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Isolation and selection of white-rot fungi for decolourisation of industrial dyes

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Palabras clave: hongos de la podredumbre blanca, colorantes textiles, decoloración, enzimas ligninolíticas.
Key words: white-rot fungi, textile dyes, decolourisation, ligninolityc enzymes.

RESUMEN. Los colorantes textiles industriales poseen estructuras aromáticas complejas que resultan tóxicas, mutagénicas carcinogénicas y ocasionan severa contaminación en los ecosistemas donde son vertidos. Los hongos de la podredumbre blanca (HPB) pueden resultar útiles en el control de la contaminación, ya que producen oxidasas no específicas capaces de degradar la lignina y otras moléculas recalcitrantes de estructura similar tales como hidrocarburos policíclicos aromáticos, plaguicidas y colorantes. Los objetivos del trabajo consistieron en aislar y seleccionar cepas de HPB con elevada capacidad para decolorar tintes, así como analizar la producción de enzimas ligninolíticas en presencia de diferentes colorantes como inductores enzimáticos. Se colectaron treinta cepas de HPB a partir de material vegetal procedente de varios ecosistemas de La Habana y Sancti Spíritus, en el período del 2000 al 2004. Los aislados correspondieron a once especies diferentes de basidiomicetos ligninolíticos, de ellos, G*anoderma* aff. *zonatum* (B-18) y *Pleurotus djamor* (B-36) fueron los más efectivos en la decoloración de once colorantes químicamente diferentes y dos efluentes textiles simulados. Como referencias en los ensayos de decoloración se utilizaron las cepas *Trametes membranacea* (B-1), *Lentinus hirtus* (B-8) y *Pleurotus djamor* (B-9) procedentes del Jardín Botánico Nacional y con probada capacidad para degradar lignina y el colorante violeta cristal. Las enzimas lacasa y manganeso peroxidasa de *G.* aff. *zonatum* (B-18) fueron inducidas por la mezcla de los colorantes reactivos Cibacron marino FN-B, Cibacron rojo FN-3G y Cibacron amarillo P-6GS y por un efluente textil simulado, lo que sugiere su participación en los procesos de decoloración.

ABSTRACT. Textile dyes have complex aromatic structures that result toxic, mutagenic, carcinogenic and cause serious pollution problems in the ecosystems where are released. White rot fungi (WRF) can be useful in the pollution control because they produce non-specific oxidases which are able to degrade lignin and other recalcitrant molecules of similar structure such as polycyclic aromatic hydrocarbons, pesticides and dyes. The purposes of this work were to isolate and select WRF strains with high capacity to decolourise textile dyes, as well as to analyze the production of ligninolytic enzymes in presence of different industrial dyes as enzymatic inducers. Thirty strains were collected from woody materia present in several ecosystems of Havana City and Sancti Spíritus, in the period of 2000 to 2004. Isolated corresponded to eleven different species of ligninolytic basidiomycetes, from them G*anoderma* aff. *zonatum* (B-18) and *Pleurotus djamor* (B-36) were the most effective in decolourisation of eleven chemically different dyes and two simulated textile effluents. As reference in decolourisation assays were used the strains *Trametes membranacea* (B-1), *Lentinus hirtus* (B-8) and *Pleurotus djamor* (B-9), from the National Botanical Garden and with proved capacity to degrade lignin and the dye crystal violet. The enzymes laccase and manganese peroxidase of *G.* aff. *zonatum* (B-18) were induced in presence of the mixture of the reactive dyes Cibacron navy FN-B, Cibacron red FN-3G and Cibacron yellow P-6GS and a simulated textile effluent, suggesting their participation in decolourisation processes.

INTRODUCTION

White rot fungi (WRF) are physiological group comprising fungi that are capable of biodegrading and mineralizing lignin. The name white rot derives from the white appearance of wood attacked by WRF, where lignin removal gives a bleached appearance. Taxonomically, WRF are mostly basidiomycetes, although few ascomycetes are also capable of white rot decay.[1] The use of this non-specificity of their extracellular ligninolytic enzyme system [mainly lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase],[3] although in some cases additional enzymes or processes may be involved in dyes degradation.[3,4] Ligninolytic enzymes are induced by lignin and several xenobiotic compound, many of which are aromatic or phenolic compound structurally related to lignin or lignin derivatives.[5] Therefore, it's

chrysosporium, *Trametes versicolor* and a few other species, thus representing only a small part of many hundreds species existing in nature. Recently there has been a growing interest in studying a wider variety of WRF with the expectation of finding better lignin-degrading systems for use in various biotechnological applications.[6] The Island of Cuba has great biodiversity of microorganisms, therefore the study of native WRF for dyes degradation may be appropriate. World production of synthetic dyes is approximately 700 000 t/year, and between 10 and 15 % are discharged to the environment mainly from textile industry.[7] These compounds have complex aromatic structures and cause pollution problems that seriously affect ecosystems, since many dyes, their precursors and degradation products are toxic, mutagenic or carcinogenic.[8] Dyes are chemically clasified as azo, antraquinone, heterocyclic, triphenylmethane or phtalocyanine according to the chromophoric group present in the molecule. Dyes also differ in the the way they interact with textile fiber during dyeing procces. For example, reactive dyes are soluble in water and contain one or two functional groups capable of forming covalent bonds with active sites in cellulose of textil fibers. Direct dyes are also soluble in water and the dyeing process occur due to the high affinity of this compounds by fibers of cotton. Vat dyes are soluble only in its reduced (oxygen-free) form. This name derives from the vessel where these types of dyes were solubilized prior to dyeing process by the addition of a reducing agent.[9] The effluents from textile industries are extremely variable and complex, containing a wide variety of dyes and dyeing additives, such as dispersants, acids, bases, salts, detergents, humectants, oxidants, etc.[10] Therefore, a fungus selected for a possible application in the treatment of these wastewater should be able to degrade chemically diverse dyes, even in presence of the compounds added during dyeing processes. Thus, the purposes of this work were to isolate WRF strains, select the strains with highest capacity to decolourise textile dyes, as well as to analyze the production of ligninolytic enzymes in presence of different industrial dyes as inducers of these kinds of enzymes.

MATERIALS AND METHODS

Isolation and identification of WRF strains

Fungal strains were obtained from 10 de Octubre and Plaza de La Revolución, Havana City and Topes de Collantes, Sancti Spíritus, in the period of 2000 to 2004. The characteristic fruiting bodies of basidiomycetes were taken from trees in humid areas and from decayed woods. Identification of fungal isolated was carried out using the keys for identification of Kreisel and Pegler.[11,12] Pure cultures were obtained as follow: the surface of each basidiocarp was ripped off with a sterile scalpel and portions of the plot of the fruiting body cultivated on malt extract agar. Purity of the cultures was checked periodically and the strains were conserved on malt extract agar slant and sterile distilled water (4 °C).[13] Strains *Trametes membranacea* (B-1), *Lentinus hirtus* (B-8) and *Pleurotus djamor* (B-9), previously isolated in the National Botanical Garden and characterized according to their ability to degrade lignin and the dye crystal violet, were used as reference in decolourisation assays.[14]

Inoculation of WRF for the assays

at 30 °C, in Kimura medium containing 20 g · L^{-1} gluc
5 g · L^{-1} bacteriological peptone, 2 g · L^{-1} yeast extract, 1 g
KH_2PO_4, 0.5 g · L^{-1} $MgSO_4$ · 7 H_2O and 15 g · L^{-1} agar y pH

Screening and selection of the strain(s) with high capacity to decolourise textile dyes

A screening strategy was done in three steps:

a) All the strains isolated as well as the strains emplo as references, were grown (20 d, 30 °C, 120 r · min Kimura liquid medium supplemented with 0.01 % (of the reactive dyes Cibacron navy FN-B, Cibacron FN-3G and Cibacron yellow P-6GS (independen Cultivation was done by triplicate, using biotic (with dye) and abiotic (without fungus) controls. Biomass weigth) and decolourisation percentages were de mined at the end of the incubation period.

b) The strains selected (in a) according to their hig capacity to decolourise three reactive dyes, were cultiva in Kimura medium supplemented separately with 0.0 (w/v) of the reactive dyes: Cibacron red FN-R, Cibacron FN-G, Cibacron scarlet C-6G, Cibacron violet WH-B, dyes: Cibanon green BF, Cibanon orange 3R, direct d Solophenyl red 7BE, Solophenyl blue BFF and Solophe red 4GE. Biotic and abiotic controls, as were descri earlier, were used.

c) Finally, the influence of dyeing auxiliary c pounds on the growth and decolourisation capacit the selected strains (in b) was determined. Cultiva was done in Kimura medium with 0.01 % of the r tive dye Cibacron red FN-3G, supplemented with mixture of the following dyeing additives: Dekol 0.0 (dispersing), Bublex H14 (antifoam) 0.003 %, Hispoga (tensioactive) 0.01 %, Tinoclarit (H_2O_2 stabilizer) 0.00 Na_2SO_4 0.9 %, in a synthetic or simulated textile efflu As biotic control the strains were cultivated in Kim medium and Kimura medium supplemented with Cibacron red FN-3G. Sterile medium of each culture v ant were used as abiotic controls. The synthetic dyes dyeing additives used in this work were kindly supp by CIBA-GEIGY and Hilatex (a Cuban textile enterpr

Influence of dye mixture and a simulated tex effluent on *G.* aff. *zonatum* B-18 growth and lacc and MnP production

After the screening, a time course analysis was car out with the best strain in the following culture med variants: Kimura medium, Kimura medium sup mented with the mixture of the dyes of Cibacron n FN-B, Cibacron red FN-3G and Cibacron yellow P-6 (0.003 % of each one) and Kimura medium with these c and dyeing additives mix. Incubation was done du 20 d, 30 °C, 120 r · min^{-1}. Samples were taken every days to determine biomass dry weight, decolourisa percentages, laccase and MnP enzyme activities.

Decolourisation percentages determination

Decolourisation percentages were determined by difference in absorbance between sample filtrates abiotic control at the corresponding maximun wa length for each dye.

$$\text{Decolourisation (\%)} = 100 - 100 \cdot \frac{OD_{\text{culture filtrate}}}{OD_{\text{abiotic control}}}$$

the mycelia were maintained during 24 h at 80 ºC until they had constant weight. The weight of the biomass was gravimetrically determined.

Enzyme assays

Laccase activity was measured with 10 mmol/L of 2,2' –azino-*bis*(3-ethylbenzothizoline)-6-sulphonate (ABTS) as substrate.[15] MnP activity was estimated by the formation of Mn^{3+}-tartrate complex (ε_{238} = 6 500 $mol^{-1} \cdot cm^{-1}$) during the oxidation of 0.1 mmol · L^{-1} Mn^{2+} ($MnSO_4$) in 100 mmol/L sodium tartrate buffer (pH 5), in presence of 0.1 mmol · L^{-1} H_2O_2. Enzyme activities were carried out at room temperature (25 °C). One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 μmol of substrate · min^{-1}.

Statistical analysis

Results are presented as the average of three replicates. Normality and variance homogeneity were investigated prior to carrying out the statistical analyses by means of the Kolmogorov-Smirnov test and the Bartlett test, respectively. Where data met these criteria, an analysis of variance of simple classification and a parametric Student-Newman-Keuls test were used. If these preliminary criteria were not met, the Kruskal-Wallis and SNK tests were used. Spearman correlation test was used to analyze the possible existence of direct relationship between biomass growth and decolourisation percentages. All data were processed with the statistical package Statistic 6.0.

RESULTS

Collection of white-rot fungi

As a result of the collect, 30 Cuban native strains of white-rot fungi, belonging to 11 species were isolated. The species more represented in our collection were *Lentinus hirtus*, with seven strains (B-27 to B-33) and *Ganoderma* aff. *zonatum*, with six strains (B-18 to B-23). Strains B-15, B-16 and B-17 were identified as *Hexagona hydnoides*. B-24 and B-25 were classified as *Pycnoporus sanguineus*. Strains B-26, B-34, B-35, B-36 belong to the species *Lentinus tigrinus, Panus crinitus, Pleurotus smithii and Pleurotus djamor* respectively. The specie *Coriolopsis floccosa* was represented in our collection by three strains (B-37, B-38 and B-39). B-40 and B-41 were classified as *Oudemansiella canarii* while B-42, B-43 and B-44 were identified as *Lenzites elegans*.

Decolourisation of the industrial dyes Cibacron Navy FN-B, C. Red FN-3G and C. Yellow P-6GS

All strains of basidiomycetes studied were able to grow in the presence of the reactive dyes Cibacron Navy FN-B, C. Red FN-3G and C. Yellow P-6GS (Fig. 1 a, b and c respectively). The mycelial biomass of 16 of them showed a grey-blue colour after 20 d of incubation in presence of Cibacron Navy FN-B. With the exception of *H. hydnoides* B-16, the biomass of all strains presented a reddish colour after 20 d of incubation in Kimura medium with Cibacron Red FN-3G. All the strains were coloured of yellow in presence of Cibacron Yellow P-6GS.

There were significant differences ($p < 0.05$) among the decolourisation percentages of Cibacron navy FN-B, Cibacron red FN-3G and Cibacron yellow P-6GS, by the strains *G.* aff. *zonatum* (B-21, B-22), with 92.2 and 91.7 % respectively.

The 33 strains analyzed were able to decolourise Cibacron Red FN-3G (Fig. 1b), *H. hydnoides* (B-16), *G.* aff. *zonatum* (B-22) and *C. floccosa* (B-37) exhibited the highest capacity to decolourise this dye with 85.7, 82.9 and 86.1 % respectively. Cibacron yellow P-6GS was the dye most recalcitrant to biological degradation, only 18 from the 33 studied strains were able to decolourise this compound (Fig. 1c), *P. smithii* (B-35) and *H. hydnoides* (B-16) presented the highest decolourisation capacity with 48.6 and 39.6 % respectively.

It is possible to observe that the strains with better capacity to decolourise C. navy and C. red did not reach high values of decolourisation for C. yellow. *P. smithii* (B-35), differed from the rest of the WRF studied because it was the strain that reached the highest decolourisation percentages for Cibacron yellow P-6GS, but did not show a good capacity to degrade the other dyes assayed. *G.* aff. *zonatum* (B-18) and *P. djamor* (B-36) are the strains most effective among the 33 WRF strains tested for decolourisation of three reactive dyes.

There was not positive correlation between the growth of the strains and the decolourisation of the three analyzed dyes.

Decolourisation of chemically different industrial dyes by *G.* aff. *zonatum* (B-18) and *P. djamor* (B-36)

G. aff. *zonatum* (B-18) and *P. djamor* (B-36) were able to completely decolourise the dyes Cibacron blue FN-G, Cibacron scarlet C-6G, Cibacron violet WH-B, Cibanon green BF, Cibanon orange 3R, Solophenyl red 7BE, Solophenyl blue BFF and Solophenyl red 4GE (less than 4 % of colour remainig in culture medium). Dye Cibacron red FN-R was partially decolourised by these two fungi (B-18 and B-36 reached 76.7 and 78.9 decolourisation percentages respectively) (Fig. 2).

Influence of dyeing additive mix on *G.* aff. *zonatum* B-18 and *P. djamor B-36* strains growth and their decolourisation ability

Besides dyes, other chemicals are present in textile effluent such as salts, chelating agents and surfactants.[1] Thus it is important to know the effect of these dyeing additives on the microorganisms that potentially can be applied in the treatment of these wastewaters.

G. aff. *zonatum* (B-18) and *P. djamor* (B-36) were able to decolourise a simulated textile effluent containing dye Cibacron red FN-3G and some dyeing additives. The presence of dyeing additives was not detrimental to fungal growth (Table 1). With the addition of the dyeing additive mixture to control medium, the decolourisation ability of the strain B-18 was reduced by 14 %, however, the decolourisation ability of the strain B-36 was not affected. Strains B-18 and B-36 reached high decolourisation percentages (76.5 and 79.0 %) in this simulated textile effluent, therefore, they appear to be promising for a practical application and should be characterized in more detail.

Influence of dye mix and a simulated textile effluent on *G.* aff. *zonatum* B-18 growth and laccase and MnP production

Strain *G.* aff. *zonatum* (B-18), was chosen to investigate its physiology and the role of the most common

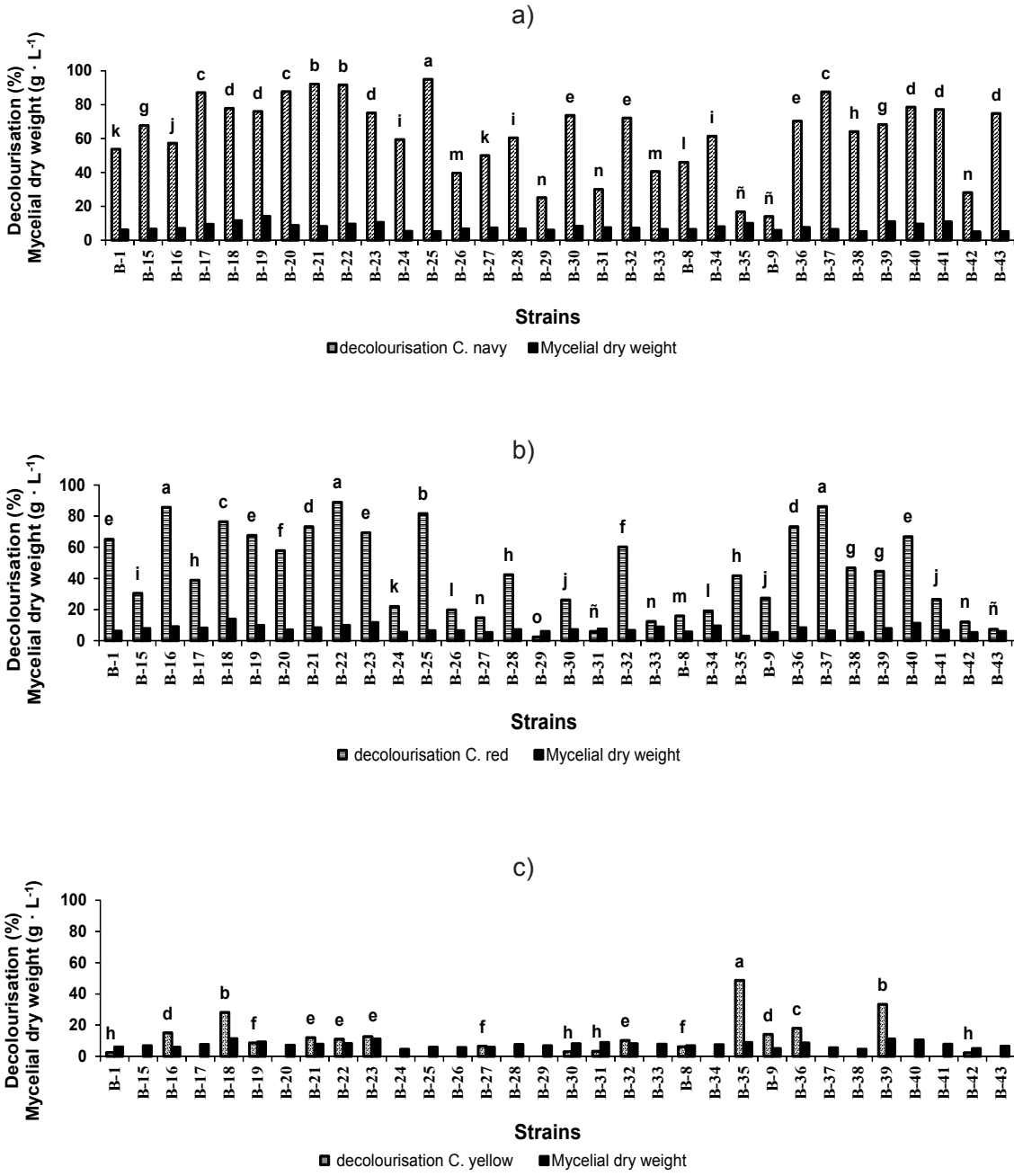


Fig. 1. *Growth and decolourisation percentages reached by different strains of white-rot basidiomycetes after 20 d of incubatio Kimura culture medium supplemented with 0.01 %. a) Cibacron Navy FN-B. b) Cibacron Red FN-3G. c) Cibacron Yellow P-*

Results are the average of three replicates. Different letters mean significant differences ($p < 0.05$) between decolourisation centages reached by WRF strains.

Strains	Species	Strains	Species
B-1	*T. membranacea*	B-34	*P. crinitus*
B-15 to B-17	*H. hydnoides*	B-35	*P. smithii*
B-18 to B-23	*G.* aff. *zonatum*	B-36, B-9	*P. djamor*
B-24, B-25	*P. sanguineus*	B-37 to B-39	*C. floccosa*
B-26	*L. tigrinus*	B-40 and B-41	*O. canarii*
B-8 and B-27 to B-33	*L. hirtus*	B-42 to B-44	*L. elegan*

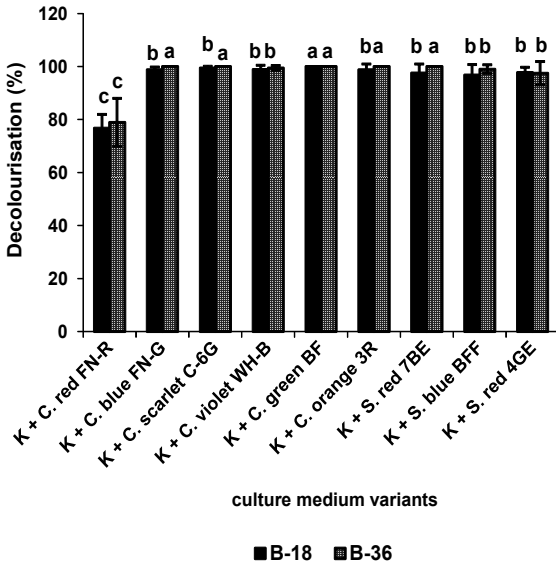


Fig. 2. *Decolourisation percentages reached by strains G.* aff. *zonatum (B-18) and P. djamor (B-36) after 20 days of incubation in Kimura culture medium (K) supplemented with 0.01 % of the different dyes assayed.* ***Reactive dyes****: Cibacron red FN-R, Cibacron blue FN-G, Cibacron scarlet C-6G Cibacron violet WH-B.* ***Vat dyes****: Cibanon green BF, Cibanon orange 3R.* ***Direct dyes****: Solophenyl red 7BE, Solophenyl blue BFF and Solophenyl red 4GE. Results are the average of three replicates and error bars represent standard deviation. Different letters mean significant differences (p < 0.05) between decolourisation percentages reached by both strains for all the dyes assayed.*

Table 1. Effect of dyeing additive mixture on the growth and decolourisation ability of *G.* aff. *zonatum* B-18 and *P. djamor* B-36.

Strains	Biomass ($g \cdot L^{-1}$)		Decolourisation (%)	
	KC	KC + DAM	KC	KC + DAM
G. aff. *zonatum* B-18	9.9 a	9.7 a	90.5 a´	76.5 b´
P. djamor B-36	8.5 a	9.7 a	79.5 b´	79.0 b´

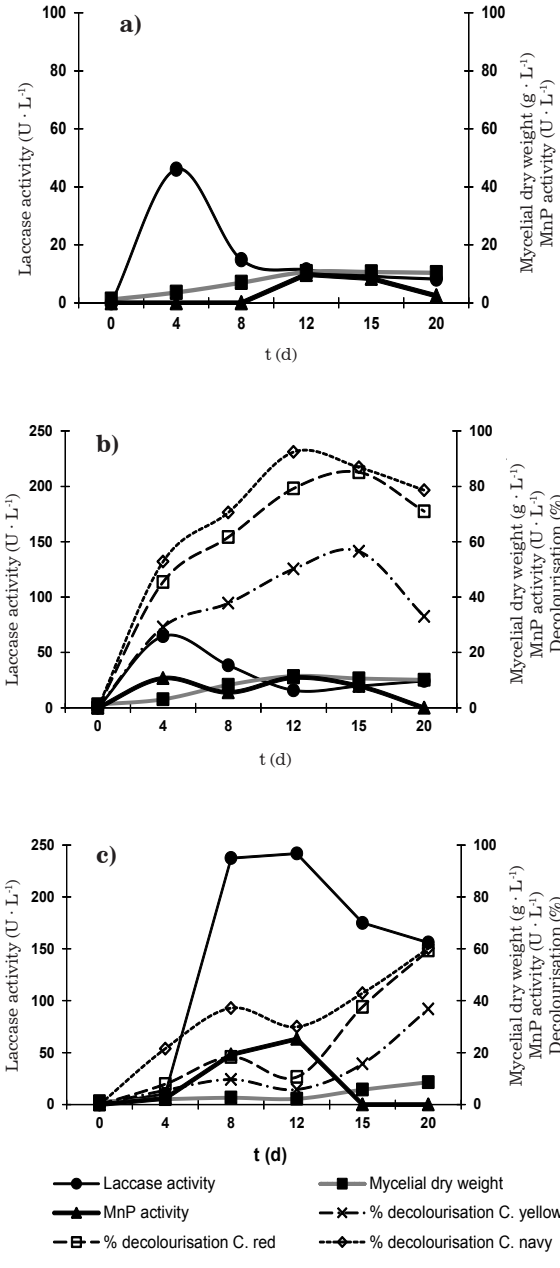
KC Kimura medium supplemented with 0.01 % of Cibacron red FN-3G. DAM Dyeing additive mixture. Different letters mean significant differences ($p < 0.05$).

Kimura medium (control), fungal growth began rapidly and reached the stationary phase at day 12 (Fig. 3a). Laccase and MnP enzymes were both detected in the culture medium. Laccase was synthesized from the beginning of the growth and reached maximum activity at day 4 with 46 $U \cdot L^{-1}$. The production of MnP began at the end of the growth phase (day 8) and reached its highest expression (10 $U \cdot L^{-1}$) in the stationary phase (day 12).

With the addition of the mixture of the dyes Cibacron navy FN-B, Cibacron red FN-3G and Cibacron yellow P-6GS to Kimura medium (Fig. 3b) the growth was not affected. However, laccase and MnP activities showed a differential behaviour in relation to the control medium.

presence of dyes induced the synthesis of MnP from the beginning of fungal growth, but there were only small increments in the highest MnP activity levels reached in this culture, compared to the control. This strain was able to decolourise efficiently the mixture of dyes. The maximum decolourisation values were 92.4 % for Cibacron navy FN-B at day 12, 85.1 and 56.5 % for Cibacron red FN-3G and C. yellow P-6GS at day 15.

In presence of the mixture of dyes and dyeing additives (a simulated textile effluent), growth was affected (Fig. 3c). Strain B-18 showed a prolonged lag phase (for 12 d)



However, during this period, laccase and MnP activity levels were much higher than the levels obtained in the other variants of culture medium. The maximum activities measured were 5.2-fold and 2.5-fold higher for laccase and MnP respectively, when compared with control treatment. Growth began after 12 d and decolourisation of dyes was increased. The decolourisation percentages at the end of fermentation were 60.2, 59.3 and 36.8 % for Cibacron navy FN-B, Cibacron red FN-3G and Cibacron yellow P-6GS respectively, high values since decolourisation conditions had not been optimized yet.

DISCUSSION

White rot fungi are considered powerful biotechnological tools to biodegrade dyes present in industrial effluents.[1,16] To assess the decolourisation capacity of different WRF we used a screening method with reactive, vat and direct dyes to assay a wider range of chemical structures and dyes with high and low solubility. These dyes have great colour stability over a wide range of pH (3-11), thermal stability and stability under culture conditions.They are commonly used in Cuba and world wide. The use of industrial dyes in screening methods was positive, it has been reported that studies on the biodegradability of model chromophoric compounds cannot be extrapolated to those used for industrial dyeing, which be expected to be more recalcitrant.[17] The use of dyeing additives in the screening strategy was practical because these are present in textile effluents, and some of these compounds can affect several WRF and their enzymes.[16]

In this work, 33 native Cuban strains of basidiomycetes belonging to 11 species were studied. With exception of *Pycnoporus sanguineus* and *Lentinus tigrinus,* the species assayed, have previously attracted only little or none research attention of other scientists, in relation with its ability to decolourise textile dyes. The selected strains *G.* aff. *zonatum* (B-18) and *P. djamor* (B-36), were effective in decolourisation of a broad spectrum of chemically different dyes, including dyes with high solubility (reactive and direct) as well as dyes with low solubility (vat) and even a simulated textile effluent. The values of decolourisation achieved by these strains are comparable to the results reported by other author using promising WRF strains for dyes degradation.[4,8] The ability of these strains to decolourise dyes are superior to the capacity of other strains that decolourise preferentially dyes with high or low solubility.[18]

The inter-generic, inter-specific and intra-specific differences found in decolourisation abilities of WRF assayed (Fig.1) are in agreement with the data reported by Claus *et al*.[19] This information suggests physiological differences in ligninolytic enzymes production, considering that decolourisation abilities of white-rot fungi are assumed to be mainly connected with their ligninolytic properties.[20] A possible explanation to the differences in decolourisation abilities detected among strains of the same species could be that they were isolated from different ecosystems where different selective pressures due to the environmental conditions in those natural habitats could provoke changes in the properties of their ligninolytic enzymes.

Fungal biomasses of many of the studied strains were coloured during decolourisation processes. This fact sug-

consistent with those of Abadulla *et al*.,[21] who repo
that some dyeing additive compounds found in
textile effluents inhibited ligninolytic enzyme
Polyporus sp. and *Trametes villos*a up to 20 %, whe
lignin modifying enzymes of *Schizophyllum comm*
were inhibited up to 70 %.

It has been shown by many authors that the ef
of different inducers supplemented to culture m
stimulates ligninolytic enzyme production. In gen
the most commonly used inducers have been phen
or aromatic compounds such as gallic acid or catec
2,5 xylidine, ρ-anisidine, veratryalcohol, guaiacol, fe
acid.[5,22] Recently there has been a growing interes
studying the effect of dyes[23] and textile effluents[2]
these enzymes. Many papers suggest a key role of
case[25] or MnP[16] in dye decolourisation. Both ligninol
enzymes were detected in all culture media assa
for *G.* aff. *zonatum* B-18 in this work. These enzy
were strongly influenced in presence of dye mix
simulated textile effluent (according to Swamy
Ramsay,[2] Wesenberg *et al.*[24]) not only because of
increment of enzyme activities due to the prese
of inducers, but also because MnP was synthethi
since the beginning of the growth in contrast with
behaviour in control medium. These findings im
that these enzymes could be involved in decolour
tion processes. However these results did not indi
a positive correlation between laccase or MnP pro
tion and dye decolourisation, which is in agreem
with results reported by Eichlerová *et al*.[23] Theref
additional enzymes or biomass associated factors c
also be involved in these degradation processes. Furt
studies that involve laccase or MnP enzyme purifica
or gene cloning to get a more powerful source of th
enzymes should be necessaries to demonstrate their
in decolourisation *in vitro*.

CONCLUSIONS

The present findings could contribute to a be
understanding of the properties of some white-rot fu
species which have not been studied in details by o
authors. *G.* aff. *zonatum* (B-18) and *P. djamor* (B-36) w
the most effective among the strains tested and also w
found to be efficient in decolourisation of nine che
cally different dyes and a simulated textile effluent
addition, the fact that *G.* aff. *zonatum* (B-18) was abl
biodegrade all these xenobiotics and that the prese
of these compounds induced the synthesis of the
ninolytic enzymes needed for their degradation, mak
this strain or its enzymes very promising candidat
for developing a biotechnological treatment of indus
textile wastewaters.

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CIBA-GEIGE and Hilatex, for supplied dyes and dye
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