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Biodegradation of the diazo dye Reactive Black 5 by a wild isolate of *Candida oleophila*

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Abstract

This work looks for a better understanding of the biodegradation of xenobiotic azo dyes mediated by yeasts. During a screening program of phenolic acid assimilating capacities it was found that a non-conventional ascomycetous yeast isolate, identified as *Candida oleophila*, efficiently decolorizes agar plates supplemented with the commercial textile diazo dye Reactive Black 5. Aerobic batch cultures of *C. oleophila* could completely decolorize up to 200 mg dye l^{-1}, an ability not yet reported for this yeast species. Moreover, this performance has been achieved in just 24 h of incubation at 26 °C in the presence of as little as 5 g glucose l^{-1} and without visible signs of dye adsorption to yeast cells. It was found that decolorization occurs during the exponential growth phase and neither laccase nor manganese-dependent peroxidase activities were detected in the culture medium. As far as the decolorization mechanism is concerned, our results indirectly suggest the involvement of an azoreductase-like activity in azo bonds cleavage.

Keywords: Azo dyes; Bioremediation; Decolorization; Wastewater; Yeasts

1. Introduction

Azo dyes are synthetic organic compounds widely used in textile dyeing, paper printing and other industrial processes such as the manufacture of pharmaceutical drugs, toys and foods including candies. This chemical class of dyes, which is characterized by the presence of at least one azo bond (–N=N–) bearing aromatic rings, dominates the worldwide market of dyestuffs with a share of about 70% [1].

Reactive azo dyes released from textile dyeing plants are highly recalcitrant to conventional wastewater treatment processes. In fact, as much as 90% of reactive dyes could remain unaffected after activated sludge treatment [2]. Therefore, alternative methods should be implemented for effective pollution abatement of dyed effluents. Besides its public acceptance, bioremediation also can be technically attractive since available physical–chemical wastewater treatment processes (e.g. adsorption, filtration, coagulation–flocculation) present some operational problems and high-costs [3,4]. Since 1990, several reports have clearly demonstrated the effectiveness of decolorization and dye removal mediated by white-rot basidiomycetous strains belonging to the genera *Phanerochaete*, *Bjerkandera*, *Phlebia*, *Pleurotus*, *Pycnoporus* and *Trametes* among others [5–9]. Fungal treatment of dyed effluents removes several chromophoric groups and thus decreases its toxicity and aesthetic impact in the receiving water bodies. However, much less work was devoted to the decolorization ability of yeasts [10], despite the obvious fact that they are also fungi. From the technological point of view, the development and maintenance of continuous stirred tank reactors operating with unicellular microorganisms, such as yeasts, is a relatively easy task. In fact, filamentous fungi are poorly adapted to a continuous wastewater treatment unit because an exuberant mycelium growth generally occurs [11,12]. Previous studies on yeast-mediated color removal (compiled by Fu and Viraraghavan [13]) revealed that azo dye adsorption to biomass was the major decolorization mechanism. More recently and according to the best of our knowledge, it was observed that few ascomycetous yeast species such as *Candida zeylanoides* [14,15], *C. tropicalis*, *Debaryomyces polymorphus* [16] and *Issatchenkia occidentalis* [17] perform a putative enzymatic biodegradation and concomitant decolorization of azo dyes.

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During a screening program for yeast isolates displaying high phenolic acid removal ability, it was observed that some of them also decolorized the diazo dye Reactive Black 5. Using molecular methods, the most promising isolate was unequivocally identified as a strain of *Candida oleophila* Montrocher. Interestingly, this species is the biological agent of the product "Aspire" currently used for the post-harvest biocontrol of fruit decay (mostly citrines and pomes) caused by *Penicillium* sp. and other molds [18]. In this work and for the first time, we report a new ability exhibited by growing cultures of a *C. oleophila* wild strain. Additionally, its performance during batch biodegradation of the industrially important diazo dye Reactive Black 5 as well as the decolorization mechanism is discussed.

2. Materials and methods

2.1. Reagents, dyestuff and microbiological media

Protocatechuic acid and *p*-hydroxybenzoic acid were obtained from Fluka and Merck, respectively. Caffeic acid, *p*-coumaric acid, syringic acid and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) were purchased from Sigma–Aldrich. The textile diazo dyestuff Reactive Black 5 (C.I. 20,505) was kindly provided by DyStar Anilinas Texteis Lda (Portugal) and used without prior purification. Yeast malt agar (YM agar) and yeast nitrogen base (YNB) was obtained from Difco. Other chemicals and medium components were at least analytical grade reagents.

2.2. Screening and yeast isolates maintenance

Several yeast strains were isolated from the wash water of olives (collected at CAOM, olive oil extraction plant located in Murça, Northern Portugal), by using the spread-plate method carried out on YM agar. Purified yeast isolates were first screened for their ability to grow in the presence of phenolic acids as sole carbon source, using the YNB medium broth supplemented with one of the following substrates: caffeic, protocatechuic, *p*-coumaric, *p*-hydroxybenzoic (1000 mg l^{-1}), and syringic (100 mg l^{-1}) acids. Phenolic acid assimilating yeasts were then inoculated into YM agar plates supplemented with the diazo dyestuff Reactive Black 5 (100 mg l^{-1}) and incubated at 25 °C. Positive isolates were maintained on YM agar slants at 4 °C and periodically subcultured. The yeast isolate (M33), which exhibited the best performance both on the ability of phenolic acid assimilation and dye decolorization was further identified.

2.3. Yeast identification

Total DNA from isolate M33 was extracted using an existing protocol [19] and its modifications [20] after culture growth on MYP agar (malt extract 0.7%, w/v; yeast extract 0.05%, w/v; soytone 0.25%, w/v; and agar 1.5%, w/v). DNA was amplified using both universal fungal rDNA primers ITS5 (5′-GGA AGT AAA AGT CGT AAC AAG G-3′) and LR6 (5′-CGC CAG TTC TGC TTA CC-3′). Cycle sequencing of the D1/D2 domain (a fragment of 600–650 base pairs at the 5′-end of the 26S rDNA) employed forward primer NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and reverse primer NL4 (5′- GGT CCG TGT TTC AAG ACG G-3′). Sequences were obtained with an Amersham Pharmacia ALF express II automated sequencer by Biopremier, Portugal. The obtained sequences were compared with those deposited at the GenBank database (National Center for Biotechnology Information, NCBI) and identified using the Basic Local Alignment Search Tool (BLAST) also available in NCBI.

2.4. Yeast growth and decolorization of Reactive Black 5

Batch cultures for growth and biodegradation experiments with the isolate M33 were conducted in minimal medium which contained the following (per litre): 5.0 g glucose, 1.0 g $(NH_4)_2SO_4$, 1.0 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.1 g yeast extract, 0.1 g $CaCl_2{\cdot}2H_2O$ and different dyestuff amounts (0, 50, 100, 200, 300, 500 mg l^{-1} final concentrations). The minimal medium and dyestuff solutions were autoclaved separately at 121 °C for 15 min. One ml of yeast suspension prepared from a recent grown culture in the same medium without dye and aseptically adjusted to an $Abs_{640} = 1.0$ was used to inoculate 250-ml Erlenmeyer flasks containing 100 ml of medium. Incubations were carried out on an orbital shaker set at 120 rpm and 26 °C. Abiotic controls (without yeast inoculation) were always included. Samples were periodically collected for determination of yeast growth, glucose depletion, enzymatic activities and color removal.

2.5. Other analytical procedures

Yeast biomass dry weight was determined by the gravimetric method after drying at 100 °C a three-fold washed yeast suspension until constant weight. Also, a calibration curve was established between biomass dry weight and culture turbidimetry evaluated by absorbance readings at 640 nm. Glucose depletion was determined by the dinitrosalicylic acid reagent [21] for reducing sugars. Culture samples were centrifuged (7500 × *g*, 10 min) and desalted by ultrafiltration before using it for enzymatic assays. The ligninolytic extracellular enzyme activities laccase (EC 1.10.3.2) and manganese-dependent peroxidase (EC 1.11.1.13) were determined as previously reported [22] using 0.1–0.4 ml of culture samples and the respective buffered substrate in 1.5 ml total reaction volume at 25 °C. Briefly, laccase was measured following the oxidation of 2 mM ABTS at 420 nm. Manganese-dependent peroxidase was measured following the formation of Mn(III)-tartrate complex at 238 nm. Color removal was determined in centrifuged (7500 × *g*, 10 min) culture samples appropriately diluted with 100 mM phosphate–citrate buffer pH 7.6. Both absorbance readings at visible maximum peak (595 nm) and scanning the UV–vis spectrum between 200–800 nm were performed using a Jasco V-530 double-beam spectrophotometer.

All values and data points presented are the means of at least three independent assays unless otherwise stated. The observed standard deviation of experimental data was always less than 7% of the reported value.

3. Results and discussion

3.1. Properties and identification of a dye-decolorizing yeast strain

When cultivated on YM agar isolate M33 developed cream colored colonies. It showed the capacity of assimilating as sole carbon and energy source some phenolcarboxylic acids such as, caffeic, protocatechuic, *p*-coumaric, *p*-hydroxybenzoic and syringic. Also, this yeast strain promoted full decolorization zones around colonies grown on YM agar plates containing 100 mg of the diazo dye Reactive Black 5 l^{-1} (Fig. 1) without visible color absorption to the biomass.

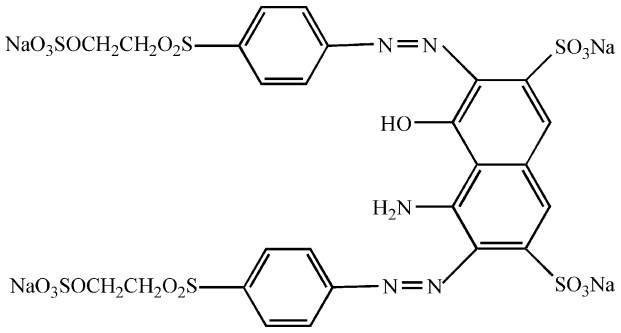

Fig. 1. Chemical structure of the diazo dye Reactive Black 5.

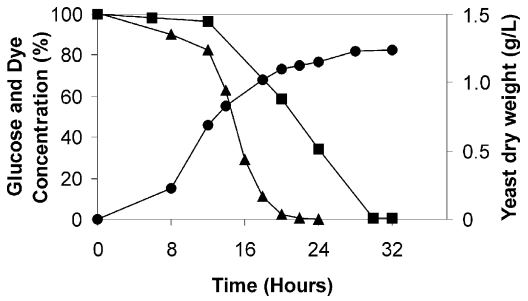

Fig. 2. Time course evolution of yeast growth (●), glucose depletion (■) and Reactive Black 5 color removal (▲) determined in batch cultures of *Candida oleophila* at an initial dyestuff concentration of 100 mg/l.

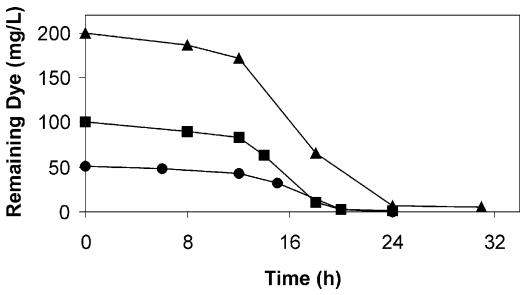

Fig. 3. Time course decolorization of Reactive Black 5 at 50 mg/l (●), 100 mg/l (■) and 200 mg/l (▲) initial concentrations in batch cultures of *C. oleophila*.

The sequence analysis of 26S rDNA D1/D2 domain showed a 100% homology to the type-strains sequences available on GenBank database. Therefore, ascomycetous strain M33 was identified to the species level as *C. oleophila* Montrocher.

3.2. Decolorization kinetics of Reactive Black 5 under batch conditions

In Fig. 2 a typical time course evolution of yeast biomass growth, glucose depletion and decolorization observed in dye-containing batch cultures of *C. oleophila* is shown. During the trophophase yeast cells depleted both glucose and color at a roughly constant rate and the beginning of exponential yeast growth coincides with the onset of decolorization. Moreover, an apparent close association between glucose depletion and color removal over time was evident. Thus, in this study, we found that growing *C. oleophila* yeast cells decolorize the dye Reactive Black 5 during primary metabolism as previously observed for other ascomycetous yeast species (*C. tropicalis* and *D. polymorphus*) [16]. Also, identical behaviour has been observed during the decolorization of different azo dyes by growing cells of the yeast species *C. zeylanoides* [14] and *I. occidentalis* [17]. From the practical point of view, and taking into account that dye decolorization is associated to primary metabolism, yeast should be kept in its exponential growth phase so that the bioremediation process occurs much more quickly. On the contrary, filamentous fungi especially of white-rot type typically remove azo dyes during secondary metabolism [4,8,22,23], which implies long incubations periods (usually several days).

Extensive decolorization (95–100%) was observed in batch cultures containing 50, 100 and 200 mg of Reactive Black 5 l^{-1} (Fig. 3). It is worth noting that *C. oleophila* achieves this performance within 20–24 h in the presence of low glucose concentration (0.5%, w/v) and without visible signs of dyestuff adsorption to yeast cells. Since preliminary assays revealed that yeast cells do not grow without glucose, culture medium supplementation with an easily metabolizable carbon and energy source is required for the decolorization process. However, preliminary assays also revealed that a phenolic effluent (olive oil mill wastewater) can be used instead of glucose. Probably, assimilation of a carbon source and its subsequent metabolism through glycolysis, tricarboxylic acids cycle and pentose phosphate pathway supplies yeast cells with reducing power (NADH and/or $FADH_2$) required for the reduction of dyestuff azo bonds [17,24]. As far as the decolorization is concerned, growing cultures of *C. oleophila* seems to exhibit an optimum ratio between the initial dyestuff concentration and biomass (dry weight) accumulated after 24 h of incubation (Fig. 4). At an initial dye concentration of 200 mg l^{-1} full decolorization occurs which corresponds to yields of 180 mg decolorized dye g^{-1} biomass (dry weight) and 40 mg decolorized dye g^{-1} glucose. These yields are substantially higher than previously reported for Reactive Black 5 decolorization by another yeast growing cultures [16]. Since higher initial dye concentrations (300 and 500 mg l^{-1}) exhibited low decolorization yields but did not significantly affected yeast biomass growth (Fig. 4), an apparent substrate (dye) inhibition occurs. Furthermore, under these conditions yeast cells have a bluish appearance, a phenomenon not observed with batch cultures containing initial dye concentrations up to 200 mg l^{-1}.

3.3. Decolorization mechanism

Several spectral scans have been performed during batch decolorization of 50, 100 and 200 mg dyestuff l^{-1} by growing cultures of *C. oleophila* (Fig. 5). Initially, Reactive Black 5 presents two main absorption peaks, one in visible region (595 nm) and another in UV region (310 nm), which can be ascribed to the presence of chromophoric azo bonds and both aryl and naphthalene-like moieties, respectively [25]. As it can be seen, significant modification occurred in the dye UV–vis spectrum versus time. Similar decay behaviour of the visible absorption band was also previously observed [16]. The presence of at least one isosbestic point near 300 nm and a gradual

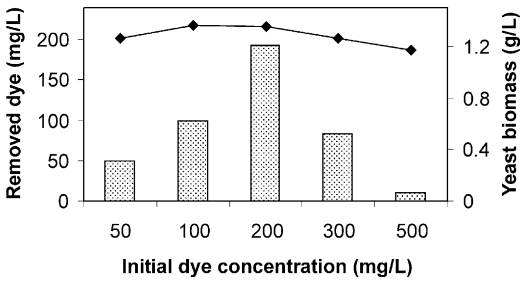

Fig. 4. Effect of different initial dye concentrations in both decolorization (bars) and yeast dry weight accumulation (line) obtained in batch cultures of *C. oleophila* after 24 h of incubation.

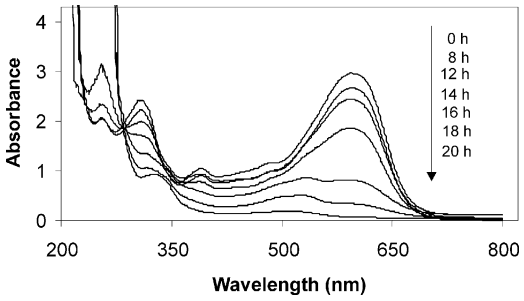

Fig. 5. Example of UV–vis spectral scans from batch cultures of *C. oleophila* containing 100 mg/l of Reactive Black 5 at different incubation times.

shift of the maximum absorption peak in the visible region from 595 to 525 nm (Fig. 5), clearly suggests that *C. oleophila* yeast cells decolorized this diazo dye through biodegradation. The fact that batch cultures turned from initial dark-blue to light-pink, which at last tend to colourless and also the absence of visible dyed yeast biomass reinforce this assumption. In order to gain additional insight into the decolorization mechanism, depletion of both color and aromaticity as well as the screening of ligninolytic enzyme activities (laccase and manganese-dependent peroxidase) were also monitored over time. While full color abatement (rupture of chromophoric azo bonds) occurs within 20 h, degradation of aromatic dye moieties proved to be a more recalcitrant process (Fig. 6). Despite manganese-dependent peroxidase might play an important role in the yeast-mediated biodegradation of Reactive Black 5 [16], we have not been able to detect any similar oxidative activity. This is not surprising since the occurrence of ligninolytic enzymes was until now just apparently reported in four yeast species [16,26,27]. It is generally accepted (at least for bacterial processes [3]) but has only recently been proven, that azoreductases play a key role during aerobic azo bond cleavage [28,29]). In addition, recent physiological and kinetic data strongly suggest that the in vivo yeast-mediated biodegradation of azo dyes [17] also could be ascribable to the activity of a putative aerobic azoreductase. In this work, the observed growth-associated decolorization and concomitant absence of extracellular oxidative enzymes in batch cultures of *C. oleophila* is consistent with an azoreductase-catalysed cleavage of azo bonds previously postulated in the ascomycetous yeast species *C. zeylanoides* [15] and *I. occidentalis* [17].

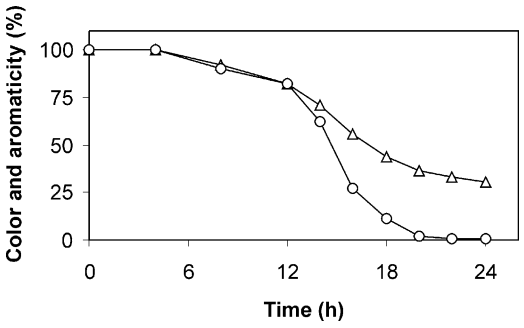

Fig. 6. Evolution of color (◯, absorbance at 595 nm) and aromaticity (△, absorbance at 310 nm) evaluated in batch cultures of *C. oleophila* from an initial dyestuff concentration of 100 mg/l.

4. Conclusions

An ascomycetous yeast strain isolated from the wash water of olives and identified as *C. oleophila* efficiently removes the color of the diazo dye Reactive Black 5. Particularly, batch cultures of this yeast species completely decolorize up to 200 mg dye l^{-1}, within 24 h of incubation without dye adsorption to the biomass. Also, we do not detect any extracellular ligninolytic activity during the decolorization time course. It was found that azo dye decolorization is associated to primary metabolism and yields of 180 mg decolorized dye g^{-1} biomass as well as 40 mg decolorized dye g^{-1} glucose were obtained. Our results, being in line with recent findings, are also consistent with a reductive mechanism of dye decolorization and constitute a new contribution to better understand the potentialities exhibited by non-conventional yeasts on the context of xenobiotic azo dye biodegradation.

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