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Dye-decolourizing yeasts isolated from Las Yungas rainforest. Dye assimilation and removal used as selection criteria

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ABSTRACT

Thirty-nine yeast isolates from "Laurel del Monte" underlying soil from "Las Yungas" rainforest (Tucumán, Argentina) were obtained following an isolation scheme on both, dye tolerance and assimilation. Fifteen isolates were selected based on their ability to decolourize agar plates containing four industrial textile dyes, either alone or as a mixture. Eight isolates were further selected according to their assimilation of dyes as sole Carbon/energy or Nitrogen source. Selected yeasts belonged to the basidiomycetous genus *Trichosporon* and the ascomycetous genera *Cyberlindnera*, *Barnettozyma* and *Candida*. Decolourization, biomass production and enzymatic production of manganese peroxidase, laccase and tyrosinase were then studied in liquid media. Isolate *Candida* sp MM 4035 was selected for future experiments and further molecular characterization and physiological tests are in progress in order to achieve the potential description of a new ascomycetous yeast.

1. Introduction

Reactive dyes are among the most recalcitrant synthetic colourants against biodegradative processes, and therefore are considered a worldwide problem. Industrial effluents containing textile dyes are usually disposed in large amounts into natural water bodies on a daily basis. Their pollution hazard is primarily based on components such as benzidine and aromatic compounds, which may be carcinogenic or toxic to living organisms (Meehan et al., 2000). Also, because of their marked colour, the effect due to light penetration reduction in lakes and rivers modifies photosynthetic activities in aquatic environments (Stolz, 2001).

Physical and chemical methods such as adsorption, coagulation–flocculation, oxidation, filtration, and electrochemical methods may be used for dye removal from industrial effluents. These approaches are expensive, have operational problems and may lead to bigger problems if their secondary products have a higher toxicity than the original effluent (Kapdan et al., 2000).

Several studies have been reported on decolourization of numerous dyes using white rot fungi (WRF). These organisms could mineralize many types of synthetic dyes through their oxidative and non-specific lignonolytic enzyme system mainly including lignin peroxidase (LiP), manganese-dependent peroxidase (MnP) and laccase (Lacc). Common WRF employed for this purpose includes *Phanerochaete chrysosporium*, *Trametes versicolor* and *Coriolus versicolor* (Yang et al., 2005). However, the strict conditions for enzyme production and the jeopardy of bacterial contamination in non-sterile conditions in dye-containing wastewaters, made difficult the application of white rot fungi for its treatment (Aksu, 2003). In addition, growth of filamentous fungi is usually slow compared with most single-cell microorganisms, and the production of mycelium often makes filamentous fungi poorly adaptable to wastewater treatments (Yu and Wen, 2005).

When compared with fungi, yeasts have many advantages. Not only because of their fast growth, but also because like filamentous fungi they have the ability to resist unfavourable environments. Besides, yeasts have been found to be very efficient in treating high-strength organic wastewaters, such as food-, molasses-, and oil-manufacturing industrial effluents (Yang et al., 2008). Ascomycetous yeasts are able to decolourize reactive dyes by biodegradation (*Pseudozyma rugulosa* and *Candida krusei*; (Yu and Wen, 2005)); reduction of azo-bond (*Candida oleophila* (Lucas et al., 2006), *Candida*

Abbreviations: WRF, white rot fungi; NDM, normal decolourization medium; HAU, halo arbitrary units; CDAU, colony dying arbitrary units; LiP, lignin peroxidase; Lacc, laccase; MnP, manganese-dependent peroxidase; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); TMPD, N,N,N′,N′-tetramethyl-p-phenylenediamine; YNB, yeast nitrogen base.

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zeylanoides (Ramalho et al., 2005), *Sacharomyces cerevisiae* (Jadhav et al., 2007)), biosorption (*Kluyveromyces marxinus* IMB3 (Meehan et al., 2000)) or catalytic degradation by ligninolytic enzymes (*Debaryomyces polymorphus* (Yang et al., 2003, 2005, 2008)).

Only a few reports involve basidiomycetous yeasts in dye decolourization, including *Rhodotorulla minuta* (Ertugrul et al., 2009), *Trichosporon multisporum* and *Trichosporon akiyoshidainum* (Pajot et al., 2007, 2008) and *Trichosporon beigelii* (Saratale et al., 2009). As previously pointed out, the apparent lack of decolourizing ability in basidiomycetous yeasts was highly surprising, taking into account the widespread distribution of ligninolytic enzymes through filamentous basidiomycetous fungi (Pajot et al., 2008).

The ecoregion of "Las Yungas" is a humid forest occurring in mountainous sectors linked to the Andes that reach the northwest of Argentina. It is one of the environments containing the highest biodiversity in Argentina. During the past years its flora and fauna biodiversity received great attention; however its microbial diversity has been scarcely explored. Like many other tropical forests, Las Yungas is at risk because of different human activities which affect their native biota, including fungal microbiota. In this context, the screening of microbiota with biotechnological potential has been previously highlighted as an important endeavour (Rovati et al., 2010).

Bio-prospecting procedures for dye-decolourizing yeasts usually employ carbon and nitrogen sources that are readily assimilable in culture media (Yang et al., 2003; Yu and Wen, 2005). Most yeasts selected in this kind of schemes are able to degrade dyes by co-metabolic processes. Such co-metabolic processes might not be economically viable and may be difficult to scale-up. In this work we propose that yeasts able to use dyes as sole carbon/energy or nitrogen can be isolated and selected from the environment by increasing the selectivity of the media. This approach might be extremely stringent and the number of isolates might be significantly reduced, but selected yeasts could present higher decolourization abilities and should not need additional substrates in order to perform decolourization of dyes.

2. Materials and methods

2.1. Samples

Based on previous works (Pajot et al., 2007, 2008, 2011), samples were aseptically collected from "Laurel del Monte" (*Phoebe porphyria*) rhizhosphere at Las Yungas rainforest (latitude 26° 43′ 20.7″S, longitude 65° 17′ 21.6″W, altitude 930 m (asl), 22.5 °C and 68% humidity) on May of 2010. In order to select only well grown, undisturbed trees, samples were taken every 10–12 m away from the soil beneath trees at least 1.5 m in diameter at chest height. The upper layer of the top-soil was removed and 10–15 g samples were taken from 5 to 10 cm deep. Five samples were collected according to this scheme, covering approximately. 0.5 ha of rainforest. Samples were taken and transported in sterile plastic containers and kept at 4 °C until they were used for subsequent microcosms experiments.

2.2. Yeasts isolation and maintenance

Samples were inoculated into 250 ml Erlenmeyer flask containing 50 ml of modified Normal Decolourización Medium (NDM), Yeast extract (YE) 2.5 g l^{-1}; KH_2PO_4 5 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.5 g l^{-1}; $CaCl_2$ 0.13 g l^{-1} (Ramalho et al., 2004). A mixture of Vilmafix® Blue RR-BB, Vilmafix® Red 7B-HE, Vilmafix® Black B-V and Vilmafix® Yellow 4R-HE (0.25 g l^{-1} each, final dye concentration 1 g l^{-1}), was used as sole Carbon/Energy, Nitrogen or both sources. The pH value was adjusted to 4.0 by addition of 1 N HCl. Microcosms were incubated at 250 rpm and 25 °C. After 24 h, 100 μl samples were plated onto agar plates with modified NDM and incubated at 25 °C until colony development. The procedure was repeated in triplicate for every microcosm. Different colony morphotypes were transferred to unmodified NDM without dyes, incubated at 25 °C and subsequently stored at 4 °C.

2.3. Dyestuff

Three azo commercial dyes; Vilmafix® Red 7B-HE (C.I. Name: Reactive Red 141), Vilmafix® Black B-V (C.I. Name: Reactive Black 5) and Vilmafix® Yellow 4R-HE (C.I. Name: Reactive Yellow 84); and a copper formazan complex dye, Vilmafix® Blue RR-BB (C.I. Name: Reactive Blue 221) were employed in this work (Fig. 1). Vilmafix® dyes were generously provided by Vilmax S.A. Stock solutions were prepared by dissolving powdered dyestuff, without prior purification, in distilled water up to a concentration of 2 g l^{-1} and filter sterilized (Millipore filter, 0.22 μm, Millipore Corp., Bedford, USA).

2.4. Dye-decolourizing ability on solid media

Decolourization screening in solid media was performed on Petri dishes containing 20 ml of NDM-agar. Dye stock solutions were added to culture media up to 200 mg l^{-1} final concentration. A mixture of all textile dyes (50 mg l^{-1} each) was used as a first approach to textile effluent simulation. Plates were inoculated with actively growing yeast from NDM-agar and incubated at 25 °C. As growth control, plates without dyes were also inoculated.

Plates were examined daily up to three days of cultivation. In every examination, production of decolourization haloes around the colony and colony dying were observed and registered. Decolourization haloes were measured and expressed as Halo Arbitrary Units (HAU). HAU took values from 0 (no halo) to 3 (extensive decolourization halo). Colony dying was also assessed by visual inspection and expressed as Colony Dying Arbitrary Units (CDAU). The CDAU value ranged between 0 (unstained colony) and 2 (highly coloured colony). Isolates displaying higher ΣHAUs and lower ΣCDAUs were selected for further studies.

2.5. Dye assimilation

Use of dyes as sole carbon/energy or nitrogen sources was assayed by the auxanogram method on solid YNB or YCB (agar–agar 20 g l^{-1}), according to standard techniques (Yarrow, 1998). Growth was recorded as weak or delayed (+) when growth was noticed after two or three weeks, positive (++) when growth was noticed in the two first weeks, or negative (−) when no growth was detected after 4 weeks. Growth controls included glucose or $(NH_4)_2SO_4$ as carbon/energy or nitrogen sources, respectively. In negative controls, no additional sources were included. Plates were incubated at 25 °C, and examined periodically up to 4 weeks.

2.6. Growth and decolourization in liquid cultures

Decolourization kinetics were evaluated in 500 ml-Erlenmeyer flasks containing 100 ml of standard NDM medium plus a single dye, or the mixture of dyes at a final concentration of 200 mg l^{-1}. 10-ml yeast suspension ($OD_{550} = 0.8$), prepared from a 24 h old NDM broth culture were used to inoculated the flasks. Incubations were carried out at 25 °C and 250 rpm for 72 h. Biotic and abiotic controls were performed using the same medium without dyes or yeasts, respectively. Samples were aseptically collected every 12 h and centrifuged for 10 min at 6500 *g*. Pellets were washed twice with sterile water and dried at 80 °C to constant weight for biomass dry weight determination. Supernatants were kept for estimating

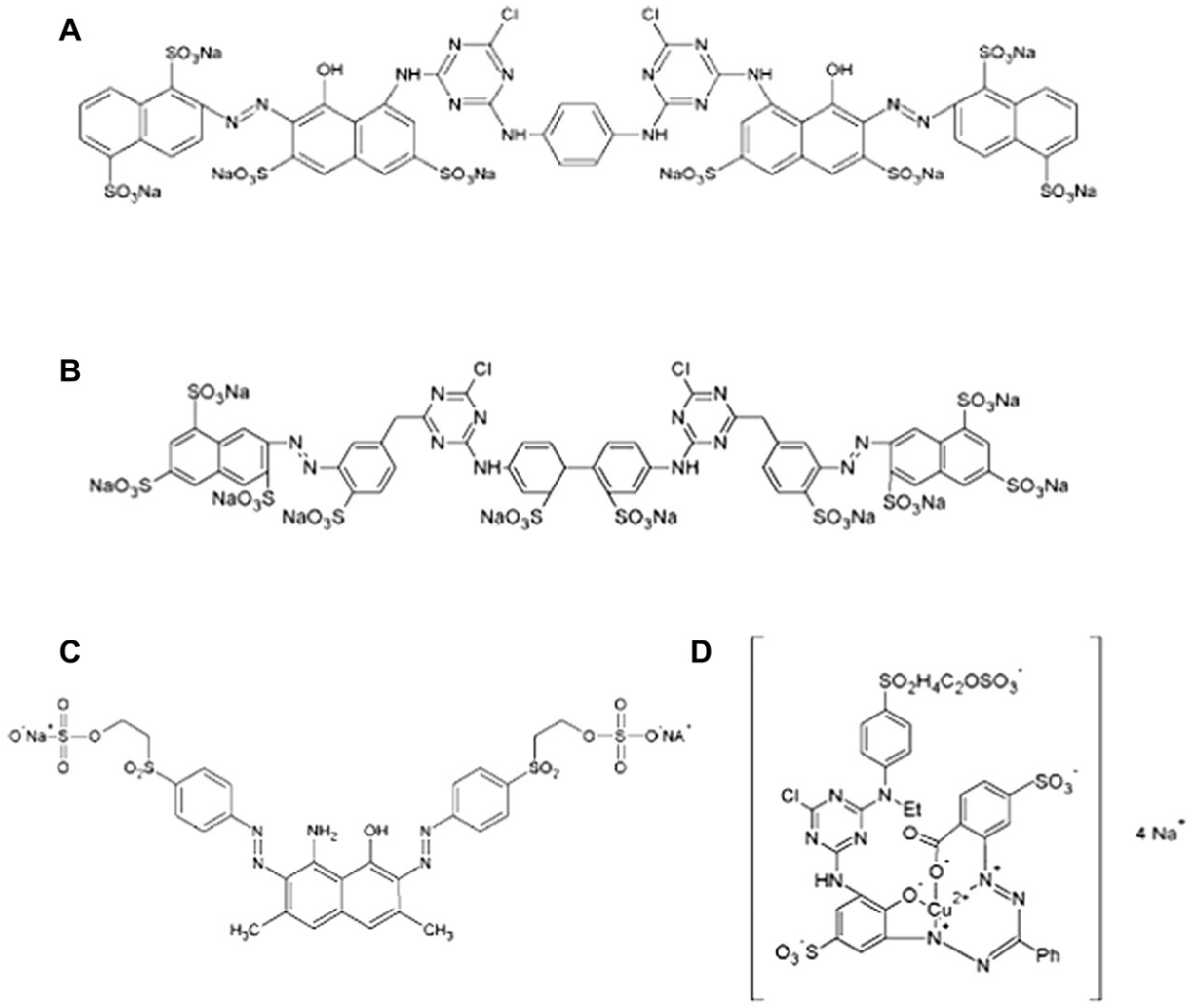

Fig. 1. Dye chemical structures of: (A) Vilmafix® Red 7B-HE (C.I. Name: Reactive Red 141), (B) Vilmafix® Yellow 4R-HE (C.I. Name: Reactive Yellow 84), (C) Vilmafix® Black B-V (C.I. Name: Reactive Black 5), (D) Vilmafix® Blue RR-BB (C.I. Name: Reactive Blue 221).

dye removal, enzymatic activities and pH. Two kinetic parameters were also calculated in order to compare the biodecolourization processes with those described in the literature: the volumetric decolourization rate (η, $mg_{dye}\,l^{-1}\,h^{-1}$) and the specific decolourization rate (ν, $mg_{dye}\,g^{-1}_{dry\ biomass}\,h^{-1}$) (Pajot et al., 2008).

2.7. Dye monitoring

Dye decolourization was monitored using a Beckman Coulter AD200 ELISA reader at each dye λ_{opt} by using culture supernatants obtained as described above. Colour removal at each dye λ_{opt} was calculated as percent decolourization, as follows: $\% = (A_0 - A_t)/A_0 \times 100$, where A_0 and A_t were the absorbance of dye-amended medium at the start point (0) and at a cultivation time (t), respectively. Additionally, absorbance spectra of culture supernatants were scanned between 350 and 750 nm in order to assay dye disappearance during cultivation.

2.8. Enzymatic assays

Colourimetric enzyme assays were performed at room temperature. All enzymatic activities were determined by using a Beckman Coulter AD200 ELISA reader, following a miniaturized procedure that was setup with a 300-μl-microwell plate. Laccase (Lacc) experiments were based on the oxidation of ABTS followed by the increase in absorbance at 420 nm in a reaction mixture containing 1.8 mM ABTS in 50 mM acetate buffer (pH 4.0) (Heinzkill et al., 1998). Mn-dependent peroxidase (MnP) experiments were based on the oxidation of MBTH/DMAB followed by the increase in absorbance at 610 nm in a reaction mixture containing 0.07 MBTH, 1 mM DMAB, 0.3 mM $MnSO_4.7H_2O$, 0.05 mM H_2O_2 in 100 mM succinate–lactate buffer (pH 4.5) (Castillo et al., 1994). Finally, Tyrosinase (Tyr) measurements were based on catechol oxidation followed by the increase in absorbance at 420 nm in a reaction mixture containing 0.9 mM of cathecol in 50 mM phosphate buffer (pH 7.4) (Bora et al., 2004).

2.9. Statistical analysis

All values and data points presented in this work are the means of at least triplicate determinations of independent assays. Data were analyzed using the GraphPad InStat Instant Biostatistics package version 3.0. Statistical analysis was conducted using Minitab (Minitab Inc., State College, PA, USA). Biomass yields differences were tested by Kruskal–Wallis test, a non-parametric one-

way ANOVA test. A critical value of 0.1 was chosen arbitrarily based on the size and type of experiment (Haaland, 1989).

2.10. Molecular identification of dye-decolourizing yeasts

Yeast DNA extraction was performed by the glass-beads method as described by Yamada et al. (2002). Molecular characterization included sequencing of 26S rRNA gene D1/D2 domain and internal transcribed spacers (ITS1–5.8S–ITS2) region, as previously reported (Pajot et al., 2008). DNA sequencing on both strands was performed by Macrogen, Korea. The sequences were registered in the GenBank Data Library under accession numbers JN122338–JN122347.

According to standard procedures, a primary identification was made by studying 26s D1/D2 domain sequences. In order to confirm those results, sequences from ITS1–5.8S–ITS2 regions were also taken into account.

All sequences were compared to the non-redundant NCBI database by using BLASTN, with the default settings used to find the most similar sequence, and were sorted by the E score. To be considered a good identification to the species level, the identification had to meet the following criteria: (i) sequence identity of $\geq$99.0%; (ii) $\geq$90.0% sequence coverage for the matching sequence; and (iii) matching sequence corresponding to a Type strain, published in a peer-reviewed journal article or submitted by ATCC, CBS, JCM, NRRL or similar culture collections.

Phylogenetic analysis employed Mega 5.0 with maximum-likelihood analysis, a heuristic search with the starting tree obtained automatically Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was $<$100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used.

Gaps were handled as missing data. Bootstrap values were based on 1000 replications; values $<$50% were not recorded.

3. Results and discussion

At the end of the isolation scheme, an average of four clearly different morphotypes could be identified coming from each soil sample. Accordingly, 39 isolates were obtained as pure cultures and maintained on NDM-agar slants at 4 °C by periodic subculturing.

3.1. Decolourization screening on solid media

According to a criterion based on higher ΣHAUs and lower ΣCDAUs values, fifteen isolates from “Las Yungas” rainforest were selected (Fig. 2). As previously described (Pajot et al., 2007), a useful association between high dye removal (according to the halo size) and low colony staining is a feature expected to guide towards the selection of yeasts with higher biodegradation than bioaccumulation potential. Nevertheless, these observations did not provide any insight into the precise colour removal rate. However they do provide some information regarding the decolourizing ability of the yeast strains tested.

Different levels of decolourization were noted according to the chemical nature of the tested dye (see Section 2.3). Vilmafix® Blue RR-BB (A copper formazan dye) was quickly decolourized. Between azo dyes, Vilmafix® Black B-V presenting the lowest molecular weight, no triazine heterocycles and a simpler structure was more easily decolourized than Vilmafix® Red 7B-HE and Vilmafix® Yellow 4R-HE dyes, that shown to be the most recalcitrant ones.

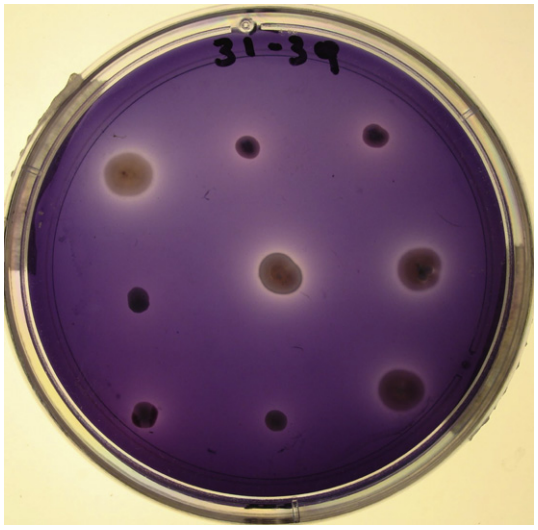

Fig. 2. Decolourization screening on solid medium for isolates MM 4033, MM 4032, MM 4031, MM 4036, MM 4035, MM 4034, MM 4039, MM 4038, MM 4037 with NDM medium plus mixture of Vilmafix® Blue RR-BB, Vilmafix® Red 7B-HE, Vilmafix® Black B-V and Vilmafix® Yellow 4R-HE (200 mg l^{-1}). Differences on colony dyeing and the presence of haloes are indicative of decolourization ability.

3.2. Dyes assimilation as carbon or nitrogen source

According to the assimilation profiles (Table 1) nine of 15 isolates (MM 4012, MM 4018, MM 4024, MM 4026, MM 4028, MM 4033, MM 4034, MM 4035 and MM 4037) were selected. We assumed weak (+) or good growth (++) when using dyes as carbon/energy or nitrogen sources, implicating the partial assimilation of these compounds. However, it may be that weak or delayed growth could be attributed to restricted uptake of dyes, to the formation of some inhibitory products or, alternatively, to the assimilation of dye impurities (Ramalho et al., 2002).

3.3. Molecular identification

The D1/D2 domain sequence of seven basidiomycetous strains (MM 4037, MM 4033, MM 4028, MM 4026, MM 4024 and MM 4012) probed to be identical. Moreover, they showed 100% identity with the D1/D2 sequence of *Trichosporon porosum* CBS 2040^T (Fig 3). All seven strains were named *T. porosum*, since it has been demonstrated that in *Trichosporon* genus there exists more variability in the D1/D2 region than in the ITS region (Middelhoven et al., 2004),

The strain MM 4018 showed 99% identity with the D1/D2 domain sequence of *Williopsis californica* NRRL Y-17395^T. Taxonomy of the *Williopsis* genus is being currently revised, since it has been demonstrated to be polyphyletic (Kurtzman et al., 2008; Kurtzman, 2011). Consequently, the creation of the genus *Barnettozyma*, including the new combination *Barnettozyma californica* has been proposed by Kurtzman et al. (2008). We have adopted this criterion and subsequently, strain MM 4018 was named *B. californica* MM 4018.

Strain MM 4034 showed 99% identity with the D1/D2 domain sequences of type strains NRRL Y-17396^T (*Williopsis saturnus* var *saturnus*) and NRRL Y-17391^T (*Williopsis saturnus* var *suaveolens*). Based on nuclear DNA reassociation and polygenic sequencing studies, it has been proposed that varieties of *W. saturnus* should be considered individual species and should be transferred to the new genus *Cyberlindnera* as *Cyberlindnera saturnus* and *Cyberlindnera suaveolens* (Kurtzman et al., 2008; Kurtzman, 2011).

Table 1
Assimilation of Vilmafix® dyes and the mixture as C and N sources in selected yeasts.

Yeast	Assimilation as C source					Assimilation as N source					Assimilation as C and N source				
	Dye 1	Dye 2	Dye 3	Dye 4	Mixture	Dye 1	Dye 2	Dye 3	Dye 4	Mixture	Dye 1	Dye 2	Dye 3	Dye 4	Mixture
4012	++	++	++	++	++	++	++	++	+	++	+	+	+	+	+
4018	–	–	–	–	–	++	++	++	++	++	–	–	–	–	–
4024	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
4026	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
4028	+	+	–	+	+	++	+	++	+	++	++	++	–	++	++
4033	–	–	–	–	–	++	++	++	++	++	–	–	–	–	–
4034	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
4035	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
4037	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
4029	–	+	–	+	+	–	–	–	–	–	–	–	–	–	
4004	–	–	–	–	–	+	++	++	++	++	–	–	–	–	–
4005	–	–	+	–	+	+	–	++	–	+	–	–	+	–	+
4009	–	–	–	–	–	–	–	–	–	++	–	–	–	–	–
4015	–	–	–	–	+	–	–	–	–	++	–	–	–	–	+
4017	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–

Dye1: Vilmafix® Yellow 4R-HE, Dye 2: Vilmafix® Black B-V, Dye 3: Vilmafix® Blue RR-BB, Dye 4: Vilmafix® Red 7B-HE.

Since the analysis of the ITS1–5.8S–ITS2 sequences showed only 95.2% identity between the strain MM 4034 sequence and its closest relative, *C. saturnus* CBS 254^T, the strain has been tentatively named *C. saturnus* MM 4034 (Fig. 3).

Strain MM 4035 could not be identified by sequencing D1/D2 domain. Its closest relative, *Wickerhamomyces quercuum* NRRL Y-12942^T showed to be 94.8% identical when considering this region (over 518 bp). The ITS1–5.8S–ITS2 region of strain MM 4035 could not be significantly aligned either. Herein, the strain was named *Candida* sp. MM 4035 (Fig. 4) and further molecular characterization and physiological tests are currently in progress in order to overcome the potential description of a new ascomycetous yeast.

3.4. Decolourization in liquid culture

Based on the identification, decolourization and assimilation on solid media assays strains *B. californica* MM 4018, *C. saturnus* MM 4034, *Candida* sp. MM 4035 and *T. porosum* MM 4037 were selected for the decolourization in liquid culture experiments. The removal of the four dyes was tested individually in liquid cultures and in every strain selected. The mixture was also tested, but colour removal was evaluated by scanning the absorbance spectra between 350 and 750 nm.

Results from abiotic controls indicated that both tested dyes remained almost intact (99 ± 1%) after 36 h incubation in NDM.

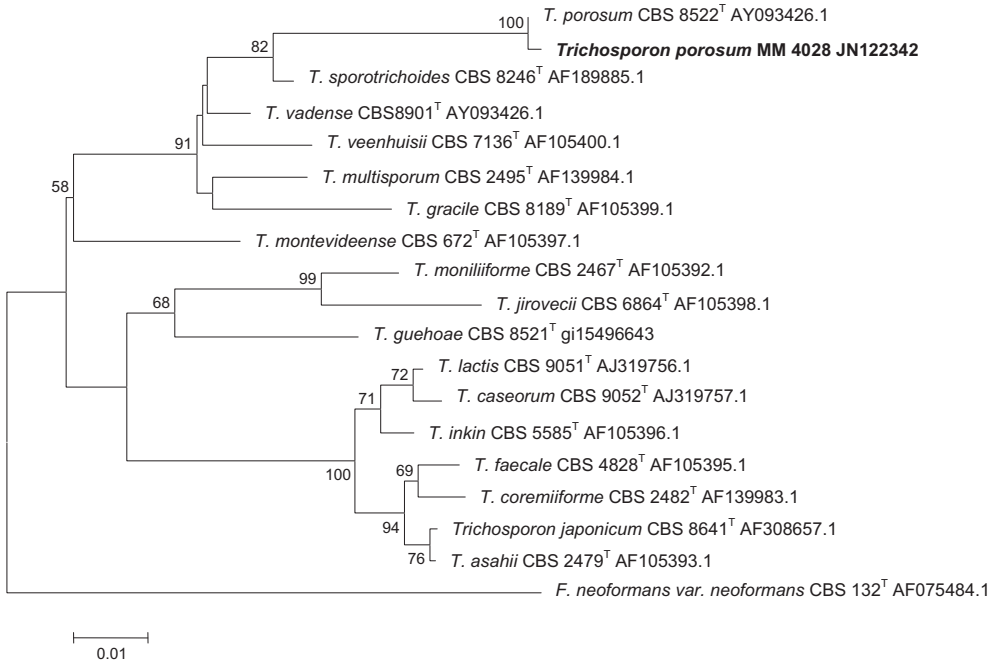

Fig. 3. Molecular phylogenetic anaylsis by maximum-likelihood method. The evolutionary history was inferred by using the Maximum-Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 21 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 518 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., in press).

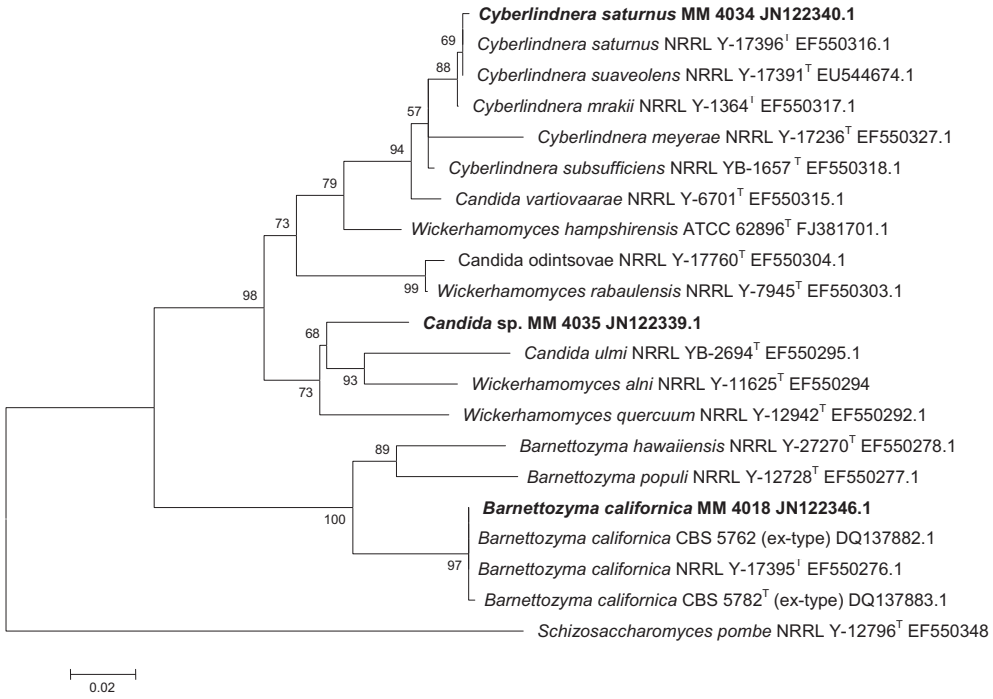

Fig. 4. Molecular phylogenetic analysis by maximum-likelihood method. The evolutionary history was inferred by using the maximum-likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 21 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 518 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., in press).

Accordingly, any reduction in the initial dye concentration for inoculated media could be confidently assigned to yeast activity.

Table 2 shows colour removal of each dye after 24 h as percentages. Based on these results, Vilmafix® Black B-V, showed to be the most easy to remove from the culture broth with removal percents higher than 90%. For isolates *B. californica* MM 4018, *Candida* sp. MM 4035 and *T. porosum* MM 4037, Vilmafix® Yellow 4R-HE was the most recalcitrant dye, showing lower decolourization percentages and biomass production. On the contrary, the lowest dye removal was observed when *C. saturnus* MM 4034 was cultured with Vilmafix® Blue RR-BB.

After 36 h incubation, there was no significant difference in biomass production for *C. saturnus* MM 4034 ($P < 0.1$) (Fig. 4) showing no growth restriction in the presence of dyes. On the other hand, isolates *T. porosum* MM 4037 showed lower biomass yields when cultured with either Vilmafix® Yellow 4R-HE or Vilmafix® Blue RR-BB. On the contrary, when cultured with Vilmafix® Red 7B-HE, biomass yields were significantly higher ($P > 0.1$). *B. californica* MM 4018 and *Candida* sp. MM 4035 growth appeared to be lower in the presence of any dye. Similar results were found for several ascomycetous yeasts including *C. zeylanoides* (Martins et al., 1999; Ramalho et al., 2004), *Geotrichum* sp. (Máximo et al., 2003), *Issatchenkia occidentalis* (Ramalho et al., 2004), *D. polymorphus* (Yu and Wen, 2005) and *C. oleophila* (Lucas et al., 2006).

Interestingly, all isolates but *B. californica* MM 4018, showed no significant growth differences when cultured with the mixture of

Table 2
Decolourization kinetic parameters of several dyes by different yeasts in liquid culture after 24 h of cultivation.

Yeast	Dye, C_i[a]	Dye removal (%)	Biomass ($g\,l^{-1}$)	Specific decolourization rate, ν ($mg\,g^{-1}\,h^{-1}$)	Volumetric decolourization rate, η ($mg\,l^{-1}\,h^{-1}$)
W. californica MM 4018	Vilmafix® Yellow 4R-HE	64.24	3.09	1.44	4.44
	Vilmafix® Black B-V	96.42	6.26	1.52	9.49
	Vilmafix® Blue RR-BB	78.25	3.20	2.03	6.49
	Vilmafix® Red 7B-HE	83.06	3.58	1.93	6.92
W. saturnus MM 4034	Vilmafix® Yellow 4R-HE	90.29	4.63	1.25	5.78
	Vilmafix® Black B-V	91.57	5.99	1.58	9.47
	Vilmafix® Blue RR-BB	67.12	5.58	0.94	5.24
	Vilmafix® Red 7B-HE	87.94	4.87	1.49	7.26
Candida sp. MM 4035	Vilmafix® Yellow 4R-HE	76.31	3.19	1.67	5.34
	Vilmafix® Black B-V	96.38	5.12	1.91	9.80
	Vilmafix® Blue RR-BB	84.24	4.29	1.59	6.81
	Vilmafix® Red 7B-HE	83.42	4.11	1.65	6.79
T. porosum MM 4037	Vilmafix® Yellow 4R-HE	66.99	2.79	1.61	4.50
	Vilmafix® Black B-V	95.82	5.67	1.69	9.58
	Vilmafix® Blue RR-BB	74.61	2.79	2.17	6.06
	Vilmafix® Red 7B-HE	91.83	7.76	2.83	6.44

dyes ($P > 0.1$). This could be explained by taking into account the final concentration of each individual dye (50 mg l^{-1}) (Fig. 5).

When decolourizing abilities were correlated with biomass production through the specific decolourization rate (v) estimation, the highest rates (1.89 and 2.17 mg g^{-1} h^{-1}) were obtained with Vilmafix® Blue RR-BB in isolates *B. californica* MM 4018, *Candida* sp. MM 4035 and *T. porosum* MM 4037. For Vilmafix® Yelow 4R-HE and Vilmafix® Black B-V, specific decolourization rates ranged between 1.44 mg g^{-1} h^{-1} and 1.67 mg g^{-1} h^{-1} and between 1.52 and 1.91, respectively. Similar values were previously reported by Pajot et al. (2007, 2008, 2011) and for well known dye-accumulating ascomycetous yeasts including *Kluyveromyces marxianus* (Meehan et al., 2000), *Candida tropicalis* (Dönmez, 2002) and *Saccharomyces cerevissiae* (Aksu, 2003).

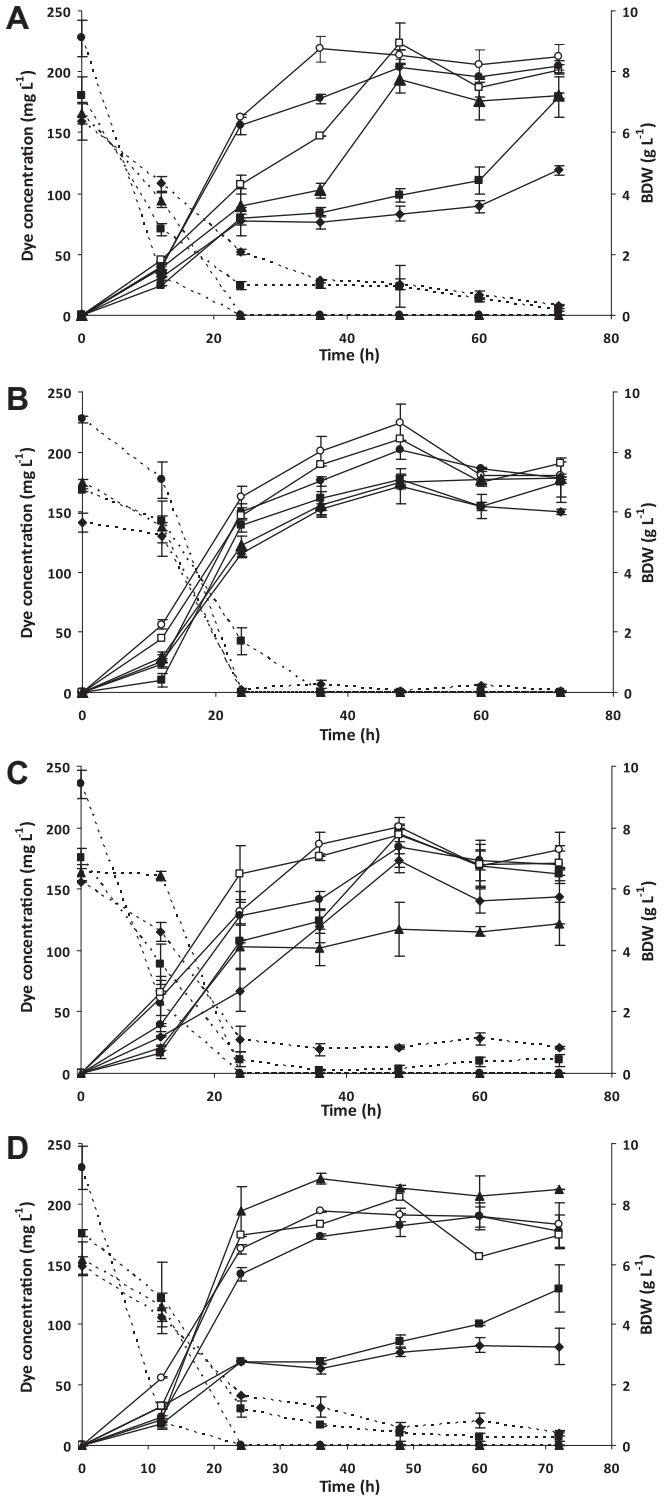

Fig. 5. Growth and decolourization kinetics in NDM medium with *Williopsis californica* MM 4018 (a), *W. saturnus* MM 4034 (b), *Candida* sp. MM 4035 (c) and *T. porosum* MM 4037 (d). Biomass in continuous line, dye removal in dotted line. (○) control, (●) Vilmafix® Black B-V, (▲) Vilmafix® Red 7B-HE, (■) Vilmafix® Blue RR-BB, (◆) Vilmafix® Yellow 4R-HE and (□) mixture.

Based on the dye removal spectra obtained for each isolate cultured with the mixture of all dyes at a 200 mg l^{-1} concentration, these could be separated in three groups. In strains *B. californica* MM 4018 and *Candida* sp. MM 4035 a general decrease of the absorbance was observed in the range analyzed, except for the region between 470 and 570 nm, which could be associated with a slower removal rate of Vilmafix® Red 7B-HE (λ_{opt} 540). For isolate *T. porosum* MM 4037, a general decrease of the absorbance was also observed, except for the region between 500 and 650 nm, in this case could be caused by a disminution in the removal rate of Vilmafix® Blue RR-BB (λ_{opt} 610) or Vilmafix® Black B-N (λ_{opt} 595). Finally for isolate *C. saturnus* MM 4034 a general decrease in all the range analyzed was observed, this may be related with adsorption processes, taking into account the staining present in the biomass (data not shown).

3.5. Enzymatic activities

Lignin modifying enzymes such as laccase (Lacc), manganese peroxidase (MnP), lignin peroxidase (LiP), tyrosinase (tyr) and some extent by N-demethylase, have been widely related to dye biodecolourization and mineralization in filamentous fungi. However, the relative contribution of each enzyme to the overall biodegradation process has proven to be different for each microorganism (Jadhav et al., 2007).

In the presence of Vilmafix® Black B-V, MnP and Tyr activities were found in all the isolates tested with a maximum Tyr activity at 24 h in *B. californica* MM 4018 (272 U l^{-1}), *Candida* sp. MM 4035 (365 U l^{-1}) and *T. porosum* MM 4037 (292 U l^{-1}), for *C. saturnus* MM 4034 the maximum activity was also reached at 24 h (322 U l^{-1}) and remainded in the same level for the rest of the cultivation time

Table 3
Enzymatic activities of the culture supernatant at 24 h of cultivation.

Enzyme activity	Vilmafix® dye	Isolate			
		W. californica MM 4018	*W. saturnus* MM 4034	*Candida* sp. MM 4035	*T. porosum* MM 4037
Laccase	Yellow 4R-HE	–	–	–	–
	Black B-V	–	–	–	–
	Blue RR-BB	–	–	–	–
	Red 7B-HE	–	–	–	–
	Mixture	–	–	–	–
Manganese peroxidase	Yellow 4R-HE	–	–	–	–
	Black B-V	++	++	++	++
	Blue RR-BB	–	–	–	–
	Red 7B-HE	–	–	–	–
	Mixture	–	+	–	–
Tyrosinase	Yellow 4R-HE	+	–	+	+
	Black B-V	++	++	++	++
	Blue RR-BB	+	–	–	–
	Red 7B-HE	–	–	–	–
	Mixture	–	–	–	–

(Data not shown). Lower Tyr activity at 24 h was found in *B. californica* MM 4018 (195 U l^{-1}), *Candida* sp. MM 4035 (108 U l^{-1}) and *T. porosum* MM 4037 (149 U l^{-1}) cultures with Vilmafix® Yellow 4R-HE. Additionally, *B. californica* MM 4018 produced Tyr activity when incubated with Vilmafix Blue RR-BB (100 U l^{-1} at 24 h of cultivation) (Table 3). MnP activities were higher at 24 h for *B. californica* MM 4018 (387 U l^{-1}), *Candida* sp. MM 4035 (447 U l^{-1}) and *T. porosum* MM 4037 (355 U l^{-1}) when cultured with Vilmafix® Black B-V. For *C. saturnus* MM 4034 the maximum activity was found at 36 h (567 U l^{-1}).

The presence of MnP and Tyr activities during Vilmafix® Red 7B-HE, Vimafix® Blue RR-BB and Vilmafix® Black B-V decolourization was previously reported for *T. akiyoshidainum* HP 2023 (Pajot et al., 2011). Meanwhile, *Devaryomyces polimorphus* also produced MnP activity when cultured with Reactive Black 5 (Yang et al., 2005).

4. Conclusion

We have previously proposed that oligotrophic yeasts with high decolourizing potential may be expected to grow after cultivation with dyes as the sole Carbon/energy source (Pajot et al., 2011).

The screening program here described, where only yeasts able to use dyes as sole carbon/energy or nitrogen could be isolated, probed to be extremely stringent. However, both asco- and basidiomycetous yeasts could be recovered.

Since most up to date known dye-decolorizing yeast are ascomycetous, it could be suggested that this prevalence, far from reflecting a real trend have been strongly influenced by the screening methodology adopted; mainly allowing only the selection of copiotrophic yeasts.

The herein present approach is not enough to rule out the possibility of a co-metabolic dye decolourization process. However, the ability of selected yeast for dye assimilation strongly suggest that it should be taken into account when planning bio-prospecting schemes pursuing the selection of yeasts with higher degradation ability through metabolic, easier to study and scale-up processes

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