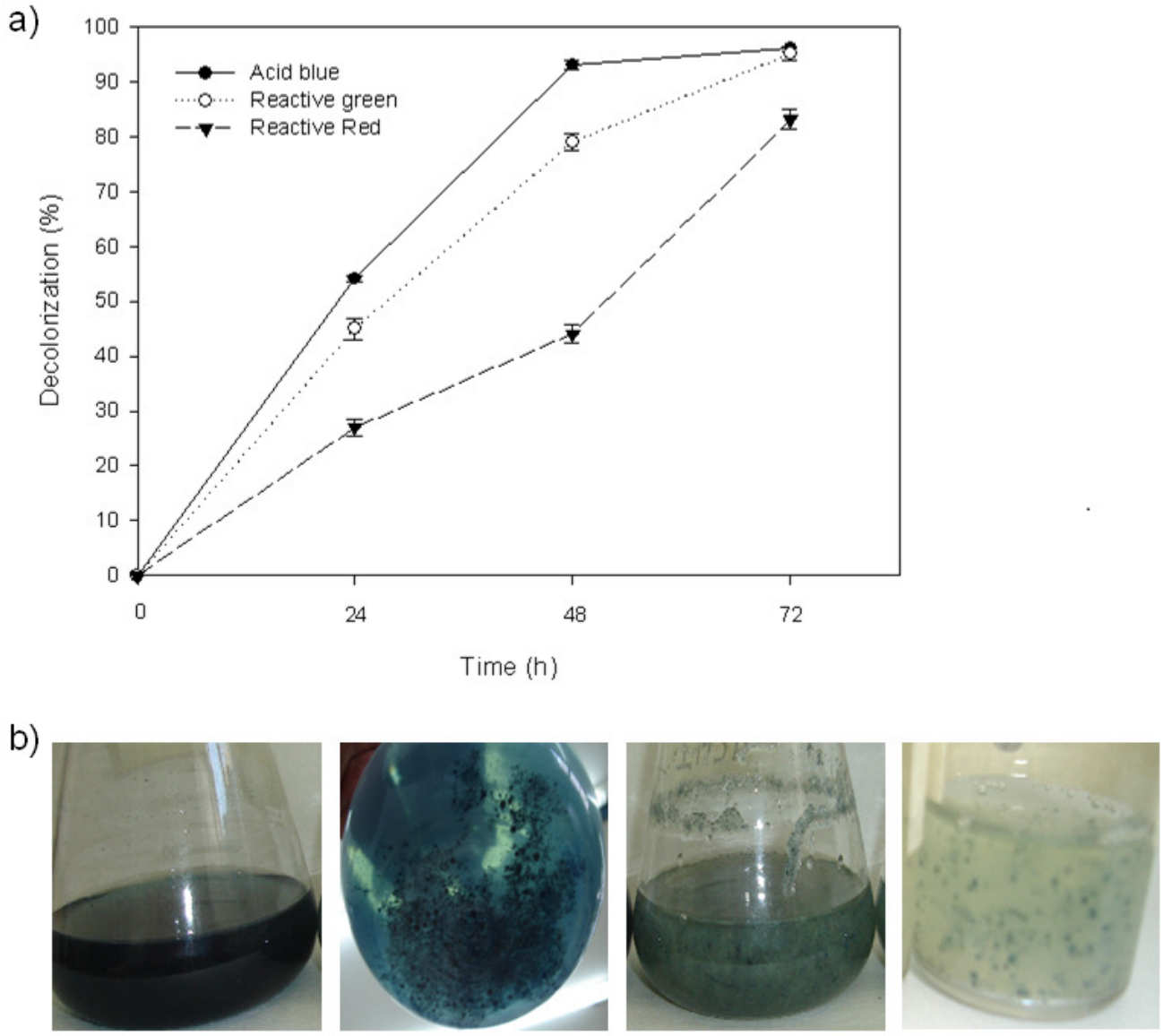

Figure 5. (a) Decolourization (%) of three commercial textile dyes (acid blue 74, reactive green 19 and reactive red 195) with Bm-2 (*T. hirsuta)*; (b) Acid blue 74 dye decolourized by Bm-2 (*T. hirsuta*).

with added green reactive 19, a decolourization of 95% was achieved at 72 h and after the same time, the medium containing red reactive 195 showed a colour removal of 83%. In the case of reactive red 195, decolourization at 24 and 48 h was two folds lower than the other dyes decolourization. Non-inoculated controls showed no colour removal.

DISCUSSION

In this study, there were identified and characterized seven fungi from different environments in Yucatan Peninsula. Few papers have been published dealing with the geographical and climatic distribution of autochthonous fungal species or describing the biodiversity in a given region for ligninolytic activity production (Dhouib et al., 2005; Saparrat et al., 2002). Most of the screening surveys for ligninolytic enzyme producers have been carried out on culture collection strains (Jaouani et al., 2005).

PCR fingerprinting approach using the simple repeat primers proved to be useful in discriminating at species and subspecies level (Van der Vossen et al., 2003), a fact also demonstrated for other author (Libkind, 2007), using the same set of primers. The variation in band patterns obtained with all AP primers used, suggests that the full set of these primers may be suitable as “universal” primer set in order to obtain white rot fungi fingerprints.

ITS sequence-based identification for fungal isolates has been successfully used for other species (Perez-Brito et al., 2007; Talhinhas et al., 2002; Than et al., 2008) and in this study, it is further supported by a phylogenetic analysis based on a maximum parsimony method.

Although, there are few reports of high laccase production for basidiomycetous fungi in initial screenings (Baldrian, 2006; Dhouib et al., 2005), it was found that the basidiomycetous strain Bm-2 was the highest laccase producer. This strain, identified as *T. hirsuta* registered a higher laccase production after 72 h without addition of mediators in comparison with other strains of the same genus reported as high producers of this enzyme (Dhouib et al., 2005).

The strains AHB-1, AHB-3 and Vic-3, identified as *C. lunatus, A. rolfsii* and *Bipolaris* sp., respectively, produced the lowest levels of laccase, possibly due to the requirement of inductors or a dependency on a physiological role for this enzyme in nature, in the host colonization process. These strains are maize phyto-pathogens and the laccases may represent factors of virulence during invasion of the plants (Baldrian, 2006).

Strains Bm-4 and Bm-5, identified as redundant isolates of *Phanerochaete chrysosporium* did not produce extracellular laccases. Oxidation zones formed in plate assays could be associated with laccases attached to the cell wall or to the presence of other ligninolytic activities such as manganese or peroxidase ligninase. Laccase production in *P. crysosporium* also needs clarification. Although, *P. crysosporium* has been reported as white rot fungi not capable to produce laccase, this enzyme has been detected in cultures of this fungus supplemented with cellulose (Srinivasan et al., 1995). However, due to the absence of laccase genes, some researchers (Larrondo et al., 2003) have associated its activity with the action of another multicopper oxidase.

Although, strains Bm-2 and AHB-6 (classified as *T. hirsuta*) shared similarities to sequences from same species they grouped in different clades. One explanation could be the differences in their habitat, because one (AHB-6) was isolated from henequen by products, this environment is characterized by very acid soils and high contents of organic acids and another (Bm-2) was isolated from wood decay in deciduous forest, suggesting that Bm-2 and AHB-6 might represent two different ecotypes of *T. hirsuta* (Kihara and Kumagai, 1994) characterized by possessing differences in extra-cellular laccase activity and textile dye decolourization rates (S. Solis-Pereira, unpublished data).

Dye decolourization by ligninolytic fungi has been associated with extracellular laccase activity in various studies (Abadulla et al., 2000; Minussi et al., 2001; Salony et al., 2006). In this study, a direct relationship between extracellular production of laccases by *T. hirsuta* (Bm-2 strain) and the decolourization of the three dyes was also found.

These findings are supported by a recent study in which *T. hirsuta* laccase was able to decolorize simulated Congo Red and Remazol Brilliant Blue R dye baths (91 and 45%, respectively) in 19.5 h without mediators (Moilanen et al., 2010) and a previous report where *T. hirsuta* and a purified laccase from this organism were able to degrade triarylmethane, indigoid, azo and anthraquinonic dyes (Abadulla et al., 2000).

The rate of decolourization of acid blue 74 (indigoid type of dye) was twofold faster than the azo dye (reactive red 195); this is in agreement with the results reported previously for *T. hirsuta* (Abadulla et al., 2000), it could be explained by the differences in chemical structure of these dyes.

In this study, the ability of *T. hirsuta* to decolorize without any mediators, three structurally different dyes were proved. These results suggest the potential of this fungus to be used in color removal of textile dye effluents. Considering that in large scale applications, the use of mediators can be expensive and to have a laccase which can work without mediators could be useful.Further studies focusing on laccase genes in order to obtain a high expression of this enzyme should be carried out.

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