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Biodecoloration of Reactive Black 5 by the methylotrophic yeast *Candida boidinii* MM 4035

María M. Martorell[1,*,**], *Hipólito F. Pajot*[2,3,**], *Pablo M. Ahmed*[4], *Lucía I.C. de Figueroa*[2,5]

1. *Antarctic Argentinian Institute, Balcarce 290, CABA, Argentina*
2. *PROIMI – CONICET, Tucumán, Argentina*
3. *Department of Products Quality IV, San Pablo University-Tucumán, Tucumán, Argentina*
4. *Agroindustrial Experimental Station Obispo Colombres, Tucumán, Argentina*
5. *Department of Superior Microbiology, Faculty of Biochemistry, Chemistry and Pharmacy, National University of Tucumán, Tucumán, Argentina*

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ABSTRACT

Azo dyes are extensively used in textile dyeing and other industries. Effluents of dying industries are specially colored and could cause severe damage to the environment. The anaerobic treatment of textile dying effluents is nowadays the preferred option, but it could generate carcinogenic aromatic amines. Recently, yeasts have become a promising alternative, combining unicellular growth with oxidative mechanisms. This work reports the characterization of the first methylotrophic yeast with dye decolorizing ability, *Candida boidinii* MM 4035 and some insights into its decoloration mechanism. The analysis of two selected media revealed a possible two stages mechanism of Reactive Black 5 decoloration. In glucose poor media, decoloration is incomplete and only the first stage proceeds, leading to the accumulation of a purple compound. In media with higher glucose concentrations, the yeast is able to decolorize totally an initial concentration of 200 mg/L. The entire process is co-metabolic, being largely dependent on glucose concentration but being able to proceed with several nitrogen sources. Manganese dependent peroxidase but not laccase activity could be detected during decoloration. Aromatic amines do not accumulate in culture media, supporting an oxidative decoloration mechanism of unknown ecophysiological relevance.

Introduction

Synthetic dyes are extensively used in textile dyeing, paper and other industries. Effluents of dying industries are specially colored and, since color reduces light penetration, the disposal of these wastes into receiving waters could cause severe damage to the environment (Sharma et al., 2009).

Several physical and chemical methods have been suggested for the treatment of dye contaminated wastewater. However, they are not widely adopted, mainly because of its high costs and secondary pollution. Consequently, bioremediation is currently considered as a promising, cost-effective and eco-friendly alternative to conventional methods, causing minimum environmental stress. Between the plethora of available options, the

* *Corresponding author*. E-mail: marmartorellsaez@gmail.com (María M. Martorell).
** These authors contributed equally to the work.

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anaerobic treatment of textile dying effluents is, by far, the preferred option (Chen et al., 2007).

Azo dyes are characterized by the presence of one or more azo linkages (–N═N–) and aromatic rings and constitute the largest and most versatile class of dyes used nowadays (Dias et al., 2010). However, anaerobic degradation of azo dyes involves the reductive cleavage of azo bonds (–N═N–) by either specific or unspecific azo reductase enzymes. Since aromatic amines are no further degraded under anaerobic conditions, treated and colorless effluents could still contain potentially hazardous-aromatic amines (Saratale et al., 2011; Rauf and Salman Ashraf, 2012; Pielesz, 1999). Thus, the employment of obligate aerobic microorganisms, mainly filamentous fungi, has attracted significant attention (Singh, 2006; Erkurt et al., 2010; Lebkowska and Zaleska-Radziwill, 2014).

Today, dye decoloration is better understood in the light of fungal lignocellulolytic potential. So, decoloration by "White Rot Fungus" (WRF) are thought to proceed through ligninolytic enzymes (*i.e.*, laccase, lignin peroxidase and manganese peroxidase) while decoloration bye "Brown Rot Fungus" (BRF) is thought to be mediated by Chelator Mediated Fenton Reaction mechanisms (Anastasi et al., 2013). The poor specificity of both mechanisms makes them able to transform, and eventually mineralize, a variety of pollutants (Martorell et al., 2012a, 2012b). The need of strict aerobic conditions, the usually rigorous conditions for ligninolytic enzyme expression (*i.e.*, C/N relation regulation, linkage to secondary metabolism), and the risk of contamination by bacteria under non-sterile conditions, make difficult the application of filamentous fungi for wastewater treatment (Dias et al., 2010).

Both asco- and basidiomycetous yeasts, on the other hand, have been successfully employed in textile dye decoloration through a variety of mechanisms, including sorption and enzymatic degradation (Dias et al., 2010; Pajot et al., 2014). Unfortunately, our present understanding of the ecophysiology of dye decolorizing yeast, and the relative contribution of diverse mechanisms to the overall process is still fragmentary. In addition, it has been proved that even when typical ligninolytic enzymes such as manganese peroxidases (MnP) and tyrosinases (Tyr) are produced by dye decolorizing yeasts as *Trichosporon akiyoshidainum*, no strong correlation could be found between enzyme activity and dye decoloration (Martorell et al., 2012a). This lack of a solid conceptual frame leaves the selection of places and methods for the bioprospection of dye decolorizing yeasts largely as a matter of personal choice.

We previously suggested (Pajot et al., 2014), in the context of dye decolorizing yeast, that the white rot/brown rot dichotomy should be replaced for a more inclusive theoretical frame: the oligotrophic/copiotrophic rationale. In general, ascomycetous yeasts tend to be fermentative, copiotrophic and specialized nutritionally. Basidiomycetous species, on the other hand, tend to oligotrophy using a wider range of carbon sources in aerobiosis and at lower concentrations. Regarding their ecology, ascomycetous yeast are often found in specialized, liquid niches involving interactions with plants, insects or other invertebrates while basidiomycetous yeasts would seem to be adapted to nutrient-poor, solid substrates (Suh et al., 2006). This work presents the polyphasic characterization of a copiotrophic/ascomycetous strain identified as *Candida boidinii* MM 4035, the first reported dye decolorizing-methanol assimilating yeast to our knowledge. The effect of culture media components over yeast growth and dye decoloration was further analyzed through a design of experiments (DOE) approach in order to gain some insights into its decoloration mechanisms and into the ecophysiology of this dye decolorizing yeast.

1. Experimental

1.1. Microorganism

Candida sp. 4035 is a litter associated yeast, previously isolated and selected based on its decoloration potential and the ability to assimilate textile dyes as sole C and/or N sources (Martorell et al., 2012a).

1.2. Morphological and physiological characterization

The phylogenetic analysis included most methylotrophic ascomycetous yeast species described in "The Yeast, a taxonomic study" (Kurtzman et al., 2011). Partial 26s sequences and complete 18s and ITS1-5.8s-ITS2 sequences where retrieved manually from GenBank (ITS1-5.8s-ITS2 sequence from *C. boidinii* type specie CBS 2428 was retrieved from CBS: http://www.cbs.knaw.nl/Collections/BioloMICS.aspx?Link=T&TargetKey=14682616000000137&Rec=16025&Revert=F).

Primers ITS1, NL4, NS1, NS8, NS20 and NS21 were employed in order to obtain the correspondent sequences from *Candida* sp. MM-4035 as previously described (Pajot et al., 2008). Complete 18s rDNA and complete ITS1-5.8s-ITS2 plus partial 26s rDNA (D1/D2 domain) were deposited in GenBank (KJ794106 and KJ794107, respectively). Physiological profiles where retrieved from "The Yeast, a taxonomic study". Morphological and physiological properties were examined in accordance to standard yeast identification methods (Kurtzman et al., 2011), as previously reported, Vilmafix® Yellow 4R-HE, Vilmafix® Black B-V (Fig. 1), Vilmafix® Blue RR-BB, Vilmafix® Red 7B-HE and a mixture of all dyes (50 mg/L each) were also tested as sole carbon, nitrogen or carbon and nitrogen sources.

DNA alignments were run in MEGA 5.2, using the MUSCLE algorithm implemented (Edgar, 2004). Alignments were manually edited to exclude regions with ambiguous alignment. Gaps were treated as missing data. The final matrix comprised 62 species and 3332 characters.

The phylogenetic searches were conducted using parsimony analyses TNT (Goloboff et al., 2008). Each run started from a Random Addition Sequence (RAS) followed by TBR. After that, the trees were submitted to a combination of Sectorial Searches (SS), Tree Drifting (TD), Ratchet and Tree Fusing (Goloboff, 1999). Multiple runs were conducted and the analysis was stopped when the minimum length was independently obtained 20 times. Clade support was assessed by absolute jackknifing frequencies with a removal probability of 0.36 (Goloboff et al., 2003). The search strategy in each jackknifing replicate comprised 30 RAS followed by TBR + SS and TD.

For microscopy purposes, cells were grown in YM broth for 3 days at 25°C and 250 r/min. Scanning electron microscopy (JSM-35CF, JEOL, Japan) and transmission electron microscopy

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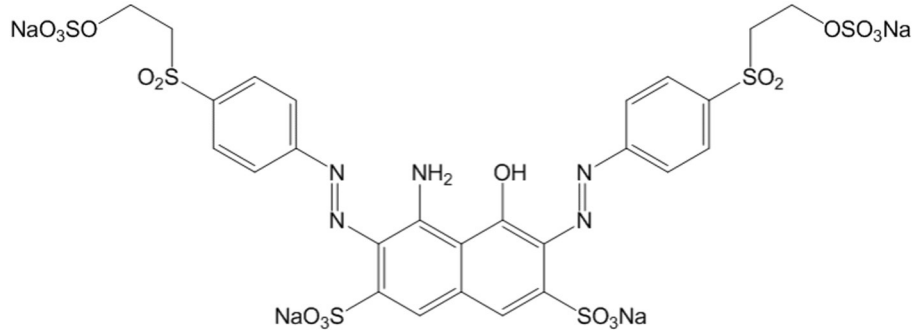

Fig. 1 – Vilmafix® Black B-V (C. I. name, Reactive Black 5) chemical structure.

(EM109, Zeiss, Germany) were performed according to routine techniques.

1.3. Original medium and dyestuff for dye decoloration

NDM (normal decoloration medium) was originally described by Ramalho et al. (2004). A modified version (without $CaCl_2$) including (in g/L): glucose, 20; yeast extract, 2.5; $(NH_4)_2SO_4$, 5; KH_2PO_4, 5; and $MgSO_4{\cdot}7H_2O$, 0.5; was employed in this work.

Reactive Black 5 (Fig. 1) stock solutions were prepared by dissolving powdered dyestuff, without prior purification, in distilled water up to a concentration of 2 g/L and filter sterilized (Millipore filter, 0.22 μm, Millipore Corp., Bedford, USA).

1.4. General growth and decoloration assays in liquid cultures

Decoloration kinetics were evaluated in 500 mL-Erlenmeyer flasks containing 100 mL of modified NDM medium or the medium under evaluation, plus Reactive Black 5 at a final concentration of 200 mg/L. The 10-mL yeast suspension (OD_{550} = 0.8), prepared from a 24 hr old NDM broth culture was used to inoculate the flasks. Incubations were carried out at 25°C and 250 r/min for 24 hr. Biotic and abiotic controls were performed using the same medium without dye or yeast, respectively. Samples were aseptically collected every 12 hr and centrifuged for 10 min at 6500 ×*g*. Pellets were washed twice with sterile water and dried at 80°C to constant weight for biomass dry weight determination. Supernatants were kept for estimating dye removal and pH.

1.5. Dye monitoring

Dye decoloration was monitored with a AD200 ELISA reader (Beckman Coulter, USA) at 595 nm by using culture supernatants obtained as above described. Color removal (R, %) at Reactive Black 5 λ_{opt} (595 nm) was calculated by Eq. (1):

$$R = \frac{A_0 - A_t}{A_0} \times 100 \quad (1)$$

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 300 and 700 in order to analyze dye disappearance.

1.6. Carbon/energy and nitrogen source selection

The effect of carbon and nitrogen sources on decoloration of Reactive Black 5 was evaluated on a full factorial design. Three carbon sources, glucose, sucrose and glycerol were assayed at equivalent carbon concentrations (0.8 g/L). Methanol was not included due to its toxic effects on high concentrations. Three nitrogen sources, $(NH_4)_2SO_4$, urea and NH_4NO_3 were also evaluated at equivalent nitrogen concentrations (0.05 g/L), resulting in nine different media. Biomass and dye removal were measured in the resulting nine media as above described.

1.7. Effects of culture media components

Single effects and interactions of glucose, yeast extract, urea, KH_2PO_4 and $MgSO_4{\cdot}7H_2O$, were measured in a five-factor fractional factorial design. Two extra replicates were included as center points, and thus a total of 34 experiments were employed in this study. In each sample decoloration percentages, biomass production and pH were evaluated after 12 and 24 hr of culture.

1.8. Decoloration process in selected media

Reactive Black 5 decoloration where compared in a carbon/energy poor medium (oligotrophic conditions, NDMp) and a carbon/energy rich medium (copiotrophic conditions, NDMr). Analysis was made comparing biomass production, dye removal, pH, total aromatic amines as determined by Pajot et al. (2008) and total aromaticity at 310 nm, according to Lucas et al. (2006). Manganese peroxidase (MnP) was measured as described by Castillo et al. (1994). Laccase was determined at pH 4.5 in 0.2 mol/L sodium acetate, or at pH 7.0 in 0.2 mol/L sodium phosphate.

Peroxidase activity was determined at pH 7.0 by monitoring the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) at 405 nm, by using a final H_2O_2 concentration of 0.1 mmol/L. Enzyme activities were expressed as units per mL, where 1 U was defined as 1 mmol of substrate oxidized per min. The peroxidase activity was always corrected for laccase activity.

1.9. Statistical analysis

All values and data points presented in this work are the means of at least triplicate determinations of independent

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assays. All the results, including those of the experimental designs were analyzed using MINITAB 16 (PA, USA). One-way analysis of variance (ANOVA) was used to test the significant differences between media evaluated. Tests were considered significantly different at $p < 0.05$.

2. Results and discussion

2.1. Morphological and physiological characterization of Candida sp. MM-4035

After 3 days of growth at 25°C in a liquid YM-medium containing, ovate budding cells and globose single cells (2.5–3 μm × 1.5–2 μm) were observed (Fig. 2).

Candida sp. MM-4035 is closely related to *C. boidinii* Y-2332^T, showing no differences in the 18s rDNA and only few differences in 26s D1/D2 domain (2 bp) and ITS1-5.8s-ITS2 region (4 bp) of rDNA. Carbon and nitrogen assimilation profiles alongside other growth characteristics are described in Tables S1 and S2.

Phylogenetic analysis either including (Fig. 3) or not physiological characters (Fig. S1), yielded highly congruent trees. Both datasets failed to resolve the subclade *Ogataea* from *Ambrosiozyma* and related species, but otherwise are in good accordance to the single-genes analyses conducted by Nagatsuka et al. (2008) and the multigene analysis as the one conducted by Kurtzman and Robnett (2010). In all cases, *Ambrosiozyma* clade is strongly supported and the *Ogataea methanolica*, *O. ramenticola*, *O. minuta* and *O. glucozyma* clusters could be easily identified. *C. boidinii*/*C. boidinii* MM-4035 form a well resolved strongly supported clade, with a basal position to the *Ambrosizyma*/*Ogataea* clade.

2.2. Carbon/energy and nitrogen source selection

After 24 hr cultivation, dye removal and biomass production were optimum with glucose as carbon/energy source, the other carbon sources evaluated, sucrose and glycerol, produced both, minimal biomass production and dye removal.

No significant differences could be attributed to the effect of nitrogen sources ($p > 0.05$) in glucose amended media (Fig. 4). Urea, providing an economic alternative to other nitrogen sources and being able of partially avoid dye sorption (by buffering the pH), was then selected for further studies in combination with glucose and carbon sources.

2.3. Effect of culture media components

Dye removal, biomass production and pH after 12 and 24 hr of culture of the 34 run fractional factorial design are shown in Table 1. All responses varied considerably under tested conditions (Table 2).

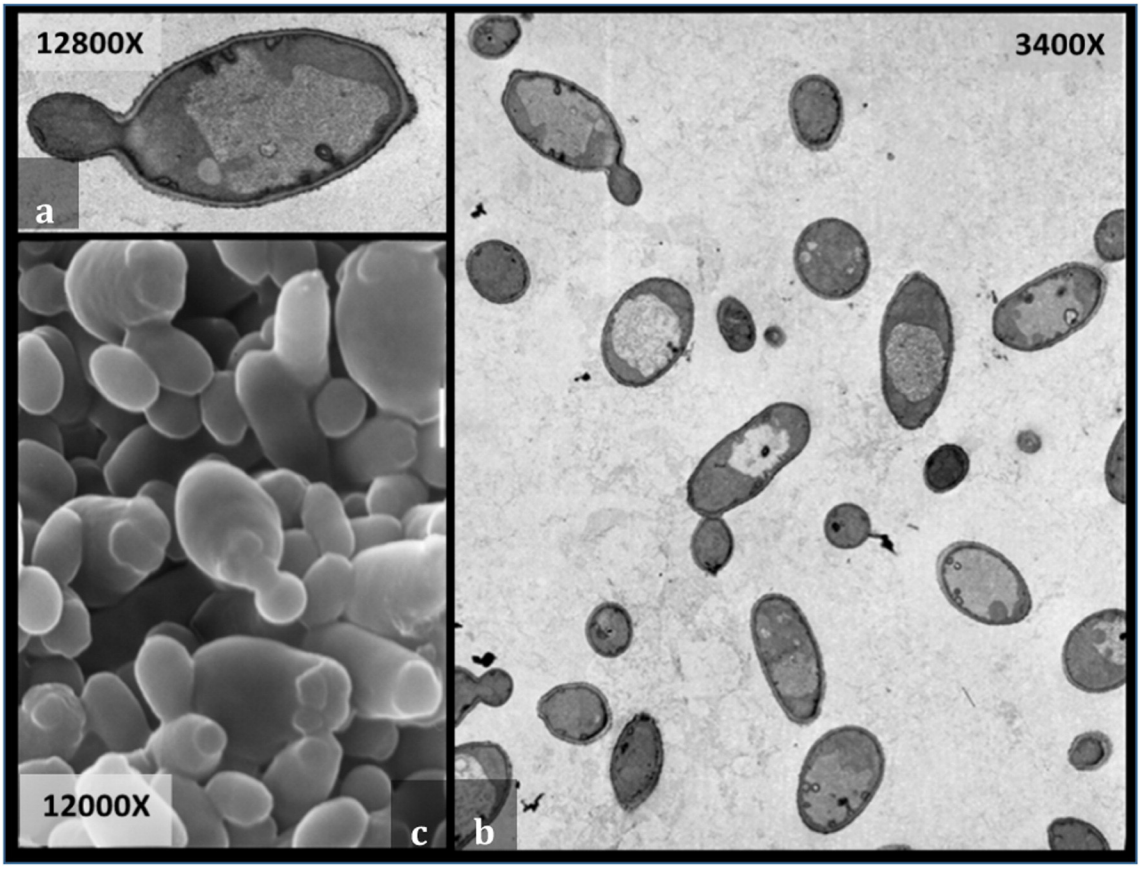

Fig. 2 – Transmission electron microscopy (TEM) (a, b) and scanning electron micrograph (SEM) (c) of *Candida boidinii* MM 4035 cells grown in YM broth for 3 days at 26°C and 250 r/min.

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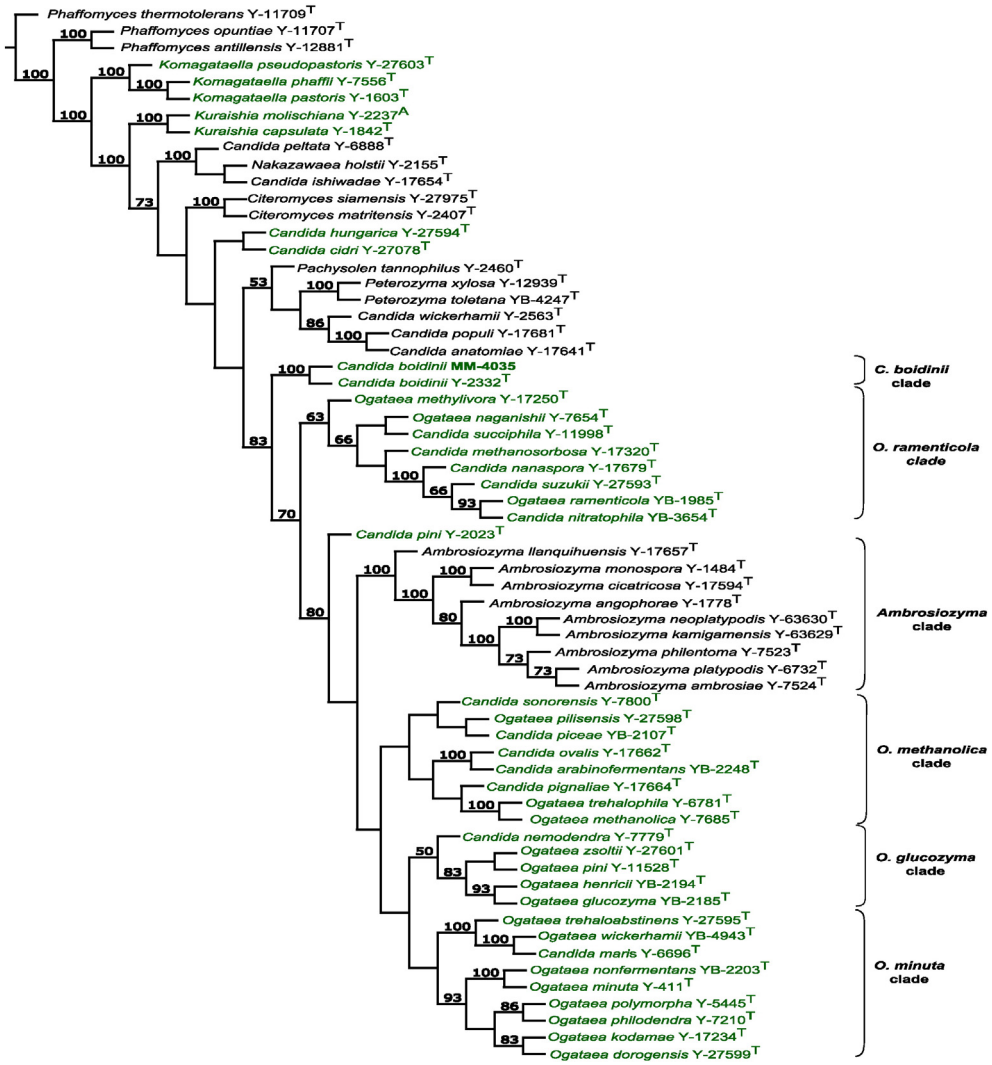

Fig. 3 – Phylogenetic relationships among methanol assimilating yeasts as represented by the strict consensus tree of 18 optimal trees obtained in the parsimony analysis of partial 26s sequences and complete 18s and ITS1-5.8s-ITS2 sequences and physiological tests. Analysis included 3332 characters, 1655 phylogenetically informative. Tree length: 6658, consistency index: 0.520, retention index: 0.694). Species in green are able to assimilate methanol.

When analyzing dye removal, all tested components showed a significant effect on dye decoloration. However, yeast extract showed a predominant effect after 12 hr while glucose exerts a bigger effect after 24 hr.

At 12 hr the maximum decoloration recorded was 91%; however at 24 hr average dye removal reached 90%, with a maximum of 100%. Surprisingly, decoloration percentages at 12 and 24 hr were not correlated ($r = 0.082$; $p = 0.643$). Thus, final decoloration could not be predicted on the basis of decoloration after 12 hr. These two responses could be satisfactorily fitted in to different models. Percentage decoloration after 12 hr model fits data with $r^2 = 0.9827$; and predicted $r^2 = 0.9307$. Meanwhile, percentage decoloration at 24 hr fits data with $r^2 = 0.9884$; and predicted $r^2 = 0.9536$ (Table S1).

In biomass analysis, all tested culture media components showed a significant effect on biomass accumulation. Yeast extract showed a predominant effect at 12 hr while glucose exerts a bigger effect after 24 hr.

Biomass production ranged from 2.51 to 3.17 g/L and from 4.71 to 9.07 g/L at 12 and 24 hr, respectively. Also in the case of biomass, values after 12 and 24 hr showed no correlation ($r^2 = 0.007$, $p = 0.968$), but data could be fitted into separated models. After 12 hr, the model fits data with $r^2 = 0.9974$ and predicted $r^2 = 0.9307$. Even better fits were attained by the model for biomass production after 24 hr of culture ($r^2 = 0.9999$; and predicted $r^2 = 0.9996$) (Table 3).

Biomass and decoloration showed a positive correlation after 12 hr ($p = 0.000$; $r = 0.675$) and after 24 hr ($p = 0.000$; $r = 0.743$). Biomass, however, remained colorless in all runs, even in runs with low decoloration percentages, supporting a cometabolic process.

Finally, for pH analysis, after 12 hr cultivation, pH values did not show great variability, ranging from 4.12 to 4.61. After 24 hr, however values ranged from 3.79 to 6.64. Values after 12 and 24 hr showed a slight correlation ($r^2 = 0.357$; $p = 0.038$), but could not be fitted to satisfactory models.

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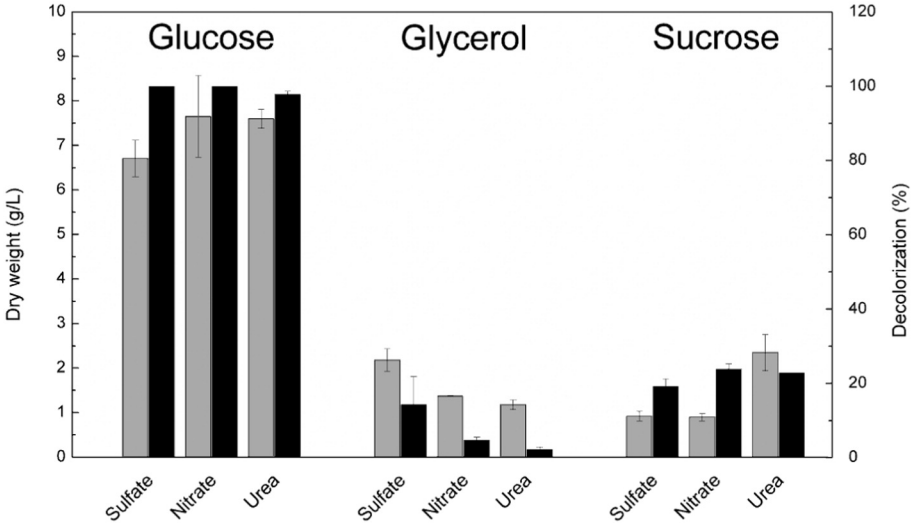

Fig. 4 – Effect of the 9 medium combinations based on the carbon and nitrogen sources evaluated on biomass (gray columns) and Reactive Black 5 (black columns) (initial concentration 200 mg/L) removal values for *Candida boidinii* MM-4035 cultures after 24 hr incubation at 25°C and 250 r/min.

Table 1 – Thirty four-run fractional factorial design for optimization of yeast dye decolorization process.

Run order	Glucose (%, W/V)	Urea (%, W/V)	Yeast extract (%, W/V)	KH_2PO_4 (%, W/V)	$MgSO_4$ (%, W/V)	Decolorization (%)		pH			Biomass (g/L)	
						12 hr	24 hr	0 hr	12 hr	24 hr	12 hr	24 hr
1	1	0.0565	0.125	0.25	0.075	73.09	86.79	5.48	4.26	5.86	2.57	4.95
2	3	0.0565	0.125	0.25	0.025	50.88	100	5.47	4.29	3.81	2.51	8.77
3	1	0.1695	0.125	0.25	0.025	54.14	71.1	5.51	4.38	6.63	2.91	4.72
4	3	0.1695	0.125	0.25	0.075	65.26	100	5.41	4.24	4.01	2.54	6.82
5	1	0.0565	0.375	0.25	0.025	79.29	82.16	5.75	4.38	6.28	3.54	5.28
6	3	0.0565	0.375	0.25	0.075	79.69	96.93	5.66	4.49	5.11	3.19	8.29
7	1	0.1695	0.375	0.25	0.075	86.79	88.5	5.72	4.46	6.4	3.9	5.27
8	3	0.1695	0.375	0.25	0.025	78.04	93.55	5.67	4.31	5.84	3.3	8.47
9	1	0.0565	0.125	0.75	0.025	51.61	69.8	5.35	4.25	5.31	2.79	5.06
10	3	0.0565	0.125	0.75	0.075	46.18	100	5.19	4.21	3.92	2.56	8.08
11	1	0.1695	0.125	0.75	0.075	62.17	78.7	5.19	4.28	5.97	2.91	4.71
12	3	0.1695	0.125	0.75	0.025	48.98	98.87	5.16	4.54	4.2	3.25	9.04
13	1	0.0565	0.375	0.75	0.075	85.12	87.15	5.35	4.44	5.55	3.76	5.14
14	3	0.0565	0.375	0.75	0.025	69.77	93.91	5.48	4.39	4.96	3.35	7.76
15	1	0.1695	0.375	0.75	0.025	79.27	81.35	5.37	4.51	5.89	3.5	4.83
16	3	0.1695	0.375	0.75	0.075	80.68	96.33	5.7	4.37	5.21	4.14	8.55
17	1	0.0565	0.125	0.25	0.075	70.66	86.64	5.5	4.31	5.99	2.62	4.98
18	3	0.0565	0.125	0.25	0.025	49.87	100	5.49	4.19	3.82	2.56	8.8
19	1	0.1695	0.125	0.25	0.025	56.41	71.09	5.53	4.3	6.64	2.96	4.75
20	3	0.1695	0.125	0.25	0.075	61.59	100	5.43	4.2	3.94	2.59	6.85
21	1	0.0565	0.375	0.25	0.025	75.91	80.35	5.77	4.41	6.2	3.59	5.31
22	3	0.0565	0.375	0.25	0.075	85.44	98.96	5.68	4.36	5.27	3.24	8.32
23	1	0.1695	0.375	0.25	0.075	91.03	92.63	5.74	4.49	6.54	3.95	5.3
24	3	0.1695	0.375	0.25	0.025	76.47	92.87	5.69	4.52	5.68	3.35	8.5
25	1	0.0565	0.125	0.75	0.025	45.66	68.37	5.37	4.35	5.45	2.84	5.09
26	3	0.0565	0.125	0.75	0.075	43.17	96.84	5.21	4.24	3.79	2.61	8.11
27	1	0.1695	0.125	0.75	0.075	62.41	77.65	5.21	4.31	5.82	2.96	4.74
28	3	0.1695	0.125	0.75	0.025	48.69	98.67	5.18	4.35	4.17	3.3	9.07
29	1	0.0565	0.375	0.75	0.075	84.8	86.89	5.37	4.25	5.54	3.81	5.17
30	3	0.0565	0.375	0.75	0.025	70.68	99.66	5.5	4.33	5.2	3.4	7.79
31	1	0.1695	0.375	0.75	0.025	74.79	79.15	5.39	4.51	5.92	3.55	4.86
32	3	0.1695	0.375	0.75	0.075	72.56	94.84	5.72	4.57	5.28	4.19	8.58
33	2	0.113	0.25	0.5	0.05	74.9	98.06	5.62	4.58	5.36	2.91	5.21
34	2	0.113	0.25	0.5	0.05	75.28	99.25	5.58	4.61	5.41	2.95	5.26

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Table 2 – Estimated regression coefficient and corresponding *T* and *p* values of biomass at 12 and 24 hr.

Term/predictor response (dye removal at)	Effect		Coef		SE coef		*T*		*p*	
	12 hr	24 hr	12 hr	24 hr	12 hr	24 hr	12 hr	24 hr	12 hr	24 hr
Constant			3.195	662.425	0.00618	0.00394	516.7	1680.77	0	0
Glucose	−0.13	32.285	−0.065	161.425	0.00618	0.00394	−10.51	409.58	0	0
Urea	0.2725	−0.114	0.13625	−0.057	0.00618	0.00394	22.03	−14.46	0	0
Y.E.	0.83	0.179	0.415	0.0895	0.00618	0.00394	67.11	22.71	0	0
KH_2PO_4	0.225	0.074	0.1125	0.037	0.00618	0.00394	18.19	9.39	0	0
$MgSO_4$	0.0525	−0.264	0.02625	−0.132	0.00618	0.00394	4.25	−33.49	0.001	0
Glucose [a] urea	0.1325	0.111	0.06625	0.0555	0.00618	0.00394	10.71	14.08	0	0
Glucose [a] Yeast extract	−0.05	−0.091	−0.025	−0.0455	0.00618	0.00394	−4.04	−11.54	0.001	0
Glucose [a] KH_2PO_4	0.215	0.194	−0.782	0.097	0.00618	0.00394	17.39	24.61	0	0
Glucose [a] $MgSO_4$	−0.0475	−0.309	−1.806	−0.1545	0.00618	0.00394	−3.84	−39.2	0.001	0
Urea [a] Y.E.	−0.0225	0.2765	−0.613	0.13825	0.00618	0.00394	−1.82	35.08	0.087	0
Urea [a] KH_2PO_4	0.0625	0.3865	0.864	0.19325	0.00618	0.00394	5.05	49.03	0	0
Urea [a] $MgSO_4$	0.08	0.1615	−0.275	0.08075	0.00618	0.00394	6.47	−20.49	0	0
Y.E. [a] KH_2PO_4	−0.02	0.3315	1.189	−0.16575	0.00618	0.00394	−1.62	−42.06	0.124	0
Y.E. [a] $MgSO_4$	0.2725	0.4915	−0.513	0.24575	0.00618	0.00394	22.03	62.35	0	0
KH_2PO_4 [a] $MgSO_4$	0.0675	0.2115	−1.403	0.10575	0.00618	0.00394	5.46	26.83	0	0
Central point			7.556	−13.893	0.0255	0.0163	−10.39	−85.49	0	0

Coef: coefficient for the regression equation; SE coef: standard error of the coef; *T*: test statistic with Student's test; *p*: *p* value associated with test statistic.

[a] Relate to the impact of both factors analyzed.

Unexpectedly, decoloration and pH resulted positively correlated at 12 hr ($p = 0.005$; $r = 0.470$), but negatively correlated at 24 hr ($p = 0.000$; $r = -0.673$).

These data, along with the colorless yeast biomass at the end of cultures, supports the idea of two slightly different cometabolic processes that have been extensively reported for ascomycetous yeasts like *Issatchenkia occidentalis* (Ramalho et al., 2004), *Candida oleophila* (Lucas et al., 2006) and *Debaryomyces polymorphus* (Yang et al., 2005); as well as for basidiomycetous yeasts like *Trichosporon akiyoshidainum* (Martorell et al., 2012b).

Two media were then selected for further analysis. An incomplete bioprocess (maximum decoloration at 12 hr), could be achieved by keeping glucose, urea and KH_2PO_4 at their lowest levels, while using higher yeast extract and $MgSO_4$ concentrations. The resulting medium was named NDMp, due to its poor glucose content.

A most extensive decoloration could be reached after 24 hr in media including glucose and $MgSO_4$ at their highest concentrations, and keeping the remainder components to their lowest levels. The resulting medium was then named NDMr, according with its higher (rich) glucose content.

2.4. Decoloration process in selected media

Kinetics of biomass production, dye decoloration, pH, total aromatic amines and total aromacity, along with laccase, peroxidase and MnP titers in the two selected media NDMp, and NDMr are shown in Fig. 5a, b. Significant differences in biomass production could be found between these media ($p < 0.05$) and it seemed to be correlated only with the initial amount of glucose. In NDMp the final pH reached 6.12 while in the NDMr, the final pH reached was 3.54. Being urea and phosphate concentrations equal in both media, pH variations could be explained in terms of acid production from glucose. In both media, dye decoloration started approximately after 3 hr incubation, increasing trough cultivation, supporting a cometabolic process. In the glucose limited NDMp medium, decoloration was incomplete and it stopped once the culture reached stationary phase (Fig. 5a).

Table 3 – ANOVA for biomass at 12 and 24 hr.

Source response (biomass at)	DF		Adj SS		Adj MS		*F*		*p*	
	12 hr	24 hr	12 hr	24 hr	12 hr	24 hr	12 hr	24 hr	12 hr	24 hr
Model	16	16	8.06819	94.395	0.52426	58.997	412.14	11,869.19	0	0
Linear	5	5	6.6675	843.474	13.335	168.695	103.68	33,938.59	0	0
2-Way interactions	10	10	1.2685	64.147	0.12658	0.6415	103.68	1290.52	0	0
Curvature	1	1	0.13219	3.633	0.13219	3.633	108.04	7308.93		
Error	17	17	0.0208	0.0085	0.00122	0.0005				
Total	33	33	8.08899	944.035						

DF: degrees of freedom; SS: sum of squares; MS: mean squares; F: test statistic with Fisher's test; *p*: *p* value associated with test statistic. Adj: adjusted; ANOVA: analysis of variance.

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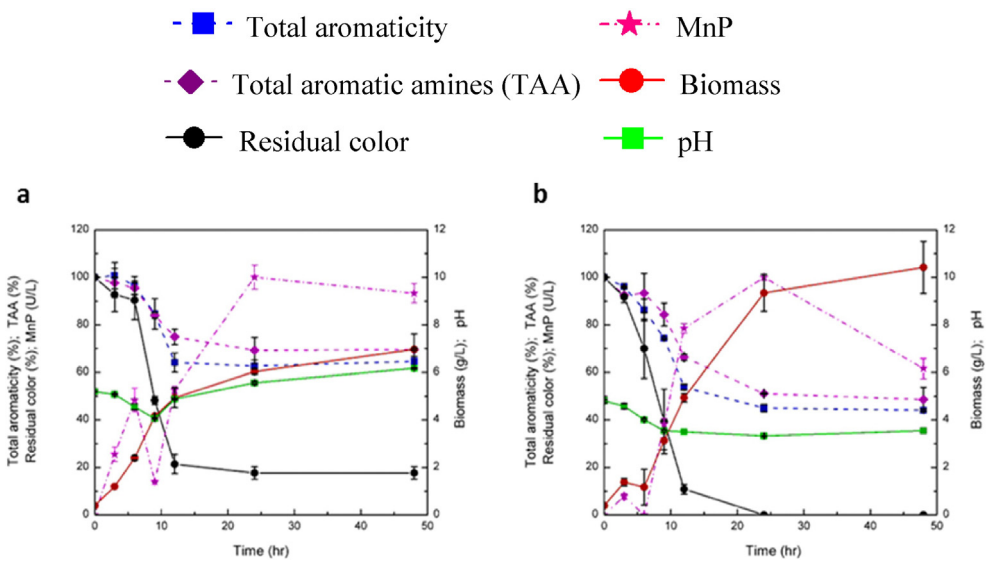

Fig. 5 – Growth and Reactive Black 5 decolorization kinetics of *Candida boidinii* MM 4035 in glucose poor medium (NDMp, a) and in carbon rich medium (NDMr, b). NDMp: oligotrophic conditions; NDMr: copiotrophic conditions.

Laccase (but not peroxidase activity) could be detected at pH 7.0 in phosphate buffer. Maximum titers reached only 0.14 U/L after 36 hr in NDMp and 0.10 U/L in NDMr, after 48 hr. No laccase activity could be detected at pH 4.5 in acetate buffer, clearly suggesting a false positive laccase reaction at pH 7.0 and the presence of a yet unidentified phenol oxidase (Mayer and Staples, 2002). Consistently, in a previous work, Mn dependent peroxidase and tyrosinase, but not laccase activities were reported during Reactive Black V dye decoloration by *C. boidinii* MM 4035 (Martorell et al., 2012a).

Mn dependent peroxidase activity was confirmed in the present work, reaching maximum titers after 24 hr cultivation in both media (0.41 and 0.33 U/L in poor and in rich media, respectively).

Absorbance spectra of partially decolorized, filtered supernatants, showed a shift of the peak of maximal absorbance from 595 nm (blue) to 540 nm (purple) (Fig. S2). In NDMp, no further decoloration was observed. Since Reactive Black 5 absorbance spectrum do not depend on the pH, such absorbance shift along with the poor dye sorption to the biomass, points to a degradative mechanism of decoloration. In NDMr, on the other hand, decoloration occurs in two stages. In the first one, the shift from deep blue to purple was observed, but there also exist a second stage, where the supernatant became colorless resembling decoloration in original NDM (Fig. 6, Fig. S2).

By taking this into account, a two-step decoloration mechanism could be proposed based on the different reactivity of the two azo-bonds in Reactive Black 5 which, in turn, could be attributed to the existence of hydroxyl (–OH) or amino ($-NH_2$) groups at the *ortho* position of azo bonds (Fig. 1) (Chivukula and Renganathan, 1995).

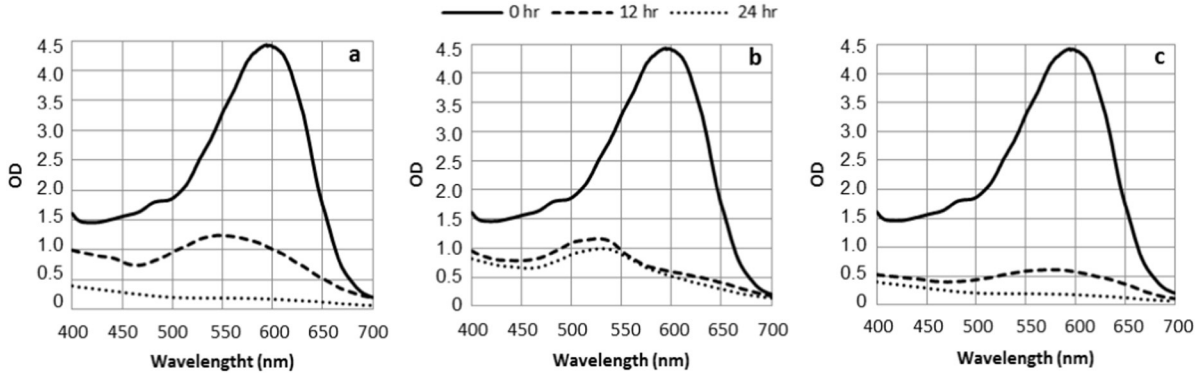

Fig. 6 – Variation of supernatant visible spectra during RB5 removal (initial concentration 200 mg/L) in original NDM (a), NDMp (b) and NDMr (c) media with *Candida boidinii* MM 4035. NDM: normal decoloration medium.

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A similar two step mechanism of decoloration of Reactive Black 5 was also reported for filamentous fungi *Phanerochaete chrysosporium* (Enayatizamir et al., 2011), *C. oleophila* (Lucas et al., 2006), *Shewanella* sp. (Pearce et al., 2006), *Pseudomonas luteola* (Chen, 2002), *Bjerkandera adusta* (Mohorčič et al., 2001), *Funaliatrogii* (Mazmanci and Unyayar, 2010), *Trametes versicolor* (Ramsay and Nguyen, 2002) and a bacterial consortium (Supaka et al., 2004).

Reduction in total aromatic amines (TAA) and total aromacity (TA) was observed during dye removal in both media. In NDMr, TAA decreased to a 49% of the original value while TA decreased to a 44%. As could be expected, minor dye degradation was related to, lower TAA and TA reductions (TA and TAA, respectively). Thus, it can be concluded that biodecoloration of azo dye Reactive Black 5 by *C. boidinii* MM 4035 did not involve the production of aromatic amines, which in turn suggests an oxidative dye degradation mechanism.

3. Conclusions

C. boidinii 4035 is, to our best knowledge, the first reported methylotrophic yeast with dye decolorizing ability. Decoloration of Reactive Black 5 proved to occur during exponential growth phase, indicating cometabolic degradation. Results showed that, even when the detailed decoloration mechanisms remain unclear, the dye decoloration process mediated by *C. boidinii* 4035 could be switched from an incomplete form in carbon poor media to a complete one in carbon rich media, as expected from a copiotrophic yeast.

The decrease in TAA, the lack of dye sorption to the biomass, the transient accumulation of a purple intermediate, and the apparent participation of a Manganese dependent peroxidase, strongly supports an oxidative decoloration mechanism. The existence of such oxidative mechanism in methanol assimilating ascomycetous yeasts raises intriguing questions about the ecological relevance of such ability. Methanol degradation involves the production of high amounts of H_2O_2, thus it could be hypothesized that methylotrophic yeasts as *C. boidinii* 4035 decolorize dyes in a similar process to the one proposed for Brown Rot Fungi, including specialized Chelator Mediated Fenton Reactions, a hypothesis supported by the measured drop in culture pH.

The herein present results encourage further study of the decolorizing ability of endophytic and litter associated yeast. Additionally, the dye decolorizing ability of *C. boidinii* 4035, encourages further studies on the scale up of the bioprocess for the treatment of industrial textile effluents.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2016.01.033.

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