International Biodeterioration & Biodegradation 65 (2011) 1199–1207

Contents lists available at SciVerse ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Evidence on manganese peroxidase and tyrosinase expression during decolorization of textile industry dyes by *Trichosporon akiyoshidainum*

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ARTICLE INFO

Article history:
Received 18 March 2011
Received in revised form
19 May 2011
Accepted 28 May 2011
Available online 14 October 2011

Keywords:
Textile dyes
Decolourization
Mechanisms
Manganese
Peroxidase
Tyrosinase
Trichosporon

ABSTRACT

Textile dyes are engineered to be resistant to environmental conditions. During recent years the treatment of textile dye effluents has been the focus of significant research because of the potentially low cost of the process. Mechanisms of biological textile dye decolorization depend greatly on the chemical structure of the dye and the microorganisms used. While basidiomycetous filamentous fungi are well recognized for dye decolorization through ligninolytic enzymes, reports on textile dye decolorization mechanisms of basidiomycetous yeasts have been scarce. Decolorization of several textile dyes by *Trichosporon akiyoshidainum* occurs during the first 12 h of cultivation. This fast decolorization process could not be solely related to siderophore production or dye sorption to biomass; it was shown to be a co-metabolic process. *T. akiyoshidainum* could use glucose, sucrose, and maltose as alternative carbon sources, and urea as an alternative nitrogen source with similar decolorization rates. The activity of two enzymes, manganese peroxidase and tyrosinase, were induced by the presence of dyes in the culture media, pointing to their potential role during the decolorization process. Manganese peroxidase titers reached 666 U l^{-1} to 10538 U l^{-1}, while tyrosinase titers ranged between 84 U l^{-1} and 786 U l^{-1}, depending on the dye tested. The present work provides a useful background to propose new eco-friendly alternatives for wastewater treatment in textile dying industries.

1. Introduction

Textile dyes are engineered to be resistant to fading on exposure to light, water, and chemicals. They are generally considered to be xenobiotic compounds and rather recalcitrant against biodegradative processes in conventional sewage treatment systems. In recent years the biological treatment of textile dye effluents has been the focus of significant research because of the potentially low cost and innocuousness of the process.

Mechanisms of biological textile dye decolorization greatly depend on the dye chemical structure and the microorganisms used. It has been demonstrated that white-rot fungi and bacteria are able to transform or even mineralize azo dyes under certain environmental conditions (Blumel et al., 2002).

Reductive cleavage of the azo bond is proposed as the initial reaction during the bacterial metabolism of azo dyes, but this reaction usually leads to the formation of potentially harmful aromatic amines. On the other hand, fungi are able to decolorize dyes through unspecific oxidative mechanisms generally associated to ligninolytic enzymes, thus avoiding the amine generation problem. However, the rigorous conditions for enzyme production in dye-containing wastewaters, as well as the risk of contamination by bacteria under non-sterile conditions, complicate the application of white-rot fungi in wastewater treatment (Dias et al., 2010).

In this context, yeasts have been successfully employed in textile dye decolorization through biosorption mechanisms. Their success is largely based on their unicellular nature, along with high growth rates (Aksu, 2003; Aksu and Dönmez, 2005). Moreover, yeasts can be easily cultivated into inexpensive growth media, and yeast biomass has potential for waste bioremediation at low pH values. Nevertheless, sorption is not usually seen as an effective attenuation mechanism, since it does not necessarily result in the destruction of contaminants or their transformation into innocuous end products.

Reports on enzymatic decolorization by yeasts have been, however, scarce (Yang et al., 2005, 2008) and information concerning the relative participation of diverse mechanisms (i.e., sorption, reduction, oxidation, etc.) is still fragmentary. The studies

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doi:10.1016/j.ibiod.2011.05.010

about copper formazan complex dye decolorization are even scarcer. The aim of this research is to explore the relative contribution of several plausible mechanisms to the decolorization of a copper formazan dye (Vilmafix® Blue RR-BB) by *Trichosporon akiyoshidainum*, a decolorizing yeast previously isolated from Las Yungas rainforest (Pajot et al., 2007, 2008).

2. Materials and methods

2.1. Dyestuff

Unless otherwise stated, the formazan, copper complex dye Vilmafix® Blue RR-BB, was employed. When studying the enzymatic decolorization of textile dyes, Vilmafix® Red 7B-HE (C.I. Name: Reactive Red 141) and Vilmafix® Black B-V (C.I. Name: Reactive Black 5), both azo dyes, were also included. They were all kindly provided by Vilmax S.A. (Fig. 1).

Stock solutions were prepared by dissolving powdered dyestuff, without prior purification, in distilled water up to a concentration of 2 g l^{-1}, and then filter-sterilizing (Millipore filter, 0.22 μm, Millipore Corp., Bedford, MA, USA).

2.2. Yeast culture and maintenance conditions

T. akiyoshidainum was previously selected based on its decolorization potential (Pajot et al., 2007); it is currently maintained in the American Type Culture Collection as ATCC MYA-4129 and in the Centraalbureau voor Schimmelcultures as CBS 10550. For routine work at lab scale, the selected yeast was maintained on normal decolorization medium (NDM) (Ramalho et al., 2002) slants incubated at 28 °C and then stored at 4 °C and subcultured at regular intervals.

2.3. General procedure

For decolorization studies, dye stock solutions were added to each culture medium up to 200 mg l^{-1} (ppm) final concentration. This was the concentration systematically used in subsequent experiments, unless otherwise stated. All media were solidified with 15 g l^{-1} of agar.

Dye decolorization kinetics was evaluated in 500-ml Erlenmeyer flasks containing 100 ml of medium. Ten milliliters of a yeast suspension ($OD_{550} = 0.8$) prepared from a fresh NDM broth culture

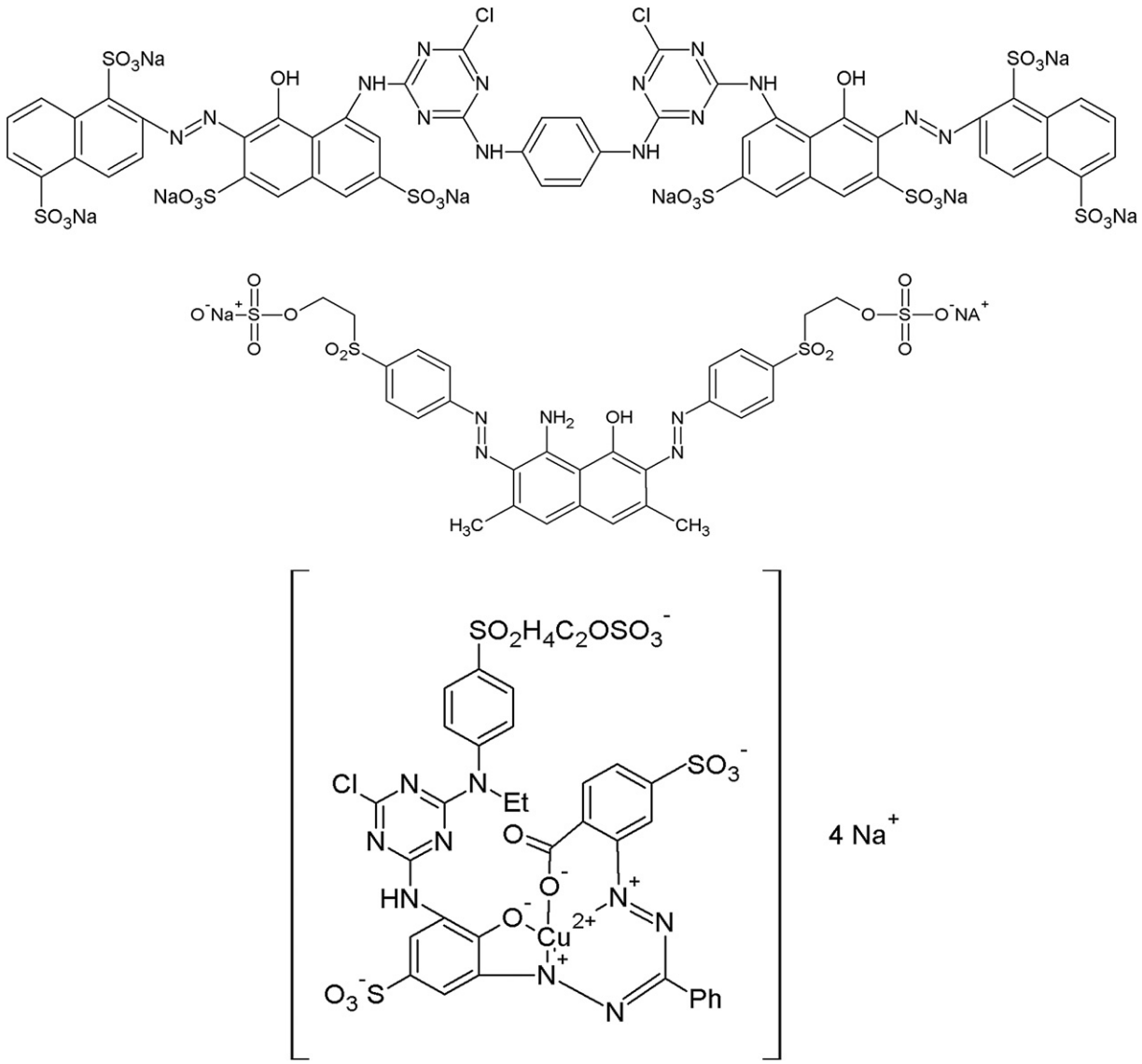

Fig. 1. Structures of (A) Vilmafix® Blue RR-BB; (B) Vilmafix® Red 7B-HE; and (C) Vilmafix® Black B-V.

were used to inoculate each flask. Pre-filtered textile dye stock solutions (Vilmafix® Red 7B-HE, Vilmafix® Black B-V, or Vilmafix® Blue RR-BB) were added in order to reach the desired concentration. Incubations were carried out at 250 rpm and 26 °C. Biotic and abiotic controls were performed using the same medium without dyes or yeasts, respectively.

Samples were periodically collected and centrifuged for 10 min at 4000 *g*. Pellets were washed twice with sterile distilled water and dried at 80 °C to constant weight for biomass dry weight determination. Supernatants were kept for analytical determinations.

2.4. Analytical procedures

When required, supernatants were tested for residual glucose (Miller, 1959), ammonia (Koroleff, 1983), and pH. Total aromatic amines were measured according to Oren et al. (1991). In this work, sulfanilic acid equivalents were chosen instead of benzidine equivalents (Isik and Sponza, 2007), because of the eventual release of sulfo-substituted amines from tested dyes. Results represent the average of at least three independent assays.

Dye decolorization was monitored with a Beckman DU640 spectrophotometer at each dye λ_{opt} by using culture supernatants obtained as described in the foregoing. Color removal was reported as mg l^{-1} removed, or as percentage decolorization $= (A_0 - A_t)/A_0{*}100$, 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.

2.5. Influence of alternative carbon/energy and nitrogen sources

The potential influence of alternative nitrogen and carbon/energy sources on Vilmafix® Blue RR-BB decolorization was assayed in modified NDM liquid cultures. Carbon/energy and nitrogen alternative sources were selected based on the physiological characterization of *T. akiyoshidainum* (Pajot et al., 2008). Glucose (20 g l^{-1} in standard medium) was replaced with one of the following as an alternative carbon source: sucrose, maltose, soluble starch, molasses, cellulose (20 g l^{-1} each), or corn steep liquor (CSL) (100 ml l^{-1}). Ammonium sulfate (2.5 g l^{-1} in standard medium) was replaced by urea (1.25 and 2.5 g l^{-1}) since the yeast is not able to assimilate nitrates or nitrites.

2.6. Initial dye concentration effect

The effect of increasing initial dye concentration (300, 500, and 700 mg l^{-1}) was determined in standard NDM liquid medium plus Vilmafix® Blue RR-BB.

2.7. Dye sorption onto T. akiyoshidainum biomass

Sorption of Vilmafix® Blue RR-BB to live or heat-killed *T. akiyoshidainum* biomass was assayed in Erlenmeyer flasks (250-ml capacity) containing 50 ml dye solution (275 mg l^{-1}) in distilled water. After addition of live or heat-killed wet biomass (final concentration 4 g l^{-1}), flasks were shaken at 25 °C and 150 rpm up to 12 h. Samples (5 ml) were taken at 10 min and 12 h and centrifuged 10 min at 4000 *g*. Supernatants were employed to analyze dye concentration. Initial dye solution pH ranged between 1.0 and 7.0, and was adjusted with either HCl or NaOH. Abiotic controls were included in order to measure the effect of pH on dye spectrum.

The following biosorption parameters were quantified: C_{eq} (residual dye concentration at equilibrium, mg l^{-1}); C_0 (initial dye concentration, mg l^{-1}); q (adsorbed dye quantity per gram of biomass at a given time, mg g^{-1}); q_{eq} (adsorbed dye quantity per gram of biomass at equilibrium, mg g^{-1}); and X (biomass concentration, g l^{-1}).

2.8. Siderophore production

The possible involvement of siderophores in decolorization via chelator-mediated Fenton reactions (CMFRs) was initially tested in standard liquid NDM cultures plus 200 mg l^{-1} Vilmafix® Blue RR-BB. Chelator-mediated Fenton reactions have been suggested as a valid alternative to overcome several limitations of classical Fenton reactions, meaning the limited or incomplete mineralization of organic compounds (depending on the amount of employed reagents and on the nature of the organic pollutant) and the narrow effective pH range (2.0–3.0) (Arantes et al., 2006; Arantes and Ferreira Milagres, 2007).

Two modified NDM media were later used as controls for siderophore production, according to the recommendation of Schwyn and Neilands (1987). In the modified media, yeast extract was replaced by thiamine (400 μg l^{-1}) and two phosphate concentrations were tested: 5 g l^{-1} ($NDM_{mod\ 5}$) and 0.3 g l^{-1}($NDM_{mod\ 0.3}$), in order to avoid phosphate interference during siderophores determination (Schwyn and Neilands, 1987). Decolorization was evaluated with Vilmafix® Blue RR-BB λ_{opt}. Biomass production was evaluated after 16 h cultivation.

Normal decolorization medium and NDM_{mod} media, with and without dye, were inoculated with yeasts obtained from a fresh NDM broth culture and incubated at 25 °C. Cultures (16-h-old), were centrifuged for 10 min at 4000 *g* and screened for siderophore production by the $FeCl_3$ test (Baakza et al., 2004), chrome azurol sulphonate (CAS) assay, and CAS agar plate test (Schwyn and Neilands, 1987). The blank reference consisted of uninoculated NDM and NDM_{mod} media. Culture broths and blanks were read at 630 nm and the percentage of siderophore units was calculated as recommended by Schwyn and Neilands (1987).

2.9. Detection of chemical nature of siderophores

Supernatants were assayed for the presence of hydroxamates and catecholates by the tetrazolium test, the Arnow's test, and the $FeCl_3$ test as described by Neilands (1981). Carboxylates were measured by the spectrophotometric test of Shenker et al. (1992). Oxalate determination was carried out by using a standard oxalate kit (Sigma Chemical Co., St. Louis, MO) with the modifications recommended by Machuca et al. (2001). Sample pH was carefully adjusted to 1.8–2.4 to ensure maximum oxalate recovery, as recommended by Parkinson et al. (1987).

2.10. Obtaining yeast cell French press lysates

Cells were harvested by centrifugation at 5000 *g* for 10 min at 4 °C, washed in distilled water, and suspended in the corresponding buffer (20% v v^{-1}). Cell suspensions were broken at 20,000 psi ($1{,}38 \times 10^5$ KN m^{-2}) and then centrifuged at 30,000 *g* for 15 min at 4 °C. Then, sample fractions corresponding to whole cells, supernatants, cell-free extract, and cell wall (Polti et al., 2010) were assayed for enzymatic activity.

2.11. Enzymatic assays

Enzymatic assays were performed at 25 °C and colorimetrically determined in a Beckman Coulter AD200 ELISA reader.

Dye peroxidase (DyP) was measured as described by Kim and Shoda (1999) using Vilmafix® Blue RR-BB (60 mg l^{-1}) in 25 mM citrate buffer, pH 3.0, and 0.15 mM H_2O_2. Dye decolorase (DyD) activity was measured in a similar way, but without H_2O_2, in order

to avoid peroxidase activities. Violuric acid, ABTS, MBTH, and TMPD (5 mM) were added to the reaction mixtures in order to study the effect of redox mediators on dye decolorization.

Laccase, Lacc (EC 1.10.3.2) was measured by using ABTS (Heinzkill et al., 1998) or syringaldazine (Palmieri et al., 1997). A general peroxidase assay was performed with 1.2 mM ABTS and 0.15 mM H_2O_2 in 30 mM phosphate buffer, pH 7.2. Lacc activity (measured in the same conditions) was subtracted in order to obtain H_2O_2-dependent activity (Heinzkill et al., 1998).

Phenol oxidase (Phox) activity was assayed with 10 mM guaiacol, in 50 mM citrate–phosphate buffer, pH 6.0 (Palmieri et al., 1997).

Tyrosinase (Tyr, EC 1.10.3.1) was measured with 0.01% w v^{-1} pyrocatechol in 50 mM phosphate buffer, pH 7.4 (Bora et al., 2004).

Manganese peroxidase (MnP, EC 1.11.1.13) was measured according to three protocols:

- Toh et al. (2003), using TMPD (0.025 mM), 0.2 mM H_2O_2, and $MnSO_4.7H_2O$ (0.1 or 1.0 mM) in 50 mM phosphate–citrate buffer, pH 5.0
- Minussi et al. (2001) including 0.01% w v^{-1} Phenol Red, $MnSO_4.7H_2O$ (0.1 or 1.0 mM), and 0.2 mM H_2O_2 in 50 mM sodium malonate buffer, pH 4.5
- Castillo et al. (1994), in a reaction mixture containing 0.07 mM MBTH, 1 mM DMAB, 0.3 mM $MnSO_4.7H_2O$, and 0.05 mM H_2O_2 in 100 mM succinate–lactate buffer, pH 4.5.

NADH-azo-reductase was measured according to the method described by Ramalho et al. (2005). Cultures were grown for 16 h in NDM medium plus either Vilmafix® Blue RR-BB or Vilmafix® Red 7B-HE, harvested by centrifugation, washed with sterile distilled water, and resuspended in an equal volume of a reaction mixture including NADH (3.5 mM), dye (60 mg/l) in either 50 mM acetate buffer, pH 4.0 (Ramalho et al., 2005) or 50 mM phosphate buffer, pH 7.2 (Blumel et al., 2002). The reaction was followed both at 340 nm (NADH oxidation) or at each dye λ_{opt}.

2.12. Production of catechol oxidase and MnP in different textile dyes

Tyrosinase and MnP production kinetics were evaluated in standard NDM media plus either Vilmafix® Blue RR-BB, Vilmafix® Black B-V, or Vilmafix® Red 7B-HE. Biomass, total ammonia, pH, total aromatic amines (TAA), and decolorization were measured as described in section 2.4. The Tyr and MnP activities were measured as stated in section 2.11.

2.13. Statistical analysis

Data were analyzed using the GraphPad InStat Biostatistics package, version 3.0.

3. Results

3.1. Decolorization in liquid cultures

Complete decolorization of Vilmafix® Blue RR-BB was achieved at 11 h of cultivation, showing faster decolorization in the late exponential growth phase (Fig. 2, control). Simultaneously with color disappearance, absorbance at 610 nm (dye λ_{opt}) decreased as a function of time. Results from abiotic controls indicated that dye remained almost intact (99 ± 1%) throughout incubation. Biotic controls showed that yeast growth was not restricted in the presence of the dye. Accordingly, any reduction of initial dye concentration could be securely assigned to yeast activity (Pajot et al., 2008).

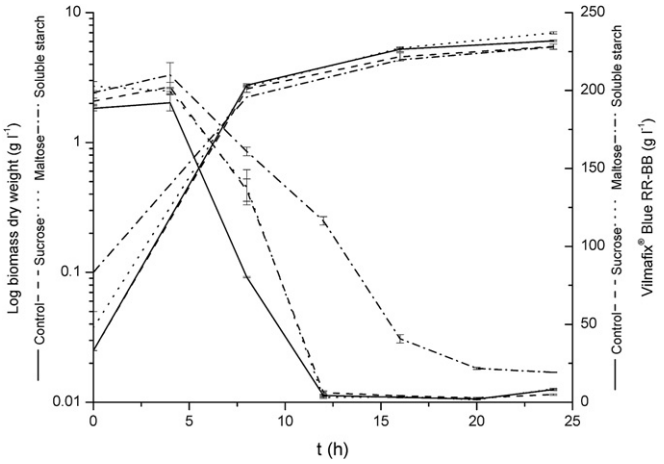

Fig. 2. Growth and Vilmafix® Blue RR-BB decolorization by *T. akiyoshidainum* with different carbon/energy sources. Control: Glucose-amended medium.

3.2. Alternative carbon/energy and nitrogen sources

Decolorization in media with soluble starch, maltose, and sucrose was similar to that reached in glucose-amended medium. However, maltose and sucrose showed slightly higher decolorization rates and may be considered the most interesting options (Fig. 2).

Urea (2.5 g l^{-1}) could be used as an alternative nitrogen source, producing biomass values (6.05 g l^{-1}) and decolorizing percentages (93.7 ± 0.9%) close to those obtained with $(NH_4)_2SO_4$ as the sole N-source. Additionally, the final pH in urea-amended media was neutral (7.0 ± 0.3) (Fig. 3).

3.3. Initial dye concentration effect

Yeast biomass production was not affected by initial dye concentrations up to 700 mg l^{-1} ($p = 0.802$). Similarly, no effects on decolorization percentages were observed for 300 mg l^{-1} (approx. 100% in 11-h-old cultures). However, higher initial dye concentrations (500–700 mg l^{-1}) caused partial reduction of the decolorization process, producing 90 and 80% decolorization, respectively. Additionally, it was observed that biomass remained undyed in media with initial dye concentrations of 500 mg l^{-1} or lower (Fig. 4).

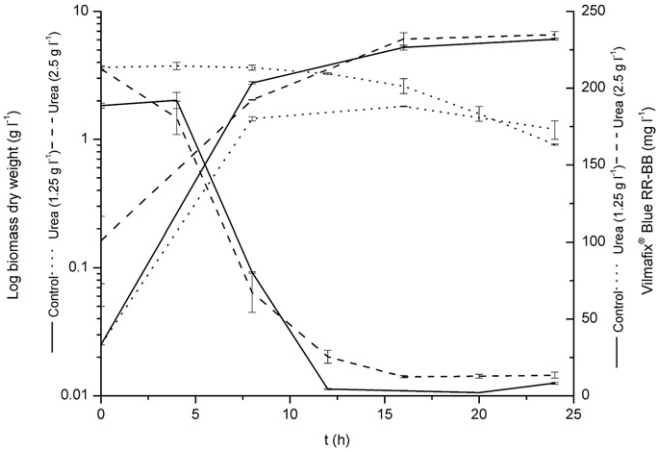

Fig. 3. Growth and Vilmafix® Blue RR-BB decolorization by *T. akiyoshidainum* with urea as N-source. Control: $(NH4)_2SO_4$-amended medium.

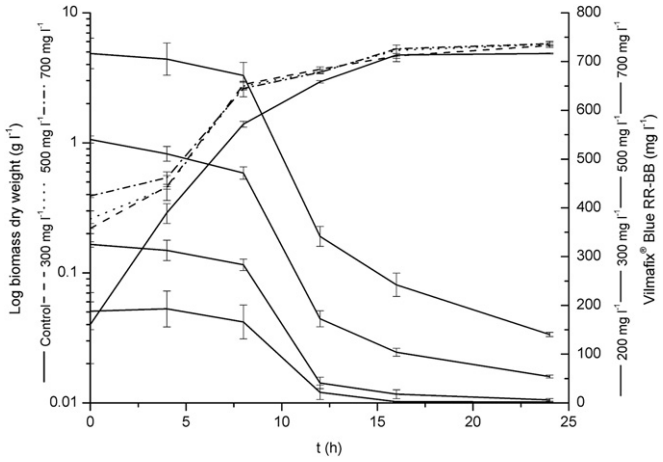

Fig. 4. Growth and decolorization profiles of *T. akiyoshidainum* in standard NDM media supplemented with 300, 500, and 700 mg l^{-1} initial Vilmafix® Blue RR-BB concentrations. Control: 200 mg l^{-1} initial Vilmafix® Blue RR-BB concentration.

3.4. Effect of initial pH, biomass viability, and incubation time on dye sorption

The dye spectrum at pH 2.0 showed an absorbance decrease at 610 nm, along with a shift in λ_{opt} from 610 to 570–575. Also, at pH 1.0, the appearance of an additional absorbance peak at 470 nm was observed. At the other pH values tested, dye spectra remained unchanged (Fig. 5).

Microscopy of heat-killed yeasts showed no apparent disruption of yeast structure and, as expected, no growth was observed in media inoculated with these treated yeasts. Dye sorption was maximal at pH 2.0 and it sharply declined with a further increase in pH, reaching minimum values at pH 7.0. The q_{eq} ($mg_{(dye)}$ $g^{-1}_{(biomass)}$) ranged between 57–64 and 9–18 mg g^{-1} depending on the assayed pH (low or high values) showing similar sorption abilities for live and heat-killed biomass (Fig. 6).

As expected, increasing incubation time from 10 min to 12 h did not produce significant changes on dye sorption to heat-killed biomass. Live biomass also adsorbed dyes quickly at low pHs. However, after 12 h incubation at pH 2.0 to 3.0, a significant increase in dye concentration could be detected in solution (Fig. 6).

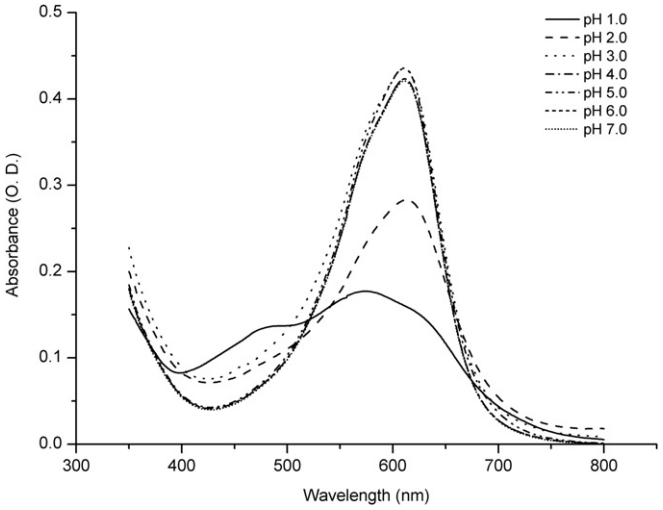

Fig. 5. Effect of pH on Vilmafix® Blue RR-BB absorbance spectra.

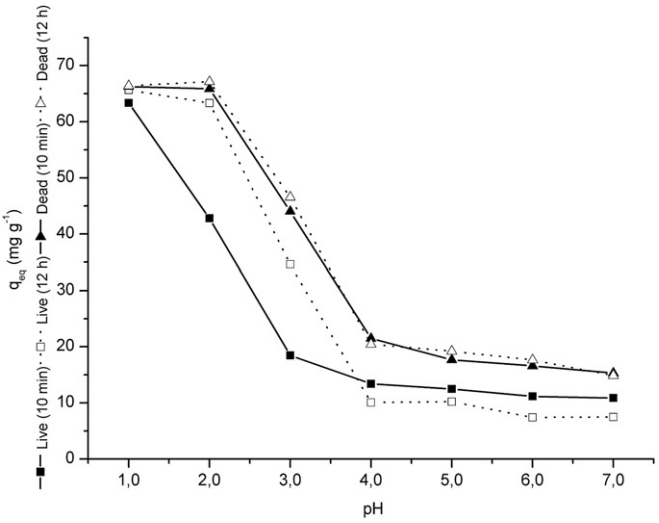

Fig. 6. pH-associated Vilmafix® Blue RR-BB sorption profiles by wet *T. akiyoshidainum* biomass (live or dead) after 10 min and 12 h of incubation in distilled water.

3.5. Siderophore production

Yeast incubation on solid $NDM_{(mod\ 0.3)}$ over 2 weeks produced yellow halos around *T. akiyoshidainum* colonies, indicating the production of siderophores. However, regardless of the assayed culture media or the detection assay employed, no siderophore production could be detected in 16-h-old liquid cultures of *T. akiyoshidainum*. Final biomass yields were affected by media composition (6.4 ± 0.4 g l^{-1} in standard NDM, 5.2 ± 0.2 g l^{-1} in $NDM_{(mod\ 5)}$ and 2.6 ± 0.3 g l^{-1} in $NDM_{(mod\ 0.3)}$). However, no significant difference could be observed in dye decolorization kinetics (Fig. 7) and no hydroxamates, catecholates, or carboxylates were detected in liquid media.

3.6. Enzymatic activities

Despite the variety of methods, media (i.e., NDM, NDM + alternative C/N sources, $NDM_{(mod\ 5)}$, $NDM_{(mod\ 0.3)}$), and conditions (i.e., media with or without dyes, redox mediators, etc.) employed, no DyD, DyP, Phox, Lacc (neither with ABTS nor with syringaldazine), or LiP activities could be found, neither in liquid supernatants

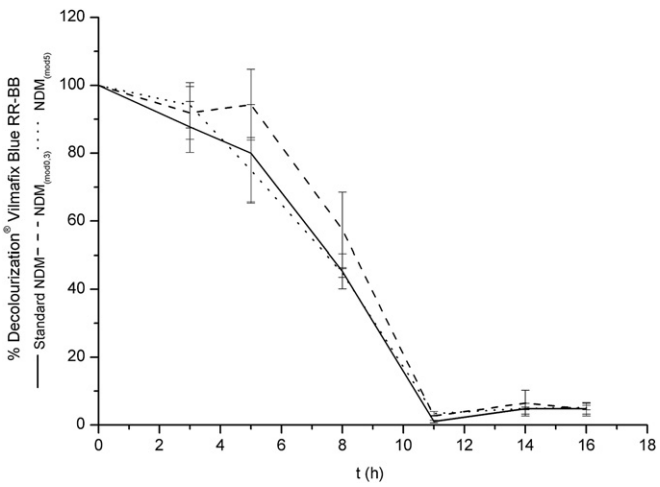

Fig. 7. Vilmafix Blue RR-BB decolorization profiles on standard NDM medium, $NDM_{mod\ 5}$ and $NDM_{mod\ 0.3}$.

nor in yeast lysates. Similarly, negative results were also obtained for MnP when assayed with phenol red or TMPD.

Direct reduction of dyes was observed when the azo-reductase assay described by Ramalho et al. (2004) was employed at pH 4.0. The effect was confirmed by both the absorbance abatement at dye λ_{opt} (dye reduction) and the absorbance increase at 340 nm (NADH oxidation). However, non-enzymatic controls (NADH + buffer + dye) showed even greater decolorization with time and, accordingly, azo-reductase activity was reported as negative.

Tyrosinase and MnP (as measured with DMAB/MBTH) activities were found only in the extracellular fraction (supernatant) of the blue, red, or black dye-amended media tested. No significant activities were detected in the intracellular fraction (lysate) or in the membrane-associated fraction of lysed yeasts. It should be noted that no significant activities of these enzymes could be detected in either supernatants or intracellular or membrane-associated fractions from dye-deprived control cultures.

In the presence of all tested dyes, MnP activity titers reached a maximum at 12 h cultivation. Maximum titers (10538 U l^{-1}) of MnP were observed in Vilmafix® Black B-V (Reactive Black 5). Titers with other dyes were significantly lower (1051 U l^{-1} in Vilmafix® Blue RR-BB and 666 U l^{-1} in Vilmafix® Red 7B-HE).

On the other hand, Tyr activity was greater on Vilmafix® Blue RR-BB (786 U l^{-1}), followed by activities on Vilmafix® Black B-V and Vilmafix® Red 7B-HE (135 and 84 U l^{-1}, respectively). Tyrosinase titers reached maximal values after 12 h in media with Vilmafix® Blue RR-BB or Vilmafix® Red 7B-HE and after 16 h in Vilmafix® Black B-V (Fig. 8B,D, and F).

The profiles of decolorization, biomass production, glucose consumption, and acidification were similar in all tested conditions. Total aromatic amine disappearance was seen to occur concomitantly with dye decolorization (Fig. 8A–F). In red- and blue-amended media, total degradation of TAA was reached after 12 and 16 h, respectively. On the other hand, a greater initial concentration of TAA was detected in black dye-amended medium (approx.700 mg l^{-1}), this probably explained why that after 16 h of cultivation, about 72% degradation could be achieved. Insignificant differences were found in ammonia consumption, while glucose depletion (and consequently biomass production) were higher in Vilmafix® Black B-V-amended medium (Fig. 8A–F).

4. Discussion

Decolorization through ligninolytic enzyme production has frequently been reported as associated with secondary metabolism in filamentous fungi. By contrast, yeasts employed in decolorization studies, i.e., *Candida zeylanoides* (Ramalho et al., 2002), *Pichia occidentalis* (Ramalho et al., 2004), *Debaryomyces polymorphus* and *Candida tropicalis* (Yang et al., 2005), *Candida oleophyla* (Lucas et al., 2006), *Trichosporon beigelii* NCIM-3326 (Saratale et al., 2009), and *T. akiyoshidainum* (this work), have usually shown maximal decolorization rates during the exponential growth phase. It has been suggested that this relationship could imply either a dependence on the production of NAD(P)H (Ramalho et al., 2002, 2004) or a true co-metabolic pathway as in *D. polymorphus* (Yang et al., 2005) and *Candida oleophila* (Lucas et al., 2006). We propose that dye decolorization by *T. akiyoshidainum* is also a classical co-metabolic process since the yeast cannot assimilate Vilmafix® Blue RR-BB (Pajot et al., 2008).

It has been proven that media composition greatly affects decolorization processes, but few studies concerning the effect of different carbon or nitrogen sources on dye decolorization can be found. Several factors, such as the existence of oxidative and reductive dye degradation mechanisms, the possible influence of media carbon/nitrogen (C/N) ratio, the exact yield of reducing power depending on the carbon/energy source used, the microorganism under study, and the cultivation conditions (i.e., aerobic vs. anaerobic), make it impossible to state any clear relationship between carbon or nitrogen sources and the dye decolorizing mechanism. In the case of *T. akiyoshidainum*, different inexpensive carbon sources could be used for dye decolorization, making it an interesting option for industrial applications.

Additional nitrogen sources are mandatory for co-metabolic processes. *T. akiyoshidainum* growth and dye decolorization were greatly restricted in media without yeast extract. However, both could be easily restored by the addition of thiamine in concentrations above 400 mg l^{-1} (data not shown). On the other hand, ammonia is an easily assimilable nitrogen source for *Trichosporon* genus members. So, the favorable effect of complex nitrogen sources (i.e., yeast extract, peptone) could be attributed to the presence of vitamins such as thiamine or other growth promoters, rather than to the assimilable nitrogen content. As an additional nitrogen source, urea is exceptionally interesting. It is an inexpensive feedstock, one that is not only able to keep the maximum decolorization and growth rates reached with $(NH_4)_2SO_4$, but also allows for the prevention of acidification of the culture broth, reducing the sorption of dyes to the biomass.

The initial dye concentration's effects on growth and dye decolorization have been widely reported. It could be speculated that shorter decolorization times (up to 1 day) could be required for oxidative enzymatically mediated mechanisms, showing little or no inhibition with relatively high initial dye concentrations (400–1000 mg l^{-1}) (Yang et al., 2005). On the other hand, growth and decolorization by bioaccumulative or reductive processes were shown to be greatly inhibited at high initial dye concentrations and usually require longer incubation times (2–3 weeks) (Dönmez, 2002; Aksu, 2003; Saratale et al., 2009). The decolorization of Vilmafix® Blue RR-BB by *T. akiyoshidainum* showed a very similar profile to the one reported for Reactive Black 5 decolorization by *D. polymorphus* (Yang et al., 2005), also suggesting an enzymatically mediated, oxidative mechanism.

The q_{eq} (sorption) values reached in this work are in good accordance with those reported for other yeasts (Aksu and Dönmez, 2005). *T. akiyoshidainum* dry biomass represents approximately 25% of the wet biomass. In *T. akiyoshidainum* cultures, decolorization occurs between 8 and 11 h of cultivation (corresponding to 1.5–3 g l^{-1} dry weight biomass; 6–12 g l^{-1} wet biomass). Taking into account the time needed to reach q_{eq}, the gradual biomass production, and the pH of cultures between 8 and 11 h of cultivation (ca. 5.5–3.8), it is hard to attribute complete decolorization to a simple sorption mechanism. Further confirmation comes from cultures in which ammonia was replaced by 2.5 g l^{-1} urea. In those cultures, complete decolorization occurs normally, but pH remains neutral. It must be noted that under such conditions, q_{eq} could only reach 20 mg g^{-1}. Finally, biomass was poorly stained in every tested condition so that the sorption mechanism could be discarded as the main decolorization strategy, even when a fast early and transient sorption of dye to the biomass could not be ruled out.

Heat-pretreatment of yeast biomass also influenced dye biosorption. Live and heat-killed biomass showed differences in the kinetics of dye sorption, especially between pH 2.0 and 4.0. When considering dead biomass, no significant differences where noted by extending exposition time. Live biomass, meanwhile, showed a significant increase in dye sorption with the increase of incubation time. At pH values over 4.0, a slight diminution of q_{eq} occurs with time, suggesting that dye sorption to *T. akiyoshidainum* biomass occurs predominantly by surface binding and that available sites on the biosorbent are the limiting factor for the biosorption. Similar conclusions were reported by Aksu and Dönmez (2003) for *C. tropicalis*.

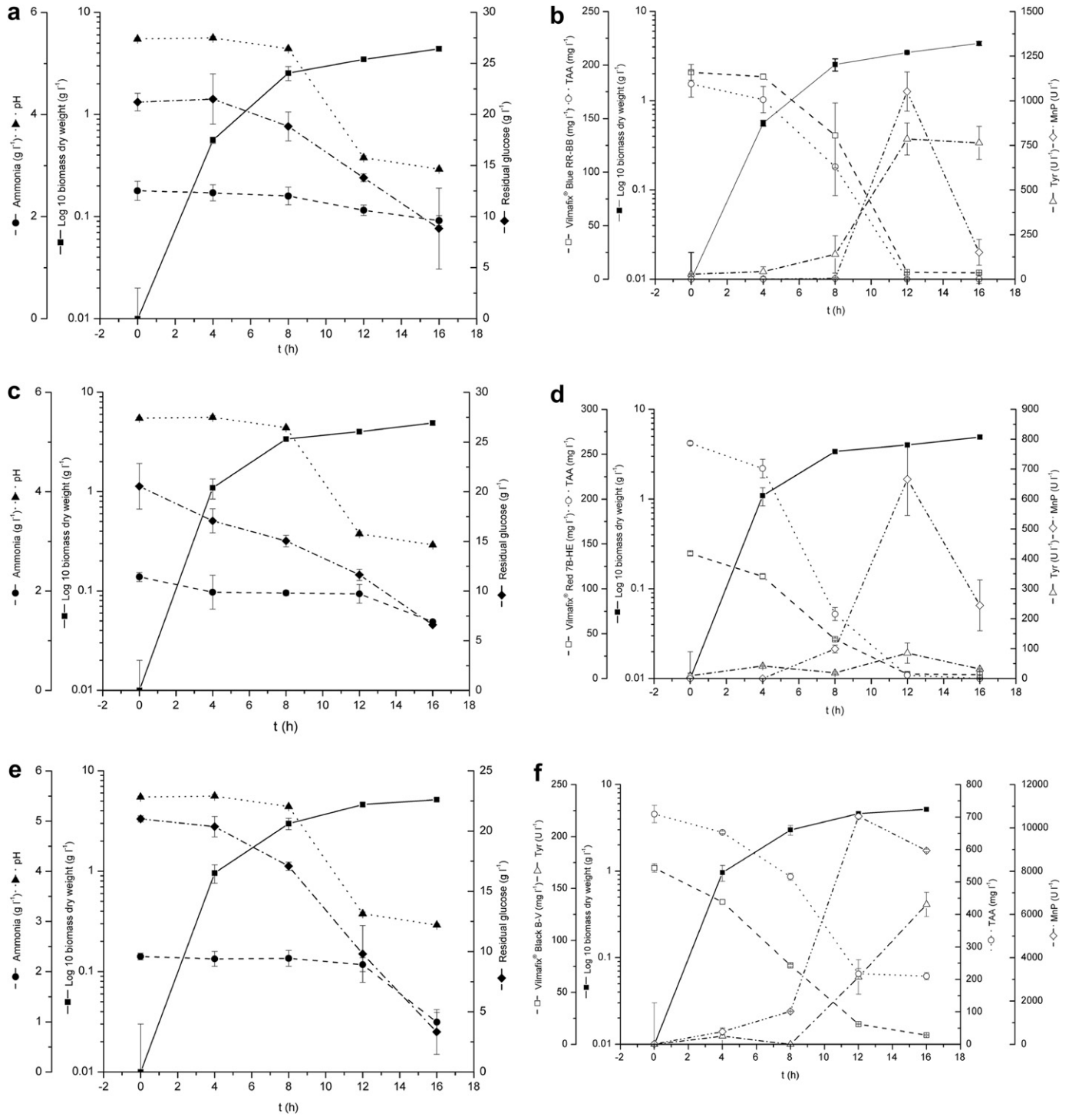

Fig. 8. Growth, decolorization, residual glucose, residual ammonia, pH, total aromatic amines (TAA), Tyr activity, and MnP activity in: (A–B) Vilmafix® Blue RR-BB (200 mg l^{-1}); (C–D) Vilmafix® Red 7B-HE (200 mg l^{-1}); and (E–F) Vilmafix® Black V-B (200 mg l^{-1}).

A possible role of siderophores from *Trametes versicolor*, *Phanerochaete chrysosporium*, and *Lentinus edodes* in decolorization processes has been proposed by Minussi et al. (2001). The authors were able to detect siderophore production in decolorized plates and proposed the participation of hydroxamates or catecholate–iron complexes via phenol oxidase-like activity or chelator-mediated Fenton reactions (CMFRs). In this work, no siderophores or oxalic acid could be detected in 16-h-old cultures of *T. akiyoshidainum*. It should be noted, however, that the acidification of the media could effectively help the occurrence of classical Fenton reactions, provided a significant amount of iron is supplied. Nevertheless, no significant amounts of iron were included in the media studied herein, so it is hard to attribute a predominant role for CMFR in the decolorization of Vilmafix® Blue RR-BB, even when a small contribution of standard Fenton reactions or other Fenton-type oxygen radical-generating systems (i.e., including Cu) could not be totally ruled out, specially at acidic pHs.

Enzymatic decolorization of textile dyes by ascomycetous yeasts has been associated with reduction of azo bonds, either via

NADH-dependent reductases (Jadhav et al., 2007) or ferric reductases with azo-reductase activities (Ramalho et al., 2005). Recently, the same enzymes have been also detected in the basidiomycetous yeast *T. beigelii* (Saratale et al., 2009). In the present work, NADH-dependent dye reduction was observed but it was attributed to a non-enzymatic reduction, as previously reported by Nam and Renganathan (2000). The existence of a NADH-dependent azo-reductase in *T. akiyoshidainum* could not be ruled out, but in any case, its influence in the decolorization process seems negligible.

There are only a few reports concerning dye decolorization by yeasts through ligninolytic enzymes. Yang et al. (2008) reported the production of MnP by *D. polymorphus* and *C. tropicalis*. Jadhav et al. (2007) ascribed the decolorization of methyl red by *S. cerevissiae* mainly to an azo-reductase, but they also reported a slight induction of other enzymes, including tyrosinase, lignin peroxidase, and NADH-DCIP reductase.

While in filamentous fungi decolorization of textile dyes through ligninolytic mechanisms is a feature largely related to basidiomycetes, in yeasts this relationship is far from settled. In this context, *T. akiyoshidainum* showed a classical basidiomycetous behavior, producing Tyr and MnP concomitantly across the decolorization process. Similarly, tyrosinases from bacteria (*Pseudomonas desmolyticum,* Kalme et al., 2007), fungi (*Aspergillus ochraceus,* Parshetti et al., 2007), yeasts (*S. cerevissiae*, Jadhav et al., 2007), and plants (*Solanum tuberosum*, Khan and Husain, 2007) appear during decolorization processes along with other ligninolytic enzymes.

However, the order and the extent of ligninolytic enzyme induction are regulated by the chemical nature of the assayed dye. The high MnP titers reached in Vilmafix® Black B-V are not a surprise. To date, to the best of our knowledge, there are only two reports on MnP-associated dye decolorization by yeasts. *Geotrichum* sp. showed higher MnP titers in Reactive Black 5- than in Reactive Red 158- and Reactive Yellow 27-amended media, but the MnP enzyme has proved to be constitutive, reaching maximum titers after 5 d of cultivation (Máximo et al., 2003). On the other hand, MnP of *D. polymorphus* and *C. tropicalis* proved to be inducible, reaching titers 15–17 times higher in media with Reactive Black 5 than in control media without dyes (Yang et al., 2003, 2005, 2008). Differences in the induction level could be ascribed to chemical differences between dyes. In this work, the most notable difference between dyes is the presence of chloro s-triazine rings in both Vilmafix® Blue RR-BB and Vilmafix® Red 7B-HE. However the effect of chloro s-triazine or its derivatives on *T. akiyoshidainum* enzyme production has not been yet proved.

5. Conclusions

It has been proved that there is no single mechanism to explain textile dye decolorization by *T. akiyoshidainum*. The decolorization efficiency of *T. akiyoshidainum* depended on a sufficient supply of carbon/energy and nitrogen sources, implying a co-metabolic pathway of degradation.

A fast first step of dye sorption to the biomass could not be ruled out. However, under standard decolorization assay conditions, no significant sorption of dyes to the biomass could be evidenced as the main decolorization strategy.

No siderophore production could be detected during dye decolorization, and no significant amounts of iron were incorporated to culture media. However, non-classical Fenton reactions could not be counted out, especially at acidic pHs.

The presence of dyes was a necessary condition for MnP and Tyr production, suggesting that MnP and Tyr may play a role in dye decolorization. However, since complete decolorization of several dyes could be reached at variable concentrations of both enzymes, their participation in the decolorization process might not be unique.

Textile dye decolorization by *T. akiyoshidainum* has proved to be a fast and reliable bioremediation process under tested conditions. The present work provides a useful background to propose new eco-friendly alternatives for the treatment of textile industry wastewater.

Acknowledgments

This work was supported by the Agencia Nacional de Promoción Científica y Tecnológica-FONCYT (PICT2005-38164), Consejo Nacional de Investigaciones Científicsa y Técnicas, CONICET (PIP 6202), and Consejo de Investigaciones de la Universidad Nacional de Tucumán, CIUNT (D-311).

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