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Unraveling the decolourizing ability of yeast isolates from dye-polluted and virgin environments: an ecological and taxonomical overview

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Abstract Microcosm assays with dye-amended culture media under a shot-feeding strategy allowed us to obtain 100 yeast isolates from the wastewater outfall channel of a dyeing textile factory in Tucumán (Argentina). Meanwhile, 63 yeast isolates were obtained from *Phoebe porphyria* (Laurel del monte) samples collected from Las Yungas rainforest (Tucumán), *via* a classical isolation scheme. Isolated yeasts, both from dye-polluted and virgin environments, were compared for their textile dye decolourization ability when cultured on solid and liquid media. Nine isolates from wastewater and 17 from Las Yungas showed the highest decolourization potential on agar plates containing six different reactive dyes, either alone or as a mixture. Five yeasts from each environment were further selected on the basis of their high dye removal rate in Vilmafix® Red 7B-HE- or Vilmafix® Blue RR-BB-amended liquid cultures. Yeasts from wastewater showed slightly higher decolourization percentages after 36 h of culture than yeasts from Las Yungas (98–100% vs. 91–95%, respectively). However, isolates from Las Yungas exhibited higher specific decolourization rates than isolates from effluents (1.8–3.0 vs. 0.9–1.3 mg $g^{-1}h^{-1}$, respectively). All selected isolates were first grouped according to microsatellite-PCR analysis and representative isolates from each group were subsequently identified based on the 26S rRNA gene sequence analysis. Yeasts from wastewater were identified as the ascomycetous *Pichia kudriavzevii* (100%) and closely related to *Candida sorbophila* (99.8%), whilst yeasts from Las Yungas were identified as the basidiomycetous *Trichosporon akiyoshidainum* and *Trichosporon multisporum*. It is suggested that findings concerning yeast selection during screening programs for dye-decolourizing yeasts may be explained in the light of the copiotroph-oligotroph microorganisms rationale.

Keywords Biodecolourization · Dyes · Yeasts · Ecology · Taxonomy

Abbreviations

WRF	White rot fungi
NDM	Normal decolourization medium
HAU	Halo arbitrary units
CDAU	Colony dying arbitrary units
LiP	Lignin peroxidase
Lacc	Laccase

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MnP	Manganese dependent peroxidase
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
TMPD	*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine
YCB	Yeast carbon base
YNB	Yeast nitrogen base

Introduction

Reactive dyes are an important group of synthetic colourants, considered xenobiotic and highly recalcitrant compounds against biodegradative processes. Effluents of dyeing industries are usually markedly coloured and their disposal into receiving waters causes damage to the environment. Since colour reduces light penetration, dye pollution may significantly affect photosynthetic activity in aquatic life. In addition, due to their own toxicity as well as because of the presence of metals, chlorides, etc., they are harmful to the in situ aquatic life and also to the living organisms drinking from these waters (Stolz 2001).

Current non-biological decolourization methods of effluents include coagulation/flocculation, oxidation or adsorption, electrochemical destruction and photocatalysis. Such methods may successfully accomplish dye removal but they could be very expensive because of the high chemical usage, costly infrastructure and high operating expenses. Moreover, accumulation of concentrated sludge becomes a new disposal problem (Aksu 2003).

Dye decolourization by white rot fungi (WRF) or their ligninolytic enzymes has been widely studied over last years, and several lab-scale bioreactors were proposed for this purpose (Yang et al. 2008). However, the rigorous conditions for enzyme production in dye-containing wastewaters, and the risk of contamination by bacteria under non-sterile conditions, retarded the application of white rot fungi for wastewater treatment. In addition, filamentous fungal growth is usually slow compared with most single-cell microorganisms, and the production of mycelium often makes filamentous fungi poorly adaptable to wastewater treatments (Assas et al. 2000).

On the other hand, yeasts have been successfully applied to treat industrial effluents such as food-, molasses-, and oil-manufacturing wastewaters, as pointed out by Yang et al. (2008). In the past few years, several yeasts were reported to be able to remove reactive dyes. Ascomycetous yeasts were reported to decolourize reactive dyes by using a variety of mechanisms including sorption (Dönmez 2002; Aksu and Dönmez 2003; Raspor and Zupan 2006), reduction of azo bonds by azo-reductases (Ramalho et al. 2005; Lucas et al. 2006; Jadhav et al. 2007) and more recently, through ligninolytic enzymes (Yang et al. 2003, 2005, 2008).

Surprisingly, only a few reports involve basidiomycetous yeasts in dye decolourization, including *Rhodotorulla minuta* (Ertugrul et al. 2009), *Trichosporon multisporum* and *T. akiyoshidainum* (Pajot et al. 2007, 2008). The apparent lack of decolourizing ability in basidiomycetous yeasts results highly surprising, taking into account the widespread distribution of ligninolytic enzymes through filamentous basidiomycetous fungi.

At present, the ecology of dye-degrading yeasts is poorly understood. Fierer et al. (2007) proposed that, by adapting the r- to K-selection continuum concept, we can understand the ecological role of these microorganisms. In general, r-strategists are adapted to maximize their intrinsic growth rate when resources are abundant, whilst K-strategists are adapted to compete and survive when populations are near the carrying capacity and resources become limited. Although r- and K-selection theory is recognized as an over-simplification, the general concept is well understood and provides a useful framework for comparing the ecological features of different taxa (Reznick et al. 2002).

Microbiologists are more likely to use the terms copiotroph and oligotroph to describe those microorganisms with ecological attributes typical of r- and K-strategists, respectively. Copiotrophs preferentially uptake easily assimilable substrates, have high nutritional requirements, and can exhibit high growth rates when resource conditions are abundant. In contrast, oligotrophs exhibit slower growth rates and are likely to out-compete copiotrophs in conditions of low nutrient availability due to their higher substrate affinities (Tate 2000). This classification is not new and similar ideas were promoted since 1920 as pointed out by Fierer et al. (2007).

In this work, results of two different approaches for the isolation of yeasts with decolourizing ability from virgin or dye-polluted environments are

described. Results from the screening procedure were compared in terms of the taxonomical features of the isolated yeasts. We suggest that the rationale of copiotroph and oligotroph microorganisms may explain the findings concerning the taxonomical affiliation of isolated yeasts throughout dye-decolourizing yeast screening programs.

Materials and methods

Sampling and yeast isolation procedures

Samples from 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) were aseptically collected from live and dead sections of "Laurel del monte" (*Phoebe porphyria*) trees and underlying soil. Samples were directly plated on acid Yeast extract-Malt extract agar (YM-agar), yeast extract 3 g l^{-1}, malt extract 3 g l^{-1}, peptone 5 g l^{-1}, glucose 10 g l^{-1}, as previously described (Pajot et al. 2007, 2008).

Polluted-site samples were collected from the wastewater outfall channel of a textile factory in Tucumán, Argentina, and used for subsequent microcosms experiments. To do this, one milliliter of previously collected samples was inoculated into Erlenmeyer flasks containing 100 ml of Normal Decolourization Medium (NDM): glucose 20 g l^{-1}; $(NH_4)_2SO_4$ 2.5 g l^{-1}; yeast extract 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) plus Vilmafix® Blue RR-BB (0.2 g l^{-1}). A surplus of dye (20 mg) was added every 36 h. Microcosms were incubated at 25°C in an orbital shaker at 250 rpm for 72 h. Samples were periodically withdrawn every 36 h and streaked out onto NDM agar plates plus Vilmafix® Blue RR-BB.

In both cases, different yeast colony morphotypes were isolated and maintained on YM-agar slants at 4°C.

Dyestuff

Six textile commercial dyes were used: Procion Red 3B-HE, Procion Blue RD-HE, Vilmafix® Red 7B-HE, Vilmafix® Blue RR-BB, Vilmafix® Green RR-4B and Vilmafix® Yellow 4R-HE. Vilmafix® dyes were kindly provided by Vilmax S.A. (Argentina) and Procion dyes were from ICI Especialidades Químicas (Brazil). Stock solutions (2 g l^{-1}) were filter-sterilized (Millipore filter, 0.22 μm, Millipore Corp., Bedford, USA) and used without further purification.

Effluent analyses

Absorbance scannings, between 200 and 800 nm, were performed to textile effluent and to each tested dye, by using a Beckman DU640 (Beckman Coulter, Brea, USA) spectrophotometer. Optimal wavelengths (λ_{opt}) were determined for each dye.

Dye decolourization was monitored at dye λ_{opt} in culture supernatants after filtering the samples with 0.45 μM Millipore filters.

Physico-chemical analysis of the effluent was performed by SAT laboratories (Sociedad Aguas del Tucumán, Tucumán, Argentina). Aromatic amines in effluent were detected by addition of Ehrlich's reagent according to standard protocols (Oren et al. 1991).

Decolourization screening on solid media

Isolates were confronted on solid NDM against six textile dyes (each at 0.2 g l^{-1}) and also to a mixture of dyes, where each dye equally contributed to the total amount of 0.2 g l^{-1}. This mixture was assayed as a first approximation to the study of decolourization in simulated textile effluents (O'Neill et al. 1999). Solid media contained 15 g l^{-1} agar.

Plates were examined for visual disappearance of colour (clear haloes around the colony) up to 72 h of cultivation. Growth controls were performed using the same culturemedium without dyes. 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) to 2 (highly coloured colony). Isolates displaying higher ΣHAUs and lower ΣCDAUs were selected for further studies.

Decolourization under submerged culture conditions

Decolourization in liquid medium was evaluated in 20-ml tubes containing 7 ml of NDM plus either, Vilmafix® Red 7B-HE or Vilmafix® Blue RR-BB as previously described (Pajot et al. 2007). Test tubes

were incubated in an orbital shaker at 25°C and 250 rpm for 36 h. Systems without yeasts or dyes were also included as abiotic or biotic controls, respectively. Tubes were periodically collected and sacrificed for analyses. Cell-free supernantants were obtained as above described and set aside for pH measurement. Pellets were washed twice with sterile distilled water and dried at 80°C to constant weight for biomass dry weight determination. All values and data points throughout this work are means of at least triplicate determinations of independent assays.

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, culture supernatants were subjected to spectral scanning between 200 and 800 nm in order to analyze dye disappearance along cultivation.

Characterization and identification of dye-decolourizing yeasts

General molecular procedures

Yeast DNA extraction was performed by the glass-beads method as previously described (Pajot et al. 2008). An initial molecular characterization was carried out by amplification of polymorphic regions of genomic DNA using microsatellite primers $(GAC)_5$ and $(GTG)_5$, following the protocol by Rodrigues and Fonseca. (2003). Further characterization included sequencing of 26S rRNA gene D1/D2 domain, internal transcribed spacers (ITS1-5.8S-ITS2) region and 18S rRNA gene, as previously reported (Pajot et al. 2008).

Sequence comparisons were performed using the basic local alignment search tool (BLAST) program within the GenBank database. The Clustal W computer program (Thompson et al. 1994) was used for alignment of multiple sequences. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.0 package (Tamura et al. 2007) by using neighbor-joining analysis (Saitou and Nei 1987). Distances were estimated according to Jukes and Cantor (1969). Gaps were handled as missing data. Bootstrap values were based on 1000 replications; values <90% were not recorded. For phylogenetic trees, only sequences belonging to type strains of closely related species, whose names have been validly published in public databases, were taken into account.

Physiological characterization

Physiological properties were examined in accordance to standard yeast identification protocols (Yarrow 1998). Nitrate and nitrite assimilation tests were assayed both, in the auxanographic and the standard liquid medium assays. Utilization of carbon and nitrogen sources was examined on solid YNB and YCB media (Difco, Detroit, MI, USA) at 25°C. Cultures were incubated up to 21 days, and examined daily. Medium pH was adjusted to 5.5, if required. Exceptionally, for galacturonic acid-, quinic acid- or potassium hemi-saccharate containing media, pH was not adjusted in agreement with the laboratory practice of CBS Delft (Middelhoven 1997).

Enzymatic assays

In the case of Lignin peroxidase (LiP), a Beckman DU640 spectrophotometer was used. Other enzymatic activities were determined by using a Beckman Coulter AD200 multiplate ELISA reader (Beckman Coulter, Brea, USA).

LiP (EC 1.11.1.14) assay followed the oxidation of veratrylic alcohol by the increase in absorbance at 300 nm (Tien and Kirk 1984). Laccase, Lacc (EC 1.10.3.2) and Mn-independent peroxidase assays were based on the oxidation of ABTS (Jarosz-Wilkołazka et al. 2002), measured by the increase in absorbance at 420 nm. Mn-dependent peroxidase, MnP (EC 1.11.1.13) assay consisted in measuring the oxidation of TMPD according to the increase in absorbance at 590 nm, as described by de Vrind-de Jong et al. (1990). All enzymatic assays were performed at room temperature.

Statistical analysis

Data were analyzed using the GraphPad InStat™ V2.04 for Windows, GraphPad Software, San Diego, CA, USA.

Results and discussion

Yeasts isolation from virgin and dye-polluted environments

Sixty-three yeast colony morphotypes could be isolated from "Laurel del monte" (*Phoebe porphyria*) and underlying soil samples from Las Yungas rainforest. Yeast isolates coming from wood-related samples were thought as potential sources of lignin-olytic organisms. On the other hand, one hundred different yeast morphotypes could be isolated from dye-microcosms experiments with textile effluents. Dye-amended microcosms were performed in order to evidence the most adapted yeast specimens to dye presence pressure.

Effluent profile

Physico-chemical analysis of textile effluent is displayed in Table 1. Consumed oxygen (CO), dissolved oxygen (DO), chemical oxygen demand (COD) and biochemical oxygen demand (BOD), and conductivity values exceeded tolerated levels according to the present legislation. Nevertheless, based on the other characterics, the effluent showed a classical profile for a partially treated effluent. These measurements indicated an anoxigenic environment with an important organic matter load (Table 1). The BOD/COD ratio (0.37) would be comparable to standard values for textile effluents, as reported by O'Neill et al. (1999), suggesting the potential biodegradability of this effluent (Chamarro et al. 2001). As pointed out by Pearce et al. (2003), when considering dye removal from textile effluents, one of the most important factors to take into account is the effect of oxygen and organic matter loads on cell growth and dye reduction.

Aerobic extracellular environments, with the presence of the high-redox-potential electron acceptor oxygen, may inhibit anaerobic biological dye reduction. On the contrary, after the reduction of azo bonds under anaerobic conditions, no further degradation of the dye molecule would be observed unless aerobic conditions are provided for its complete mineralization.

With regard to aromatic amines, they were detected in the effluent (20 mg l^{-1}) presumptively due to the presence of dye synthetic intermediaries or partial degradation products of dyes.

Table 1 Physico-chemical analysis of textile industry effluent

Parameter	Units	Effluent	Parameter	Units	Effluent
Total alcalinity	mg l^{-1}	370	Aluminium	mg l^{-1}	0.098
Conductivity	us cm^{-1}	1010	Ammonia	mg l^{-1}	0.49
BOD	mg l^{-1}	630	Arsenic	mg l^{-1}	<0.020
COD	mg l^{-1}	1700	Cadmium	mg l^{-1}	<0.003
Total hardness	mg l^{-1}	1160	Calcium	mg l^{-1}	28
Consumed oxygen	mg l^{-1}	190	Cyanide	mg l^{-1}	<0.020
Dissolved oxygen	mg l^{-1}	0	Chlorides	mg l^{-1}	70
pH		7.2	Copper	mg l^{-1}	<0.2
Turbidity	UNT	58	Chromium	mg l^{-1}	<0.010
Total residues	mg l^{-1}	895	Fluorine	mg l^{-1}	0.204
Fixed solids	mg l^{-1}	605	Phosphates	mg l^{-1}	0.462
Volatile solids	mg l^{-1}	290	Iron	mg l^{-1}	0.38
Suspended solids	mg l^{-1}	200	Magnesium	mg l^{-1}	21.8
Fixed suspended solids	mg l^{-1}	80	Manganese	mg l^{-1}	<0.020
Volatile suspended solids	mg l^{-1}	120	Mercury	mg l^{-1}	<0.001
Settleable solids (2 h)	ml l^{-1}	3	Nitrates	mg l^{-1}	4
Colour		Greenish blue	Nitrites	mg l^{-1}	0.02
			Lead	mg l^{-1}	<0.015
			Sulfate	mg l^{-1}	100
			Sulfides	mg l^{-1}	<1

Agar-plate screening for decolourization

Following the criterion of higher ΣHAUs and lower ΣCDAUs values, sixteen out of the 63 yeast isolates from Las Yungas unpolluted environments, and nine out of 100 isolates from microcosms experiments with textile effluents were selected. 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.

Yeasts from Las Yungas preferentially decolourized formazan-triazine dye Vilmafix® Blue RR-BB and disazo-triazine dye Vilmafix® Red 7B-HE, whilst isolates from polluted environments showed major decolourization of disazo-triazine dye Procion® Red 3B-HE and the non azo, anthraquinonic-triazine dye Procion® Blue RD-HE. Such differences might be attributed to the chemical nature of dyes.

These results confirmed that a single rule should not be adopted in order to predict the degradability of particular dyes by newly isolated yeasts. In addition, the herein selected isolates were able to decolourize several dyes in simulated dye effluent agar plates. This ability would be particularly valuable at industrial level, since the inapplicability to a variety of dyes has been one of the main drawbacks for the implementation of certain biodecolourization treatments (Kaushik and Malik 2009).

In order to test yeast isolates from both virgin and dye-polluted environments under submerged culture conditions, two dye models were chosen: Vilmafix® Blue RR-BB, more susceptible to decolourization, and Vilmafix® Red 7B-HE, a moderately recalcitrant azo dye.

Decolourization under submerged-culture conditions

Results from abiotic controls indicated that both tested dyes remained almost intact (99 ± 1%) after 36 h incubation in NDM. Accordingly, any reduction on the initial dye concentration for inoculated media could be securely assigned to yeast activity.

Selected yeasts from the agar plate screening were evaluated by taking into account their kinetics of dye decolourization and the extent of dye removal in liquid cultures. According to these criteria, five yeasts from Las Yungas (out of the 16 agar-plate selected isolates) named HP-2014, HP-2023, HP-2030, HP-2118 and HP-2184, and other five yeasts from textile-dye polluted environments (out of the 9 agar-plate selected isolates) named HP-3045, HP-3061, HP-3065, HP-3094 and HP-3098, were selected for further studies.

After 36 h of cultivation, dye removal ratios between Vilmafix® Blue RR-BB and Vilmafix® Red 7B-HE under submerged culture conditions were close to the unit for those yeasts isolated from textile effluent, giving evidence of similar decolourization rates for both tested dyes. Similar decolourization rates could be attributed to unspecific decolourization

Table 2 Percentage of decolourization after 36 h of cultivation in NDM medium

Isolate #	Vilmafix® Blue RR-BB	Vilmafix® Red 7B-HE	Ratio[a]
HP-3061	**98.03**	**99.68**	**1.0**
HP-3098	**97.60**	**99.87**	**1.0**
HP-3045	**95.98**	**99.42**	**1.0**
HP-3094	**95.57**	**98.81**	**1.0**
HP-3065	**95.52**	**96.61**	**1.0**
HP-2023	**91.43**	**95.30**	**1.0**
HP-3027	89.18	94.67	0.9
HP-3018	87.93	93.86	0.9
HP-2184	**85.49**	**75.26**	**1.1**
HP-2030	**84.30**	**68.6**	**1.2**
HP-2118	**80.44**	**63.51**	**1.3**
HP-2014	**78.74**	**52.90**	**1.5**
HP-2036	76.96	58.58	1.3
HP-2178	74.98	39.92	1.9
HP-2136	74.24	37.45	2.0
HP-2147	74.03	32.07	2.3
HP-2056	72.44	71.16	1.0
HP-3078	72.25	88.04	0.8
HP-2028	70.75	52.69	1.3
HP-3019	65.77	92.19	0.7
HP-2021	65.13	51.85	1.3
HP-2006	64.70	61.15	1.1
HP-2019	62.46	45.81	1.4
HP-2025	58.16	30.32	1.9
HP-2048	50.55	32.47	1.6

[a] Ratio was estimated as % removal blue dye/% removal red dye. Selected isolates at the end of the screening scheme are indicated in bold

mechanisms, such as biosorption. Meanwhile, isolates from Las Yungas showed ratios between 1.0 and 1.5 denoting some differences in the decolourization ability depending on the dye nature (Table 2), a behavior usually observed in enzyme mediated degradation mechanisms.

When biomass production was taken into account (Fig. 1), isolates from dye-polluted effluent showed to produce, in average, two-fold more biomass (measured as dry weight) than those isolates from Las Yungas. Moreover, comparison with biotic controls showed that yeast growth was not restricted in presence of dyes. On the contrary, isolates HP-3098 and HP-2118 showed slightly increased biomass yields when cultured in presence of dyes ($P < 0.014$). Isolates from Las Yungas HP-2023 and HP 2184 also showed higher biomass yields when cultured with Vilmafix® Blue RR-BB ($P < 0.034$) and isolates from polluted environments showed a similar response when cultured with Vilmafix® Red 7B-HE ($P < 0.018$). Only for the isolate HP-3094, growth appeared to be lower in the presence of Vilmafix® Red 7B-HE, as compared to the dye-deprived control ($P = 0.016$). Similar results were found for several ascomycetous yeasts including *Candida zeylanoides* (Martins et al. 1999; Ramalho et al. 2002), *Geotrichum* sp. (Máximo et al. 2003), *Issatchenkia occidentalis* (Ramalho et al. 2004), *Debaryomyces polymorphus* (Yang et al. 2005) and *Candida oleophila* (Lucas et al. 2006).

Considering dye removal under submerged culture conditions, different decolourization kinetic parameters such as volumetric decolourization rate (η, $mg_{dye}\ l^{-1}\ h^{-1}$) and specific decolourization rate (v, $mg_{dye}\ g^{-1}_{dry\ biomass}\ h^{-1}$), were taken into account for comparison purposes. After 36 h of cultivation yeasts from Las Yungas showed volumetric decolourization rates between 2.9 and 5.4 for Vilmafix® Blue RR-BB, and between 3.7 and 4.1 for Vilmafix® Red 7B-HE. Isolates from microcosm-enriched textile effluent showed similar volumetric decolourization rates with values ranging between 4.2 and 4.8 for the blue dye, and between 4.2 and 4.8 for the red dye. However, when decolourizing ability was correlated to biomass production, i.e. through the specific decolourization rate estimation, isolates from Las Yungas showed better performances. Yeasts from Las Yungas exhibited values between 2.0 and 3.0 for Vilmafix® Blue RR-BB, and between 1.8 and 2.5 for Vilmafix® Red 7B-HE. Isolates from textile dye effluents showed lower specific decolourization rates, ranging between 0.9 and 1.1 for the blue dye, and between 1.0 and 1.3 for the red dye. Similar values were previously reported for well known dye-accumulating

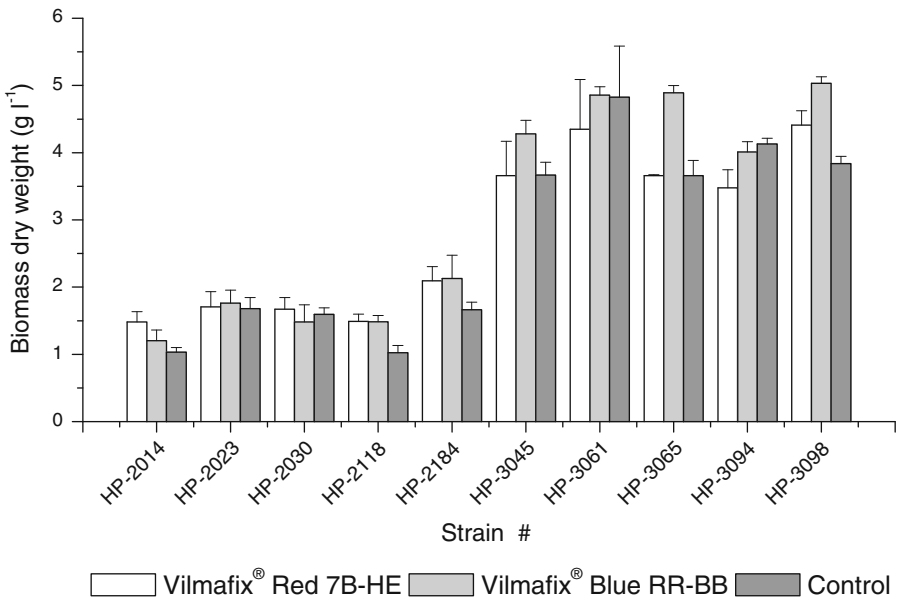

Fig. 1 Biomass yields (expressed as dry weight) of selected decolourizing yeasts after 36 h of cultivation in NDM medium (Control), NDM plus Vilmafix® Blue RR-BB (200 mg l^{-1}) or NDM plus Vilmafix® Red 7B-HE (200 mg l^{-1})

ascomycetous yeasts including *K. marxianus* (Meehan et al. 2000), *C. tropicalis* (Dönmez 2002) and *S. cerevissiae* (Aksu 2003).

Enzymatic activities

Previous detection of Lacc and MnP in yeasts isolated from Las Yungas, when grown on solid cultures, required between 2 and 4 weeks for positivation (Pajot et al. 2007). However, in this work, none of the isolates revealed positive results for the evaluated enzyme activities under the assayed conditions. It is possible that, if present, the reaction conditions were not suitable for the production of these enzymes, the production level was too low, or the enzymes were not released to the supernatant. Different cultivation conditions, for instance in the presence of other dyes (Yang et al. 2003; Yang et al. 2005) or longer incubation times, may enhance enzymatic production up to readily detectable levels.

Characterization and identification of dye-decolourizing yeasts

Results from MS-PCR allow us to group Las Yungas isolates within two groups: Group I) isolates HP-2023, HP-2030 and HP-2184, and Group II) isolates HP-2014 and HP-2118. Similarly, yeasts from textile effluents could be divided into 3 groups: Group A) isolate HP-3045, Group B) isolates HP-3061, HP-3065 and HP-3098, and Group C) isolate HP-3094.

As previously found for isolate HP-2023 (Pajot et al. 2008), based on the 26S D1/D2 rRNA gene domain sequence analysis, HP-2023-related isolates (group I) could be also identified as *Trichosporon akiyoshidainum*. According to the same criterion, yeasts HP-2014 and HP-2118 (group II) were identified as *T. multisporum* (Fig. 2).

Isolate HP-3045 (group A) and HP-3098-related isolates (group B) where identified as *Pichia kudriavzevii*, (previously *Issatchenkia orientalis,* Kurtzman et al. 2008), showing 100% similarity (0 differences in 522 bp) when the D1/D2 domain was analyzed. This species belongs to the *Pichia membranifaciens* clade (Fig. 3). These results were all confirmed by sequencing the ITS1-5.8S-ITS2 region and the 18S rRNA gene, displaying 99.05% and 100% similarities, respectively.

When D1/D2 26S rRNA gene domain sequence of isolate HP-3094 (group C) was analyzed, 98–99% similarity with *Candida* genus members was found, being *C. sorbophila* (99.81% similarity;1 difference in 527 bp) and *C. infanticola* (98.86% similarity; 6 differences over 527 bp) the closest relatives (Fig. 4). It is widely accepted that members of a species show no more than 0 to 2 nucleotide substitutions in the D1/D2 domain (Kurtzman and Robnett 1997). On the other hand, while no differences were found between ITS1-5.8S-ITS2 sequences from HP-3094 and its closest relatives, 18S rRNA gene sequence analysis showed only 5 differences between HP-3094 and *C. sorbophila* (99.69% identity). The latter sequences could not be compared with the ones from *C. infanticola* since they are not available in public databases. Further molecular characterization will be necessary in order to overcome the potential identification of a new *Candida* species closely related to the *C. sorbophila–C. infanticola* clade.

Physiologic characterization of yeasts

All the yeasts isolated from Las Yungas produced positive reaction to urease and DBB tests, but they were not able to ferment glucose (Table 3), typical features of basidiomycetous yeasts. Only minor differences were found between isolates HP-2023, HP-2030 and HP-2184 (group I, *T. akiyoshidainum)* and the related species *T. multisporum* and *T. laibachii*. Group I Las Yungas yeasts were able to assimilate salicin but not mannitol as C-source, whereas isolates HP-2118 and HP 2014 (group II, *T. multisporum*) could assimilate mannitol; but salicin could be only weakly assimilated.

With regard to the isolated yeasts from textile effluents, HP-3045 and HP-3098 (groups A and B) showed assimilation profiles almost identical to the *Pichia kudriavzevii* NRRL-Y5596^{T}, showing only minor differences in D-glucosamine assimilation and their growing ability in 10% NaCl + 5% glucose.

Isolate HP-3094 could be differentiated from *C. sorbophila* by the assimilation of L-sorbose as sole C-source, and could be also differentiated from *C. infanticola* according to the results in L-sorbose, soluble starch, DL-lactate and ethanol assimilation, as well as the ability to grow in 10% NaCl + 5% glucose (Table 3). These discrepancies along with the analysis of D1/D2 domain sequences did not allow us

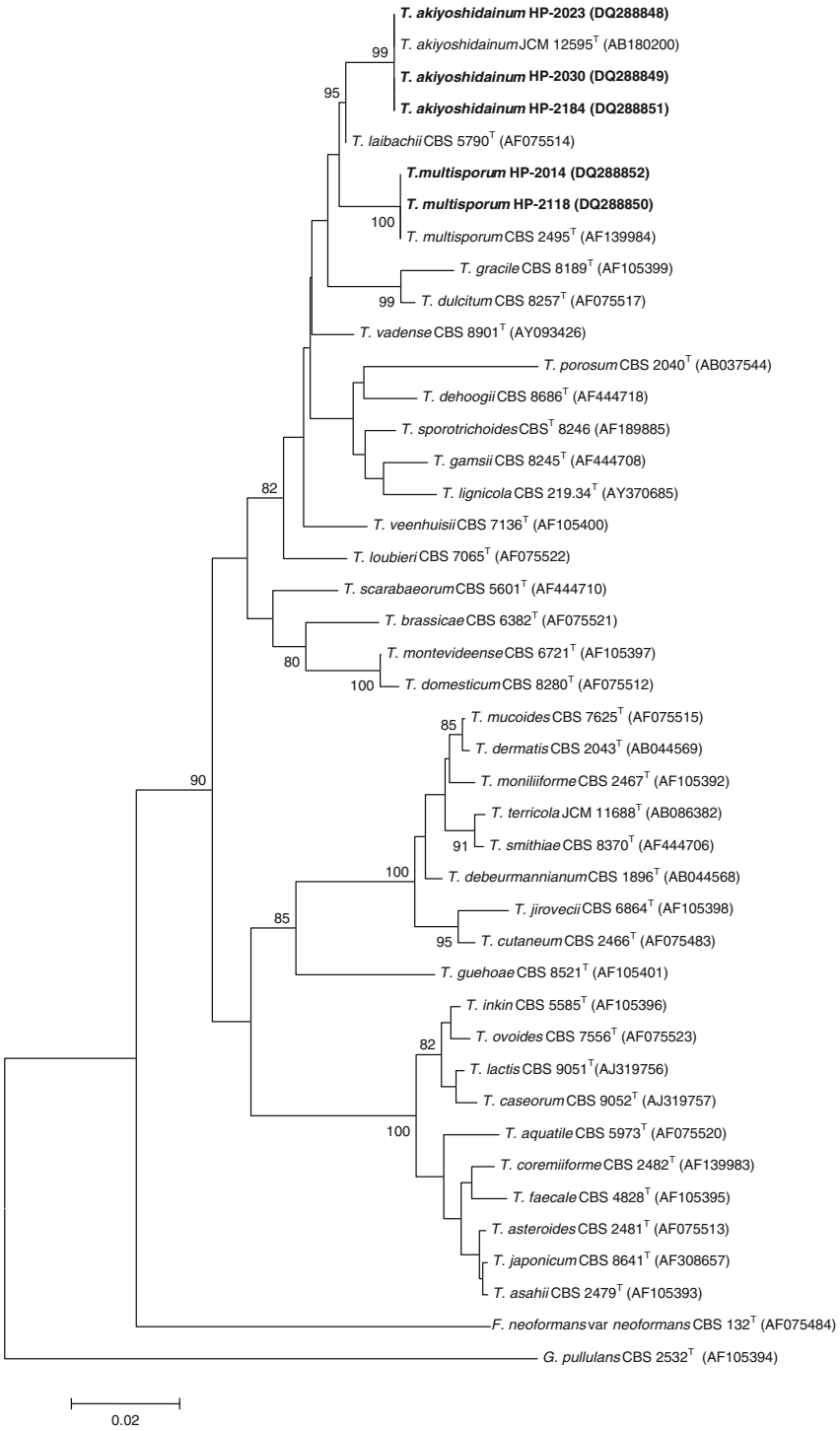

Fig. 2 Phylogenetic tree based on 26S rRNA gene D1/D2 domain sequences for species of the genus *Trichosporon*. The tree was constructed by the neighbor-joining method and the Jukes-Cantor model. Numerals represent the confidence level from 1000 replicate bootstrap samplings (frequencies less than 80% are not indicated). Bar indicates the distance corresponding to two-bases change per hundred nucleotide positions. *Filobasidiella neoformans* var. *neoformans* and *Guehomyces pullulans* were used as outgroup

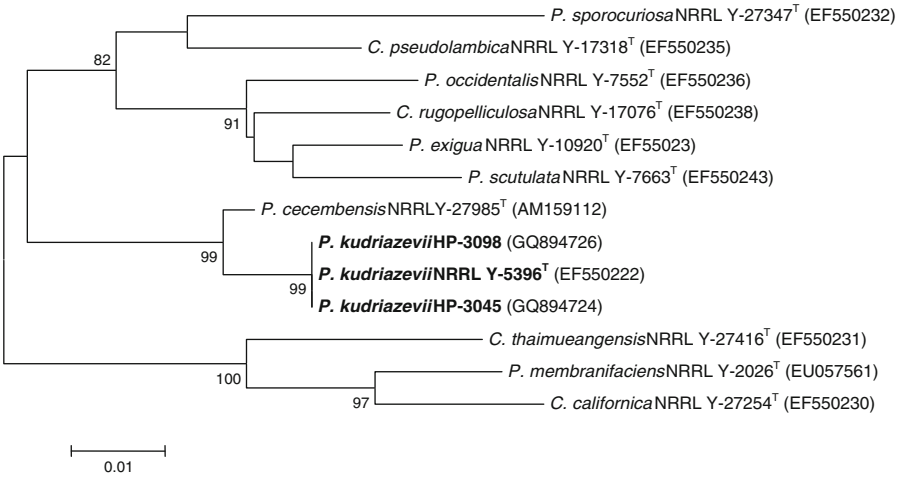

Fig. 3 Unrooted phylogenetic tree based on 26S rRNA gene D1/D2 domain sequences for *P. kudriazevii* HP-3045 and HP-3098 and closely related species. The tree was constructed by the neighbor-joining method and the Jukes-Cantor model. Numerals represent the confidence level from 1000 replicate bootstrap samplings (frequencies less than 80% are not indicated). Bar indicates the distance corresponding to one-base change per hundred nucleotide positions

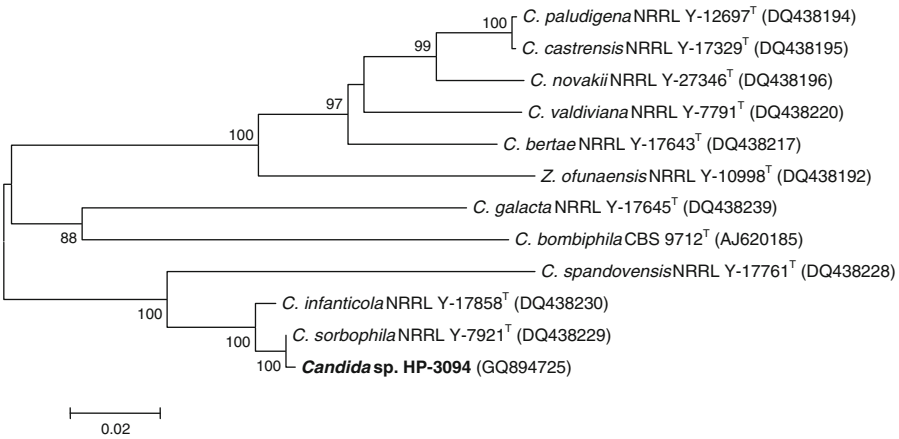

Fig. 4 Unrooted phylogenetic tree based on 26S rRNA gene D1/D2 domain sequences for *Candida* sp. HP-3094 and closely related species. The tree was constructed by the neighbor-joining method and the Jukes-Cantor model. Numerals represent the confidence level from 1000 replicate bootstrap samplings (frequencies less than 80% are not indicated). Bar indicates the distance corresponding to two-bases change per hundred nucleotide positions

to unambiguously identify the isolate HP-3094. In accordance, the denomination *Candida* sp. was kept for this isolate.

There is a generally accepted relationship between the taxonomy and the probable mechanisms of decolourization in filamentous fungi. Whilst White Rot Fungi (WRF) are mainly basidiomycetes and are usually associated with the production of lignin-degrading enzymes (i.e. LiP, MnP, Lacc, etc.), ascomycetous fungi are frequently associated with different decolourizing mechanisms, especially biosorption (Wesemberg et al. 2003; Chander and Arora 2007; Barrasa et al. 2009; Kaushik and Malik 2009).

In yeasts, this relationship is far from clear yet. Ascomycetous yeasts have shown the abovementioned ascomycetous dye-sorption decolourizing mechanism (Aksu 2005; Barrasa et al. 2009) but also, azoreductase (Ramalho et al. 2005; Lucas et al. 2006; Jadhav et al. 2007), and ligninolytic activities, such as MnP (Villas-Bôas et al. 2002; Máximo et al.

Table 3 Physiological properties of selected isolates

	HP-2014 and 2118	HP-2023, 2030, 2184[a]	HP-3045	HP-3094	HP-3061, 3065, 3098
Assimilation of carbon compounds					
D-Glucose	+	+	+	+	+
D-Galactose	+	+	−	+	−
L-Sorbose	+	+	−	−	−
D-Glucosamine	+	−	−	−	−
D-Ribose	+	+	−	−	−
D-Xylose	+	+	−	−	−
L-Arabinose	+	+	−	−	−
D-Arabinose	−	−	−	−	−
L-Rhamnose	w	w	−	−	−
Sucrose	+	+	−	−	−
Maltose	+	+	−	−	−
D-Trehalose	+	+	−	−	−
α-Methyl-D-glucoside	+	+	−	−	−
Cellobiose	+	+	−	−	−
Salicin	−, w	+	−	−	−
Melibiose	+	+	−	−	−
Lactose	+	+	−	−	−
Raffinose	+	+	−	−	−
Melezitose	−,w	−, w	−	−	−
Inulin	−	−	−	−	−
Soluble starch	+	+	−	+	−
Glycerol	+	+	+	+	+
Erythritol	w	w	−	−	−
Ribitol	−	−	−	−	−
Xylitol	−	−	−	−	−
L-Arabinitol	w	w	−	−	−
D-Glucitol	w	w	−	+	−
D-Mannitol	+	−	−	+	−
Galactitol	+	+	−	−	−
myo-Inositol	+	+	−	−	−
D-Gluconate	+	+	−	−	−
Glucuronate	+	+	−	−	−
DL-Lactate	+	+	+	+	+
Succinate	+	+	+	+	+
Citrate	+	+	−	−	w
Methanol	−	−	−	−	−
Ethanol	+	+	+	+	+
Fermentation					
D-Glucose	−	−	+	−	+
D-Galactose	−	−	−	−	−
Maltose	−	−	−	−	−
Sucrose	−	−	−	−	−
Lactose	−	−	−	−	−

Table 3 continued

	HP-2014 and 2118	HP-2023, 2030, 2184[a]	HP-3045	HP-3094	HP-3061, 3065, 3098
Assimilation of nitrogen compounds					
Ammonium sulfate	+	+	+	+	+
Nitrate	−	−	−	−	−
Nitrite	−	−	−	−	−
Miscellaneous					
Vitamin-free	−	−	+	w	+
0.1% Cycloheximide	+	+	w	w	w
0.01% Cycloheximide	+	+	+	+	w
DBB test	+	+	−	−	−
50% glucose	−	−	−	−	−
10% NaCl + 5% glucose	−	−	−	−	−

+: growth within 7 days; −: no growth after 21 days; w: weak growth response

[a] Data from Pajot et al. (2008)

2003; Yang et al. 2005, 2008). The herein isolated ascomycetous yeasts from textile dye effluents (*Candida* sp. and *Pichia kudriavzevii)* showed specific decolourization rates similar to other well known dye-accumulating ascomycetous yeasts. In addition, these yeasts showed in average higher ΣCDAUs and lower ΣHAUs values, this feature being indicative of a higher bioaccumulation potential.

On the other hand, basidiomycetous yeasts have been much less studied concerning decolourization. It has been reported that *Rhodotorulla mucilaginosa* is able to decolourize Remazol Blue through sorption (Ertugrul et al. 2009), whilst azoreductase activity has been recently reported as a decolourizing mechanism in *Trichosporon beigelii* (Saratale et al. 2009). At the same time, the involvement of ligninolytic enzymes has been more recently speculated in basidiomycetous yeasts related to dye decolourization (Pajot et al. 2007).

Taxonomy of selected yeasts and the possible implicated mechanisms of decolourization may be frequently biased according to the screening program adopted. That may explain why schemes including enrichment cultures with easily assimilable C-sources usually result in the selection of copiotrophic (fast growing) yeasts (Fierer et al. 2007). In agreement with this assumption, it was not surprising the selection of ascomycetous yeasts after microcosms enrichment, since they normally exhibit higher growth rates when consecutively exposed to readily assimilable and/or relatively abundant C-sources.

Martins et al. (1999) have reported a *Candida zeylanoides* strain isolated from dye-polluted soils near a textile finishing plant by using dye-amended YEPD plates. Also, Yang et al. (2003) have isolated *Debaryomyces polymorphus* and *Candida tropicalis* (both ascomycetous yeasts with MnP activity) by a combination of enrichment cultures and microcosms assays (including 5 g l^{-1} glucose and 1 gl^{-1} $(NH_4)_2SO_4$), selecting those yeasts because of their high Reactive Black 5 decolourization rates. *Candida oleophila*, another ascomycetous yeast isolated from olive mill wastewaters in a plant in Portugal was selected by enrichment culture with phenolic caffeic, protocatechuic, *p*-coumaric, *p*-hydroxybenzoic (1 g l^{-1}), and syringic (0.1 g l^{-1}) acids as C-sources, showing a marked ability for Reactive Black 5 degradation (Lucas et al. 2006).

Meanwhile, more classical yeast isolation procedures not including enrichment strategies (Yarrow 1998; de Figueroa et al. 2004) usually allow the isolation of both copio- and oligotrophic yeasts. On this basis, the selection of basidiomycetous yeasts (usually oligotrophic) such as the *Trichosporon* isolates herein described after preliminary growth on solid acid YM and subsequent exposure to dye-amended NDM, represents an unsurprising finding after this screening procedure. Therefore, this selection scheme appears to be useful for isolating decolourizing yeasts that may be missed if the microcosm strategy were applied at the beginning of the screening with an additional C-source.

Oligotrophic yeasts can usually thrive in environments with relatively low nutrient concentrations, but generally at low growth rate, low rates of metabolism, and low population density. For that reason, when competing with copiotrophic organisms, oligotrophic population can result eliminated.

Particularly for environmental samples, preliminary growth under not selective conditions, with a moderate concentration of the C-source, and on media where growth can be controlled (solid media), seems to be suitable for promoting the development of yeasts with different growth profiles. In a second stage, a more diverse population would be available for the selection based on decolourizing properties. Supporting this idea, MacGillivray and Shiaris (1993) were able to isolate (in acid YM medium) and select several yeasts belonging to ascomycetous (*Candida* and *Torulopsis*) and basidiomycetous (*Cryptococcus, Rhodotorulla and Trichosporon*) genera, according to their ability for degrading polycyclic aromatic hydrocarbons.

Based on a different strategy, oligotrophic yeasts with decolourizing potential may be expected to grow after cultivation with dyes as the sole C-source. However, these conditions could be extremely recalcitrant and the number of selected isolates may be significantly reduced. In accordance, to our knowledge, there is no report of yeasts being isolated by using this kind of isolation scheme.

In conclusion, the present taxonomical findings, far from reflecting their ecological precedence, would be strongly influenced by the screening methodology adopted. As previously emphasized, oligotrophic microorganisms are usually related to the ability of degrading a broad spectrum of substrates, whilst copiotrophic microorganisms are related to the efficient degradation of easily accessible substrates (Fierer et al. 2007). Accordingly, as further demonstrated by the present research, it may be suggested to take into account these premises when planning isolation and selection schemes pursuing to maximise the selection of yeasts with higher degradation ability.

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