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RESEARCH ARTICLE

Enhanced textile dye decolorization by marine-derived basidiomycete *Peniophora* sp. CBMAI 1063 using integrated statistical design

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Abstract In the present study, the biotechnological potential of the marine-derived fungus *Peniophora* sp. CBMAI 1063 was investigated in relation to Reactive Black 5 (RB5) dye decolorization and degradation using an integrated statistical design composed of Plackett-Burman design (P&B), central composite design (CCD), and response surface methodology (RSM). RB5 dye was effectively decolorized (94 %) in saline conditions, without any detection of mutagenic compounds, and simultaneously, 57 % of total organic carbon (TOC) was removed in 7 days. The activity of lignin peroxidase (LiP) was not detected during the process. The gene expression of laccase (Lac) and manganese peroxidase (MnP) enzymes produced during the process was evaluated, and results from this experiment coupled with LC-MS analyses revealed that in the early stage of dye decolorization, a higher MnP gene expression and significant enzymatic activity was detected in *Peniophora* sp. CBMAI 1063 with the formation of p-Base and TAHNDS compounds. This paper reports innovative data related to the textile dye decolorization by the marine-derived basidiomycete *Peniophora* sp. CBMAI 1063, showing the metabolites formed and enzymatic action throughout the process in saline condition. The strategy used showed to be an efficient statistical approach that provides an attractive solution for the screening and simultaneous optimization of the degradation process.

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Introduction

Textile dyeing wastewater has a large amount of complex components with a high concentration of organic and high-color compounds. Some dyes used in textile industries are problematic for various aquatic microorganisms, plants and fish and can also cause human health disorders and severe damage to important human organs. Even today, color treatment and removal of these effluents can be considered a biotechnological challenge (Khan et al. 2013).

Reactive Black 5 (RB5) belongs to the group of reactive azo dyes and has been extensively used in textile industries all over the world, transported to rivers as part of industrial effluents. The exposure to this dye can cause human allergenic problems (respiratory tract), and some of the metabolites generated from its degradation can be carcinogenic (Usha et al. 2006). Additionally, the presence of RB5 dye in effluent can cause eutrophication of rivers and lakes due to the oxygen deficiency, resulting in algae growth and death of aquatic life, as well as, in a reduction of the photosynthesis process efficiency (Bardi and Marzona 2010, Hadibarata et al. 2013).

Dye decolorizing ability of ligninolytic fungi has been extensively studied in terrestrial basidiomycetes (Kaushik and Malik 2009); however, studies related to marine-derived fungi are still scarce. Enzymes produced by fungi from marine origin can offer different properties from their terrestrial counterparts (Trincone 2010). The adaptability of these microorganisms to oceanic conditions can be considered an attractive point in the field of fungal marine biotechnology, including the treatment of industrial colored effluents, which usually present extreme pH values and high amounts of salts (Bonugli-Santos et al., 2015). Sodium even in small concentrations is toxic to most of the living cells that inhabit terrestrial and freshwater environments (Raghukumar et al. 2008).

Nutrients and physical parameters have a significant effect on dye decolorization (Singh et al., 2013). Statistical tools are considered as an efficient approach to determine the best culture conditions in biotechnological processes and are also useful for environmental studies (Pearce et al. 2013, Zeng and Arnold 2014). Experimental designs were successfully applied for the optimization of the ammonium removal process in wastewater (Yingling and Zhengfang 2013), to enhance the chrysene degradation (Ghevariya et al. 2011), and for the optimization of biohydrogen production (Liu et al. 2011).

In this context, the purposes of the present study are to investigate the enhancement of the RB5 decolorization by the marine-derived basidiomycete *Peniophora* sp. CBMAI 1063 using integrated statistical design and also to assess the contribution of ligninolytic enzymes during RB5 dye decolorization, as well as to evaluate the metabolites generated during the degradation process.

Materials and methods

Microorganism

The marine-derived fungus *Peniophora* sp. CBMAI 1063 was isolated from the Brazilian sponge *Amphimedon viridis* collected in the town of São Sebastião, São Paulo State, Brazil (Menezes et al. 2010) and taxonomically identified as previously reported by Bonugli-Santos et al. (2010). The strain was deposited at the Brazilian Collection of Microorganisms from Environment and Industry—CBMAI.

Fungal preparation and growth

Peniophora sp. CBMAI 1063 was cultivated in 2 % (*w/v*) malt extract agar (MA2) for 7 days at 28 °C (Table 1). For the experimental design, two to four fungal culture plugs (0.5 cm diameter) from the edge of the colony were transferred to 250-mL Erlenmeyer flasks containing 50 mL of liquid medium according to Table S1 and S2 (Supplementary material) for Plackett-Burman design (P&B 16 and 12) and to Table S3 (Supplementary material) and Table 2 for Central Composite Design (CCD). In the P&B, the assays were incubated at different temperatures (supplementary material, Table S1) and in the CCD at 28 °C. After 72 h, the Reactive Black 5 (RB5) dye was added to the assays at different concentrations as showed in Table S1 (P&B 16). For all other experimental designs, 200 mg/L of RB5 was used. All assays were performed under saline condition according to the volume of artificial seawater (ASW) added to the medium (Bonugli-Santos et al. 2010). In P&B 16 (Table S1), three different salinity conditions were used. Salinity corresponds to the ASW concentration (4 %) and was measured using a salinity meter. Three different volumes of ASW were added to P&B 16 in order to achieve 1.2 % salinity (15 mL of ASW and 35 mL of distilled water), 2.6 % (32.5 mL of ASW and 17.5 of distilled water), and 4 % (50 mL of ASW). For all other experimental designs and in the validation assay, the salinity was 1.2 %.

Assays were incubated for 7 days at 140 rpm and 28 °C. Cultures were harvested by centrifugation at 12,074×*g* for 30 min (Eppendorf Centrifuge 5804R), and the supernatant was used for the enzymes quantification, evaluation of the dye decolorization, and degradation and the AMES assay.

After experimental design, the optimized conditions were the following: three fungal culture plugs in 50 mL of liquid medium prepared with 3 g/L of malt extract, 3 g/L of wheat bran, 15 mL of ASW, and pH 6.25. The assays were carried out at 28 °C and 140 rpm. After 72 h of incubation, 200 mg/L

Table 1 Second central composite design (CCD) matrix, experimental data, and predicted values by the response surface analysis of RB5 decolorization by *Peniophora* sp. CBMAI 1063 incubated at 140 rpm for 7 days

Run	Variables [a,b]		Decolorization (%)
	Malt extract	Wheat bran	
1	0.065(−1)	0.079(−1)	57
2	0.14(+1)	0.079(−1)	90
3	0.065(−1)	0.22(+1)	88
4	0.14(+1)	0.22(+1)	62
5	0.05(−1.41)	0.15(0)	89
6	0.15(+1.41)	0.15(0)	90
7	0.1(0)	0.05(−1.41)	70
8	0.1(0)	0.25(+1.41)	69
9	0.1(0)	0.15(0)	89
10	0.1(0)	0.15(0)	86
11	0.1(0)	0.15(0)	86
12	0.1(0)	0.15(0)	87

[a] In parenthesis, coded values

[b] Values in g/50 mL

of RB5 was added to the medium. The validation assay was performed in triplicate, and the supernatant was obtained as described above.

Experimental design and optimization of dye decolorization by *Peniophora* sp. CBMAI 1063

The strategy used in the experimental design was composite by two P&B (matrix 16 and matrix 12), two CCD, and the validation assay. The integrated statistical design was initialized by the selection of significant factors using P&B model. The P&B 16 was used to evaluate 12 independent factors, with initial values determined by preliminary experiments based on literature reviews (Table S1). Three assays on center point were added to the matrix in order to determine the standard error. The design matrix created by the statistical STATISTICA 7 from Statsoft Inc. (2325 East 14th Street, Tulsa, OK, 74104, USA) is also represented in Table S1. After standardized effect (estimate) by statistical analysis of results from P&B16, new ranges of some independent factors (variables) were again evaluated, and other variables were eliminated or fixed in the P&B 12 matrix (Table S2). The standardized effect was based on the following first-order polynomial model:

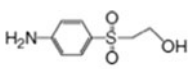
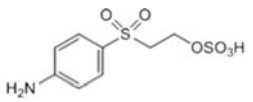
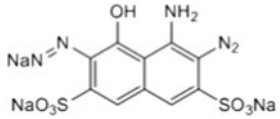
$$y = \beta_0 + \sum \beta_i x_i \quad (1)$$

where y was the predicted response, β_0 was the model intercept, β_i was the linear coefficient, and x_i was the independent variable level.

Following the strategy based on the results of P&B, the experiment was further expanded to a central composite design CCD (Table S3) with three variables: glucose, malt extract, and wheat bran. A 2^3 randomized factorial central composite design (CCD) with six axial points $\alpha = (2^3)^{1/4}$ and four replicates at the center points leading to a total 18 experiments was employed to optimize the decolorization of RB5 dye *Peniophora* sp. CBMAI 1063. These experiments were performed to obtain a second-order model to predict the percentage of decolorization on functions of different variables. The quadratic model for predicting the optimal point was expressed as follows:

$$y = \beta_0 + \sum \beta i x i + \sum \beta i i x 2\, i + \sum \beta i j x i x j \quad (2)$$

Table 2 Metabolites identified according molecular weight by LC-MS analyses

Compounds	Molecular weight (m/z)	Structure and chemical formula
p-Base	201	 p-Base (PB) [M-H]⁻ *m/z*=201,2
p-Base	281	 Chemical Formula: $C_8H_{11}NO_6S_2$ Exact Mass: 281,00278
Triaminohydroxynaphthalene disulfonic acid (TAHNDS)	439	 Chemical Formula: $C_{10}H_5N_5Na_3O_7S_2$ Exact Mass: 439,93235

where y was the predicted response, β_0 was the model intercept, x_i and x_j were the independent variable levels, β_i, β_{ii}, and β_{ij} were the linear quadratic and interaction coefficients, respectively.

Since the results demonstrated that the rate of decolorization was similar to P&B 12 and this test has not achieved optimal conditions, a new CCD was carried out. The second CCD (Supplementary material, Table S4) evaluated two variables (malt extract and wheat bran). This CCD was performed as the first CCD, and a 2^2 randomized factorial central composite design (CCD) with four axial points, $\alpha = (2^2)^{1/4}$, and four replicates at the center points leading to a total 12 experiments were employed to optimize the decolorization of RB5 dye by *Peniophora* sp. CBMAI 1063.

The quality of fit of the model equation was expressed by the coefficient of determination R^2, and its statistical significance was determined by F test (analysis of variance—ANOVA). The results were analyzed by the software STATISTICA 7. A significant level of 10 % ($P > 0.1$) was considered for the variables screened and 5 % ($P > 0.05$) for the central composite design.

To confirm the model equation adequacy, confirmatory experiments under the optimized condition were carried out. All the confirmatory experiments were conducted in triplicate, and the values predicted by the optimization model were set as controls.

Evaluation of RB5 dye decolorization and degradation by UV/VIS and LC-MS analyses

The evaluation of RB5 dye decolorization and degradation consisted of four trials using UV/VIS and LC-MS analyses allowing more complete coverage of the study: percentage of decolorization and kinetics, absorbance spectrum, quantitative degradation kinetics by LC-MS, and qualitative evaluation by LC-MS.

For the percentage and kinetics of the decolorization and absorbance spectrum analyses, the supernatants were diluted tenfold with distilled water prior to UV/VIS spectrophotometric analysis (Shimadzu UV-1240, Kyoto, Japan). The assays were performed as previously described (Bonugli-Santos et al. 2012). Color reduction was followed spectrophotometrically, and decolorizing activity was calculated from the decrease in the maximum absorption of RB5 peak. Absorption spectra were read in the wavelengths 300, 400, 500, 600, and 700 nm and dye decolorization at 598 nm (maximum absorption peak of this dye). To discard the mycelium participation in dye decolorization (dye adsorption by mycelium), the fungus was incubated for 7 days in the same conditions and without adding the RB5 dye. After this period, the assays were autoclaved, 200 mg/L of RB5 dye was added, and the assays were again incubated at 28 °C and 140 rpm after 7 days, the experiments were centrifuged according to standard procedures, and supernatant absorbance was measured as described above.

For the analyses using the liquid chromatography system and mass spectrometric detection (LC-MS), 2 mL of the supernatants were dried using the system speed vacuum for 12 h. After the samples were diluted in 1 mL of methanol and injected onto a HPLC (Agilent 1260 series) connected to a Q-TOF mass spectrometer (Agilent 6520), samples were resolved by Agilent Zorbax Eclipse C-18 (2.1 mm × 150 mm × 3.5 μm) HPLC column using the following elution system: Mobile phase was solvent A 2 mM ammonium acetate and solvent B 1 % acetonitrile using a gradient (0–5 min 100 % solvent A, 5–7.5 min 99 % solvent A, 7.5-12 min 5 % A, 12–15 min 99 % A,), which was pumped through the system at 0.5 mL min-1. Eluent from the HPLC column was directed through a diode array detector (DAD) set to monitor at 254, 665, 270, 238, 597, 600, 310, and 350 nm. After passing through the DAD, 100 % of the eluent was directed into the electrospray ionization source of the Q-TOF mass spectrometer. The mass spectrometer was operated in negative ionization mode scanning from 50–1100 m/z. Drying gas flow rate, temperature, and nebulizer pressure were at 6 L min-1, 325 °C, and 30 psi, respectively. Fragmentor and skimmer voltages were kept at 175 and 65 V, respectively (Xingzu et al. 2008).

For quantitative analysis, concentrations of 0.5 to 10 mg/L of BR5 dye were applied as standard. The peak area corresponding to the dye was calculated and used to determine the calibration curve. The dye concentration in each sample (first to seventh days of incubation) was achieved by applying the mathematical expression obtained from the standard curve ($R^2 = 99$ %):

$$y = 15.757\,\mathrm{x} + 3.6171 \quad (3)$$

In the evaluation of metabolites formed during the degradation, the chromatographs were obtained with five different wavelengths (310, 597, 270, 238, and 254 nm) monitored by the detector diode. ESI scan mode and UV-visible ranges from 190 to 700 nm of each peak were used to determine the molecular mass of possible metabolites.

The total carbon reduction (TOC) analysis was performed with a total organic carbon analyzer, TOC-L Shimadzu. TOC measurement is based on the combustion of the total carbon (TC) by catalytic oxidation at high temperature. The equipment was set to provide a variation coefficient lower than 2 % for each sample (in triplicates). The %TOC reduction was calculated using Eq. (4)

$$\%\,\mathrm{TOC\,reduction} = \left(\mathrm{TOC_{initial}} - \mathrm{TOC_{(t)}}\right)/\mathrm{TOC_{initial}} * 100 \quad (4)$$

where $\mathrm{TOC_{(initial)}}$ and $\mathrm{TOC_{(t)}}$ represent the initial TOC value and the TOC value at time "t" (h), respectively. Abiotic

controls (without microorganism) were included during the experimental investigation.

Contribution of ligninolytic enzymes in the RB5 dye decolorization

Enzyme assays

All enzyme activities were measured spectrophotometrically (Shimadzu UV-1240, Kyoto, Japan) in triplicate. MnP activity was determined using manganese sulfate as substrate (Giardina et al. 2000). The reaction mixture contained 0.5 mM manganese sulfate and 0.1 mM H_2O_2 in 50 mM sodium malonate buffer, pH 4.5. Oxidation of $Mn2^+$ to $Mn3^+$ was followed by absorbance increase at 270 nm (e270 = 11590 M-1 cm-1) due to the formation of malonate-$Mn3^+$ complex. LiP activity was determined by the oxidation of veratryl alcohol as previously described (Tien and Kirk 1984). The mixture reaction was composed of 0.5 mL enzyme extract, 1 mL sodium tartarate buffer (125 mM pH 3.0), 0.5 mL veratryl alcohol (10 mM), and 0.5 mL hydrogen peroxide (2 mM). The reaction was initiated with hydrogen peroxide, and the appearance of veratraldehyde was measured at 310 nm. Laccase activity was determined using 2.2′-azinobis-(3-ethylbenzothiazoline (ABTS) as previously described by Buswell et al. (1995). The mixture was composed of 0.3 mL sodium acetate buffer (0.1 M pH 5.0), 0.1 mL ABTS solution (0.5 mM), and 0.6 mL enzyme solution. ABTS oxidation was measured by monitoring the increase in absorbance at 420 nm.

One enzyme unit was defined as 1.0 μmol of product formed per minute under the assay conditions, obtained from calculations performed from equation 5, derived from the Beer-Lambert Law:

$$\mathrm{U\,L^{-1}} = \Delta \mathrm{A} \times \mathrm{V} \times 10^6 / \varepsilon \times R \times t \qquad (5)$$

ΔA	Difference between the final and initial absorbance
V	Reaction volume (total assay volume)
10^6	Conversion of moles from ε to μmols
ε	Extinction coefficient (M^{-1} cm^{-1})
R	Amount of enzyme in the broth (L)
T	Reaction time (min)

Enzymes expression

The expression of genes, encoding laccase and MnP, was assessed by a semi-quantitative method. The total RNA was obtained from optimized assay (item 1) at times: zero and after second, fifth, sixth, and seventh days. The cells were filtered through cheesecloth to remove any liquid sample. The cells were then transferred to a mortar previously cleaned with 70 % ethanol and NaOH in an ice bath. Then, a small aliquot of liquid nitrogen was added to the cells, and they were macerated with the aid of a pestle. This step was repeated until the formation of a powder (approximately three times). The macerated cells were transferred to polypropylene tubes and stored frozen at −80 °C. RNA extraction was performed using the RNeasy Plant Mini kit (Qiagen) according to manufacturer's instructions. To eliminate contaminating genomic DNA during the extraction, DNase was used for digestion. The extracted RNA was quantified spectrophotometrically (260 nm), and the RNA quality assessed electrophoretically in 0.8 % agarose gel. For synthesis of the first cDNA strand, High Capacity RNA-to-cDNA kit (Applied Biosystems) was used.

Relative expression was performed by PCR using degenerate primer AMP 2'-(5'-ACG AGS TCM TCC GTC TKA CKT TCC-3') and AMP-6R (5'-GTT SAC GAA GCC CTG CCA GAW GC-3') as previously described (Morgenstern et al. 2008), Cu1AF (5'-ACM WCB GTY TGG CAY CAY GG-3') and Cu2R (5'-G CCA GAA GTA GTG RCT NGT NCC-3') as previously described (Kellner et al. 2007) and ITS1-5.8S-ITS2 for normalization (relative activity), as previously described (Sette et al. 2006). The relative levels of mRNA were determined by densitometric quantification using the Image J program of RT-PCR products obtained by electrophoresis on 1.2 % agarose gel stained with ethidium bromide (1 μL.100 mL). The results were expressed in arbitrary units.

Results

Optimization of RB5 dye decolorization by *Peniophora* sp. CBMAI 1063 using experimental design

The influence of 12 independent factors on RB5 dye degradation was investigated using P&B 16, and results are showed in Table S1. The decolorization ranges were from 10 to 70 %, and the optimal result was obtained in assay five, 70 %. The effects of the variables on the response and significant levels ($p < 0.1$) are shown in Fig. 1. Based on the statistical analysis, the factors having the greatest impact were peptone, yeast extract, dye concentration, and $CuSO_4$, all with negative effect.

In order to improve the percentage of dye decolorization as well as to reduce the number of variables and to simplify the cultivation medium, the fungus was subjected to another P&B (matrix 12), where eight variables were evaluated (Table S2), including significant variables in the first matrix (Fig. 1) whose concentrations were reduced, and the dye that was set at the center point (200 mg/L). Some variables that were not significant were set to: pH 6.25,

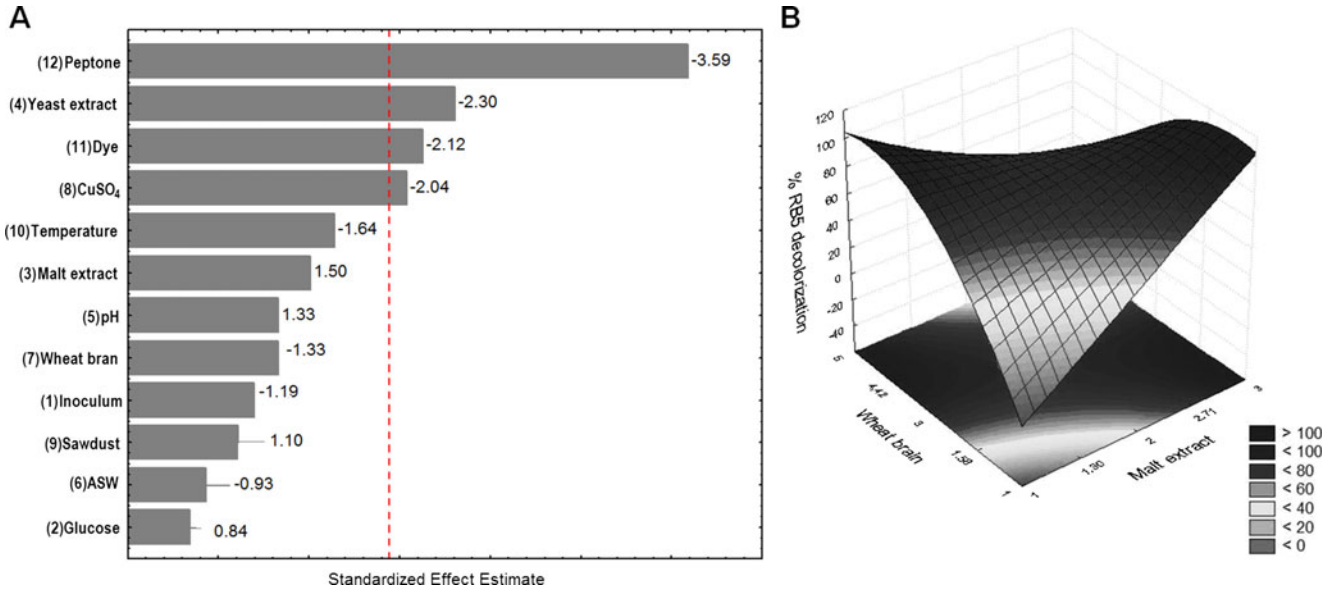


Fig. 1 Results from integrated statistical design. **a** Standardized Pareto chart showing the effects of the 12 independent factors on the RB5 dye decolorization. **b** Response surface curve and contour plots of the quadratic model for RB5 decolorization by *Peniophora* sp. CBMAI 1063

ASW 15 mL (1.2 %), and inoculum (two plugs). Temperature, glucose, malt extract, wheat bran, and sawdust were again evaluated in other zones to confirm their relevance in the study.

In the second P&B, there was an improvement in the percentage of decolorization, where 91 % was achieved in assay three (Supplementary material, Table S2). However, none of the variables were considered significant (date not shown). So, in the design of the next experiment, new values were defined taking into account the culture conditions of the assay that derived the highest rate of decolorization (assay three, Table S2). The variable temperature was set at 28 °C, and the variables yeast extract, peptone, and $CuSO_4$ were removed from the experiment, since the value was zero in assay three (Table S2). Sawdust was also excluded from the study because it is a substrate with large raw material variation, which can lead to changes in the results. Thus, glucose, malt extract, and wheat bran were selected to continue the optimization using a central composite design (CCD).

Results from the first CCD showed that the rate of decolorization was similar to P&B 12 (Supplementary material, Table S3), not reaching the optimal conditions, even though some factors were highly significant (Table 1). Therefore, a new CCD was undertaken removing the variable glucose, which was not significant, and working on a narrower range of the significant variables malt extract and wheat bran. In this second CCD (Table 1), 90 % of decolorization was achieved (assay two and six), and the significant variables were wheat bran quadratic and the interaction of malt extract with wheat bran (Supplementary material, Table S5). By applying multiple regression analysis to the experimental data, the second-order polynomial function was established (Table S5):

$$\text{RB5 decolorization by } \textit{Peniophora} \text{ sp. CBMAI 1063} = 89.91 - 9.99\,\text{wheat bran}^2 - 14.99\,\text{malt extract versus wheat bran} \quad (6)$$

The statistical significance (Supplementary material, Table S6) was checked by an F test (ANOVA). As the F test, value (98.7) for the regression was highly significant (higher than the F tabulated (3.01)), and the percentage of variation explained by the model was suitable ($R^2 = 96$ %), the model could be considered to be predictive and was used to generate a contour plot and response surface (Fig. 1). The results derived from the response surface show that the study was close to the optimization point, working with a maximum of malt extract (3 g/L) and the minimum of wheat bran (x axis) or at the other extreme with a maximum of bran wheat (5 g/L) and malt extract at minimum (y axis).

To confirm the adequacy of the model equations, confirmatory experiments under the optimized condition were carried out. In the model, validation 94 % of RB5 dye was decolorized after 7 days of incubation.

Evaluation of RB5 dye degradation and mutagenicity

Data from LC-MS confirm the reduction of 98 % in the dye concentration after 7 days of fungal incubation, and in the model validation experiment, the absorption spectrum showed that no new peak was formed during the process (Fig. 2). Interestingly, after 24 h of the RB5 addition, the dye concentration was reduced from 200 to 41 mg/L, meaning that

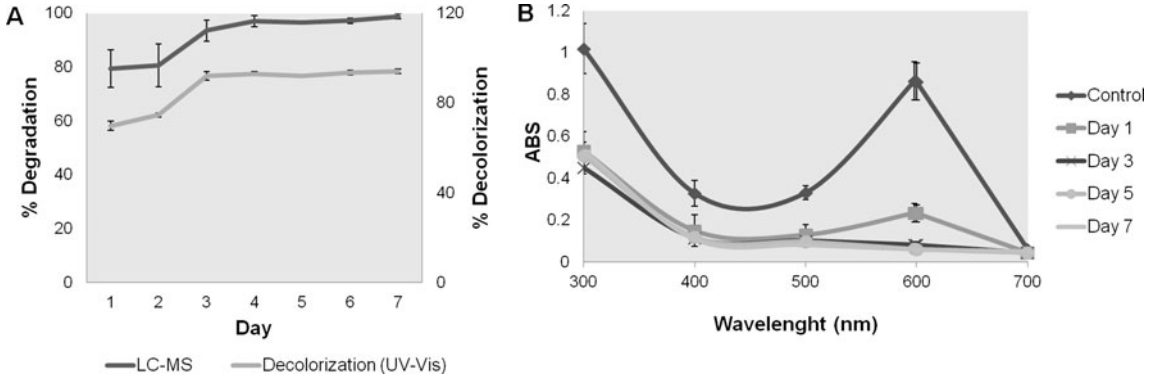


Fig. 2 **a** RB5 decolorization and degradation (LC-MS) percentages after *Peniophora* sp. CBMAI 1063 treatment for 7 days. **b** Absorbance spectrum in the wavelengths 300, 400, 500, 600, and 700 nm after fungal treatment of RB5 for 7 days. *Control line* represents the spectra of dye-containing assay without the fungus

79.5 % of the dye was consumed or biotransformed in the initial process. After 7 days, 57 % TOC reduction was observed (798 mg/L). The TOC of initial medium was around 1838 mg/L (dye TOC ~ 265 mg/L).

LC-MS monitoring identified mainly three metabolites after 7 days with molecular weight: 201, 281, and 439 m/z (Table 2). These metabolites present similar m/z and structure with p-Base and triaminohydroxynaphthalene disulfonic acid (TAHNDS) compounds that show variable molecular weights.

Results from AMES test (Maron and Ames 1983, supplementary material) revealed that no samples showed a mutagenic ratio (value higher than 2) and no statistical differences were obtained in ANOVA test against the negative control. These results indicate that the untreated RB5 dye and its metabolites do not exhibit mutagenic risk even after metabolism by the S9 fraction.

Contribution of MnP and laccase in the RB5 dye decolorization

MnP and Lac (Fig. 3) were produced during the decolorization but LiP was not detected. Lac- and MnP-specific PCR using complementary DNA (cDNA) template from RNA samples after the addition of RB5 dye expression increased, especially MnP (Fig. 3). The level of MnP expression was higher than Lac, and MnP activity was detected from the second day. Lac was detected later, after the fifth day. A decrease of expression and extracellular MnP activity was observed after the fourth day, when the rate of decolorization was stabilized.

The analysis of the active fraction responsible for the dye decolorization "in vitro" (Supplementary material) showed that in conditions where Lac activity was evident, no decolorization was obtained in all of the days evaluated. On the other hand, under the conditions where MnP activity was evident, 73.4, 34.2, and 1.6 % of decolorization were observed on days 3, 5, and 7, respectively, only in the assay with $MnSO_4$ and H_2O_2.

Discussion

Decolorization of RB5 by the marine-derived basidiomycete *Peniophora* sp. CBMAI 1063 could be considered highly promising since the process was efficient and occurred under saline condition. Considering that 92 % of RB5 decolorization was achieved by the basidiomycete *Phanerochaete chrysosporium* in a previous study after 4 days using a lower concentration of the dye (100 mg/L, 50 % less than that used in the present study) and without saline condition (Enayatizamir et al. 2011), the results obtained in the present study demonstrate the potential of fungus from the marine environment for application in saline processes, such as textile

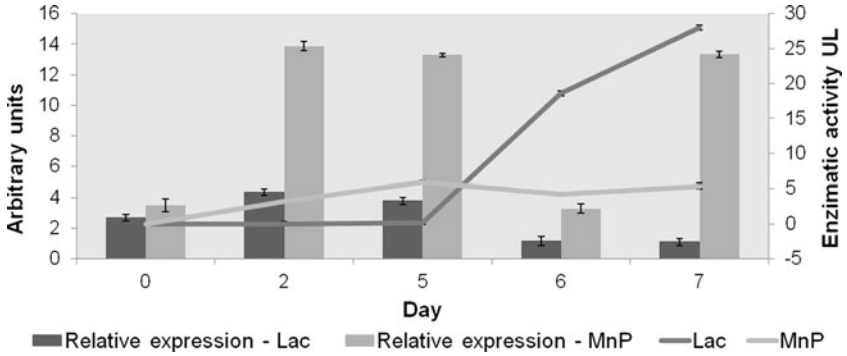


Fig. 3 Relative levels of laccase and MnP genes from *Peniophora* sp. CBMAI 1063 messenger RNA (mRNA) versus extracellular enzymes activities in the RB5 decolorization assay

dyes degradation. The statistical methodology applied allowed a positive result in the optimization of RB5 decolorization by *Peniophora* sp. CBMAI 1063. The application of statistical experimental design techniques in decolorization process can result in improved removal, reduced process variability, closer confirmation of the output response to nominal and target requirements, and reduced development time and overall costs (Srinivasan and Murthy, 2009).

Data derived from AMES test showed that no mutagenic compounds were formed during RB5 dye decolorization by *Peniophora* sp. CBMAI 1063. This result reinforces the potential of this marine-derived fungus for biotechnological application (textile dye degradation), since it was able to highly degrade RB5 dye without generation of dangerous compounds. RB5 dye presented a moderate toxicity rate (using Microtox) even after degradation by the basidiomycete *Trametes versicolor* (Ramsay and Nguyen 2002).

During the process of RB5 degradation, no new peak was observed, indicating that decolorization process by the fungus involves the same biotransformation metabolites. Once the degraded percentage was very similar to the percentage of decolorization, results from degradation by LC-MS actually may represent the decolorization. The degradation occurred in two phases. In the first, the dye concentration decreased rapidly (24 h), and the color of the solution remained purple. In the second, the dye concentration decreased slowly until it became colorless (144 h). According to Ip et al. (2010), complete decolorization involves two steps: (1) reduction of azo bond, resulting in a purple intermediate that still has a remaining azo bond (p-intermediate), and (2) further reduction of p-intermediate azo bond. The first step is much faster than the second one, confirming the profile found in the present study. This two-step decolorization in RB5 is attributed to the reactivity difference between the hydroxyl (−OH) and the amino ($-NH_2$) groups, which are the activating groups of aromatic electrophilic substitution at the ortho position related to the azo bonds (Enayatizamir et al. 2011). TAHNDS and p-Base compounds were identified during RB5 decolorization/degradation. TAHNDS are the main products obtained in a study related to RB5 anaerobic degradation using a bacterial culture and which have also been found in aerobic degradation processes (Rehorek and Plum 2006). These compounds are formed during reduction when cleavage of both azo bridges occurs (Rehorek and Plum 2006). To the best of our knowledge, this is the first report related to TAHNDS metabolites produced by filamentous fungus during RB5 dye degradation. In addition, the TOC analysis confirmed the degradation potential of RB5 by *Peniophora* sp. CBMAI 1063 since approximately 60 % was reduced in only 7 days. Even though results from mutagenicity and LC-MS indicate that RB5 was degraded during the process (7 days), a longer process (assay) would be interesting for TOC stabilization. A higher TOC reduction could be obtained after more than 7 days of incubation.

Ligninolytic enzyme systems, particularly MnP, Lac, and LiP have been considered as the main factors responsible for the decolorization of dyes by white-rot basidiomycetes. As previously discussed, the first stage of RB5 degradation is a result of high oxidative action that leads to the generation of purple color compounds. Based on relative expression, probably this oxidation could be a result derived from the action of MnP, which despite the lower extracellular activity compared with Lac, could directly oxidize this dye by transferring electrons from the protein surface (Heinfling et al. 1997). The analysis of the active fraction responsible for the dye decolorization confirmed this profile. In "in vitro" decolorization conditions, where Lac activity was evident, no decolorization was obtained in any of the days evaluated.

It is important to highlight that the expression of Lac was obtained from the first day, but its extracellular activity was detected only on day 5, suggesting the existence of Lac fractions associated with cells not detected in supernatants (Solé et al. 2008). In this sense, the activity of Lac in decolorization of the RB5 dye cannot be ruled out. However, since the best rate of decolorization was observed between the second and third days (during the peak of expression and activity of MnP), it is reasonable to believe that MnP is more involved mainly in the early stages of RB5 decolorization by the fungus *Peniophora* sp. CBMAI 1063. MnP has been reported as the major enzyme responsible for RB5 and other textile dyes decolorization by various microorganisms (Tang et al. 2011, Yang et al. 2003).

Conclusion

In the present study, integrated statistical design was successfully used to optimize the RB5 dye decolorization by *Peniophora* sp. CBMAI 1063, resulting in a very high rate of dye decolorization and degradation in saline conditions. The methodology provides an attractive solution for the screening and simultaneous optimization of the degradation process. Additionally, this study reports for the first time the metabolites generated during RB5 dye degradation by a basidiomycete fungus and the timing involvement of ligninolytic enzymes, including its gene expressions. The effectiveness of the dye biodegradation by *Peniophora* sp. CBMAI 1063 can also be proven by production of non-mutagenic metabolites, as observed in the AMES assay and by TOC reduction.

Additionally, results from phylogenetic analysis (supplementary material, Figure 4) revealed that ITS sequence of marine-derived *Peniophora* sp. CBMAI 1063 clustered with ITS sequences from fungal isolates from South America and Antarctic continents and formed a group clearly separated from the ITS fungal isolates from North America and Europe, a point to be explored in the sense of diversity and biotechnological potential of this genus.

In conclusion, the polyphasic approach used in this study was successfully applied and highlights the potential of marine-derived fungus *Peniophora* sp. CBMAI 1063 for biotechnological application, stimulating future studies related to the treatment of textile effluent. This fungus could be considered as a target genetic resource for environmental pollutants degradation, especially in usages where land counterparts are deficient, such as in saline processes.

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