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# Relationship of chemical structures of textile dyes on the pre-adaptation medium and the potentialities of their biodegradation by *Phanerochaete chrysosporium*

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**Abstract**

Azo dye derivatives of azobenzene constitute the largest group of dyes used in the textile industry and possess recalcitrant chemical groups, such as those of azo and sulphonic acid. Some microorganisms are able to degrade these aromatic compounds. In the present work, decolourisation of culture media containing azo dyes by the ligninolytic fungus *Phanerochaete chrysosporium* was achieved under nitrogen-limited conditions. The dyes used in the study are derivatives of *meta-* or *para-*aminosulphonic or aminobenzoic acids and include in their structures groups such as guaiacol or syringol, which are bioaccessible to the lignin degrading fungus *P. chrysosporium*. The aim of this study was to pre-adapt the microorganism to the structure of the dyes and to establish the relationships of the chemical structure of the dye present in the pre-adaptation medium with the chemical structure of the dye to be degraded. The azo dye used in the pre-adaptation medium that gave the best overall decolourisation performance was a *meta*-aminosulphonic acid and guaiacol derivative. The azo dye derivative of a *meta*-aminobenzoic acid and syringol showed a better performance in the decolourisation assays. Preliminary GC-MS studies indicated the formation of a nitroso substituted catechol metabolite, a precursor of aromatic ring cleavage, which was confirmed to occur by an enzymatic assay. The presence of this type of metabolite allows the establishment of a possible metabolic pathway towards mineralisation. © 2002 Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

*Keywords:* *Phanerochaete chrysosporium*; Pre-adaptation; Decolourisation; Biodegradation; Dyes chemical structure

## 1. Introduction

The synthesis of azo dyes is very well established and each year new azo dyes are being developed [46]. Coloured effluents from dyestuff and textile industries, the major producers and users of azo dyes, not only produce visual pollution but can also be detrimental to life, as they are usually resistant to biological treatment. Living organisms generally do not synthesize azo linkages and aromatic sulphonic groups and our knowledge about biodegradation of these compounds in nature is limited. Commonly used waste removal treatments do not adequately eliminate many azo dyes from the effluent waters and costly physical-chemical decontamination processes are often the only treatment alternative for such wastewaters. However, different biological effluent treatments have been developed, including the biotreatment of azo dyes [27].

Many microbial strains have been isolated to degrade this kind of aromatic compound [22,24,33,34,41]. Most of the metabolic studies have been limited to bacterial genera; however, since azo dyes are considerably recalcitrant [28, 38] several fungi able to degrade xenobiotics by cometabolic reactions have been studied [15,23,36]. White rot wood basidiomycetes degrade cellulose and lignin, a relatively recalcitrant aromatic polymer, more extensively than any other group of organisms [12,16,20]. The basidiomycete ligninolytic fungus *Phanerochaete chrysosporium* is capable of oxidizing different chemical compounds, degrading several dyes, including azo dyes [6,23,26], and mineralising persistent aromatic pollutants [1,2,11,14,44,45]. The ability to degrade such a diverse group of compounds depends on the non-specific fungal ligninolytic enzymatic system, presenting lignin peroxidases, manganese peroxidases and laccases [4,13,32], although *P. chrysosporium* seems not to be

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a laccase producer [31]. The activities of both peroxidases depend on $H_2O_2$ produced by enzymes such as glyoxal oxidase [17]. Lignin peroxidases activity can often not be detected in the culture supernatants [19], but this could be explained by the presence of proteases as the major cause [3,7, 35] although a minimum of peroxidases activity seems to be enough to obtain considerable percentages of biodegradation or decolourisation [25]. Notwithstanding this, under nutrient and energy-limiting conditions, the different contributions of the pollutant compounds as a carbon source could be related to a clearcut production of catalytic enzymes and a low increase in biomass [42]. Nevertheless, these enzymes are inducible under specific conditions such as low nutrients and the presence of inducer substances [9,16]. Aerobic azo dye biodegradation can also be enhanced, modifying the chemical structures of commercial dyes by linking bioaccessible groups present in the lignin, such as guaiacol or syringol, that seemed to provide an access point for the fungal ligninolytic enzymes [23,29,39], without an appreciable change in their properties as dyes [30]. Furthermore, the influence of electron distribution and charge density and of steric factors has also been pointed to as a possible cause of differing extents of biodegradation [21].

To protect the environment, strategies that extend the range of xenobiotic compounds degraded in wastewater treatment or the capacities of degradation of microorganisms are required. Bearing this in mind, the azo dyes used in the present work include reactive acid groups that provide covalent bonds with the textile fibres, avoiding the loss of large amounts of dyes after the dyeing process, and bioaccessible groups to the enzymatic system of the fungus, guaiacol and syringol. Furthermore, in order to provide new approaches to enhancing the biodegradation of these pollutant compounds, the relationship between the potentialities of degradation of *P. chrysosporium* and the chemical structure of the dyes either in the liquid culture medium or in a pre-adaptation medium used as inoculum source were studied.

## 2. Materials and methods

### 2.1. Azo dyes

Eight azo dyes (Fig. 1) were synthesized by diazotation of *meta*- or *para*-aminobenzoic or aminosulphonic acids, as diazo components and coupling with 2-methoxyphenol (guaiacol) or 2,6-dimethoxyphenol (syringol) as coupling components [23].

### 2.2. Microorganism

*Phanerochaete chrysosporium* Burds, MUM 95.01 used in this study was obtained from Micoteca da Universidade do Minho (MUM) culture collection. Stock cultures were maintained on TWA cellulose medium at 4 °C, with periodic transfer.

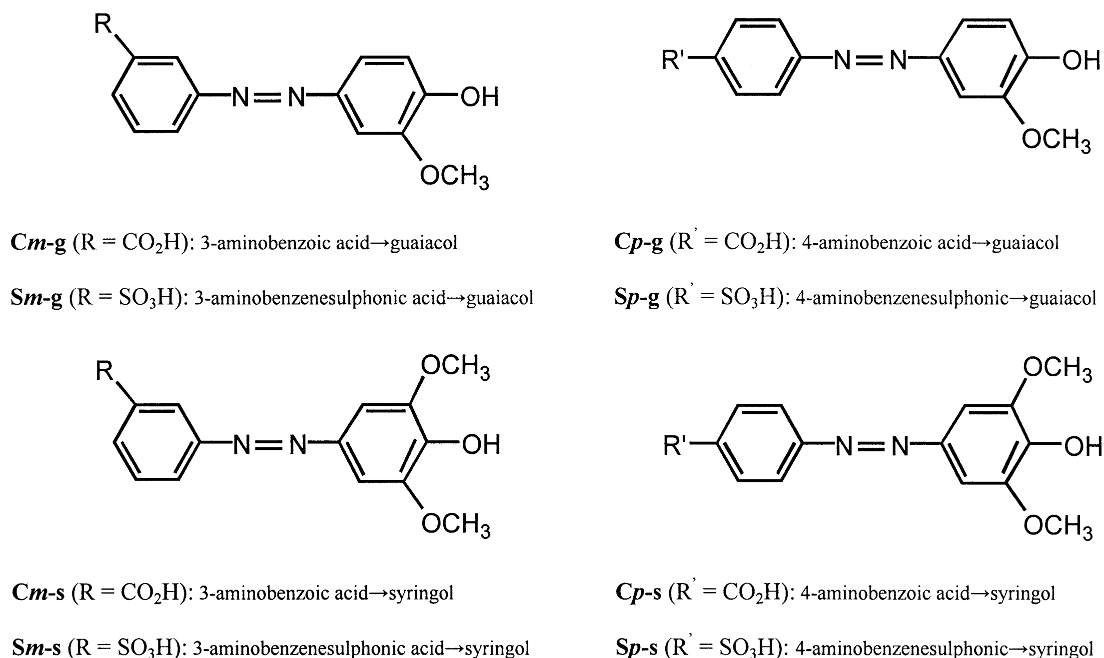


Fig. 1. Structure of the eight synthesized azo dyes. The nomenclature adopted, expressing the diazo component → coupling component, is used in the textile chemistry to suggest the synthesis process. The sigla used refer to the kind of acid (carboxylic – C or sulphonic – S), its position relative to the azo bond (*meta* – *m* or *para* – *p*) and also the coupling component (guaiacol – g or syringol – s). In the present paper, these sigla may be in normal typeface or underlined, referring to the dyes used in the decolourisation assays or the dyes used in the pre-adaptation medium, respectively.

### 2.3. Media

The liquid culture medium, LCM (sucrose 5 g/l, ammonium sulphate 0.5 g/l, YNB – yeast nitrogen base without amino acids and ammonium sulphate 1.7 g/l, L-asparagine 1 g/l and one of the synthesized azo dyes 50 mg/l) was used in all the assays. The final pH was 4.5. Stock solutions of the dyes (100×), YNB (10×) and L-asparagine (10×) were filter-sterilized using 0.45-μm membranes. The pre-growth solid medium used to grow inocula for the experiments (pre-adaptation medium) PAM had the same chemical composition as LCM but was solidified with agar (15 g/l). TWA cellulose, tap water agar (agar 15 g/l in tap water) with a strip of cellulose paper, was used as an inducer medium of the enzymatic system of the fungus.

### 2.4. Culture conditions

An 8-mm diameter plug, cut with a sterile cork borer from the periphery of a 7-day-old colony grown on a TWA cellulose culture plate, was inoculated at the centre of a pre-adaptation culture plate (PAM). Incubation was carried out for 7 days at 30 °C. For the assays, 100 ml of LCM medium in 250 ml flasks were inoculated with five plugs of 8 mm from the periphery of a pre-adaptation culture plate. The liquid cultures were incubated at 30 °C in a Certomat rotary shaker at 150 rpm. Samples were collected every 7 days for a total of 28 days. Controls were carried out in the same conditions but without dyes or inoculum in order to compare, respectively, the variation of biomass and dye concentrations, with the results of the assays. Dead-organism controls, prepared with fungal culture inoculum killed by autoclaving, were also included in the experiments to follow the possible contribution of adsorption dyes to fungal mycelium.

Results presented are the means of at least two independent assays, and in the graphics, vertical error bars indicate the standard deviations from the average.

### 2.5. Dyes, biomass and sucrose concentrations

The concentration of dye in the samples was determined in a spectrophotometer UV/VIS Jasco 7850 ranged from 250 to 500 nm, measuring the decrease in the intensity of absorption at the maximum wavelength ($\lambda_{max}$) of the dye. It was established that the absorbance value at $t_0$ corresponds to 100% of dyes. The spectra were obtained from 1 ml of supernatant samples diluted with 2.5 ml of the appropriate buffer solution of citric acid/sodium hydrogenphosphate, pH 4.4 [23].

Biomass concentration was determined by dry weight measurement after drying fungal cells at 105 °C for 24 h on a 0.45 μm preweighed membrane.

The sucrose concentration was determined colorimetrically, using the sucrose/D-glucose kit/test (Boehringer Mannheim, cat. N° 139041). The kit was used as recommended by the manufacturer with the following modifications: (i) the blank sucrose sample was prepared with the sample solution but without the kit enzyme suspension; (ii) a miniaturized procedure was set up using a 300-μl microwell plate in an ELISA spectrophotometer SLT Spectra; (iii) to obtain the sucrose concentration a standard curve was previously constructed using the same sugar as standard. It was established that the initial sucrose concentration present in LCM corresponded to 100%.

### 2.6. Enzymatic assays

The supernatant of the samples of 1, 3, 5, 7, 14 and 28 days of incubation of the LCM containing C*m*-s was stored at −20 °C until analysed for proteases, ligninolytic enzyme activities and for testing the possible cleavage of catechol.

The *meta*/*ortho* cleavage of the catechol test was based on the detection of the mechanisms of cleavage of the aromatic ring [40]. The reaction mixture contained in about 3 ml: 600 μl of supernatant samples, 200 μl of toluene (Sigma), 200 μl of a 0.1 M solution of catechol (Sigma), 1 ml of citric acid/sodium hydrogenphosphate buffer solution of pH 8.0. This mixture, without a yellow colour characteristic of *meta*-cleavage, was incubated at 30 °C, at 150 rpm, for 1 h; 0.5 g of ammonium sulphate (Merck), 100 μl of a 5 N solution of ammonium hydroxide (Sigma) and 10 μl of a saturated solution of sodium nitroprusside (SNP, sodium nitroferricyanide, Sigma) were added to visualize the possible formation of a purple colour characteristic of *ortho*-cleavage.

All the enzymatic assays were done at room temperature and, determined colorimetrically, using a spectrophotometer UV/VIS Jasco 7850 in the case of the LiP assay, or an ELISA spectrophotometer rainbow Tecan-Sunrise, following a miniaturized procedure that was set up with a 300-μl microwell plate with some adaptations to the references procedures.

The lignin peroxidase, LiP (EC 1.11.1.14) assay was based on the oxidation of veratrylic alcohol by the increase in absorbance at 310 nm [43]. The reaction mixture contained, in 3 ml: 1 ml of supernatant samples, 0.33 ml of 2 mM solution of veratrylic alcohol (3,4-dimethoxybenzylalcohol, Sigma-Aldrich) 0.33 ml of 0.15 g/l 30% solution of hydrogen peroxide (Merck) and, 1.34 ml of citric acid/sodium hydrogenphosphate buffer solution of pH 4.4.

The glyoxal oxidase, GLOX, was based on the oxidation of methylglyoxal [18] by the increase in absorbance at 436 nm. The reaction mixture contained, in 300 μl: 10 μl of supernatant samples, 10 μl of a stock solution (100×) of 2.8 mM guaiacol (Sigma-Aldrich); 10 μl of 1 mg/l solution of peroxidase (Merck), 50 μl of methylglyoxal (acetylformaldehyde, Sigma) and, 120 μl of citric acid/sodium hydrogenphosphate buffer solution of pH 6.0.

The laccase, Lcc (EC 1.10.3.2) assay was based on the oxidation of syringaldazine [10] by the increase in ab-

sorbance at 525 nm. The reaction mixture contained in 300 µl: 10 µl of supernatant samples, 90 µl of a 0.11 mM solution of syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde azine, Sigma) in ethanol absolute (Merck) and, 200 µl of citric acid/sodium hydrogenphosphate buffer solution of pH 6.0.

The Mn-dependent peroxidase, MnP (EC 1.11.1.13) assay was based on the oxidation of MBTH [5] by the increase of absorbance at 590 nm. The reaction mixture contained in 300 µl: 10 µl of supernatant samples, 10 µl of 0.23 g/l 30% solution of hydrogen peroxide (Merck), 120 µl of 0.07 mM solution of MBTH (3-methyl-2-benzothiazoline hydrazone, Sigma), 10 µl of 0.3 mM solution of manganese (Merck) and, 150 µl of citric acid/sodium hydrogenphosphate buffer solution of pH 4.4.

The protease assay was based on the oxidation of azoprotein [37] by the increase in absorbance at 440 nm. The reaction mixture contained in 300 µl: 10 µl of supernatant samples, 30 µl of 2% solution of azoprotein (sulfanylic-acid-azoalbumin, Sigma) in 50–100 mM buffer, 100 µl of a 10% solution of trichloroacetic acid (TCA, Riedel-de-Häen), 160 µl of citric acid/sodium hydrogenphosphate buffer solution of pH 6.0.

For each enzymatic activity assay, the same reaction mixture containing boiled supernatant samples was employed as a blank.

One unit (U) of enzyme activity was defined as the amount of the enzyme that caused a change in absorbance of 0.01 per minute under the assay conditions.

### 2.7. Extraction and derivatization procedures for GC-MS analysis

GC-MS analysis was performed on the biodegradation assay of C*m*-s at day 7, with 400 ml of LCM in a 1000-ml flask, in order to obtain a significant amount of organic extract. The supernatant, after filtration (0.45 µm), was extracted with dichloromethane (3 × 150 ml). The organic extract was dried ($Mg_2SO_4$) and the solvent was removed under reduced pressure.

About 20 mg of the dichloromethane extract were trimethylsilylated according to the literature [8]; the residue was dissolved in pyridine 250 µl and compounds containing hydroxyl and carboxyl groups were converted into trimethylsilyl (TMS) ethers and esters, respectively, by adding bis(trimethylsilyl)trifluoroacetamide (BSTFA) 250 µl and trimethylchlorosilane (TMSCI) 50 µl. After the mixture had stood at 70 °C for 30 min, the derivatized extracts were analysed by GC-MS.

### 2.8. GC-MS analysis

GC-MS analyses were performed using a Trace Gas Chromatograph 2000 Series with a Finnigan Trace MS mass spectrometer and equipped with a DB-1 J&W capillary column (30 m × 0.32 mm i.d., 0.25 µm film thickness). The chromatographic conditions were as follows: 35 cm/s as the carrier gas (He) flow rate; 80 °C as initial temperature for 5 min; 4 °C/min as temperature rate; 285 °C as final temperature for 10 min; 290 °C as injector and transfer-line temperature; 1:100 as split ratio. Compounds were identified, as TMS derivatives, by comparing their mass spectra with the GC-MS spectral library (Wiley-NIST).

## 3. Results

The results obtained in the biodegradation assays were: (i) decolourisation of the LCM (Fig. 2); (ii) disappearance of sucrose after 7 days of incubation where a high percentage

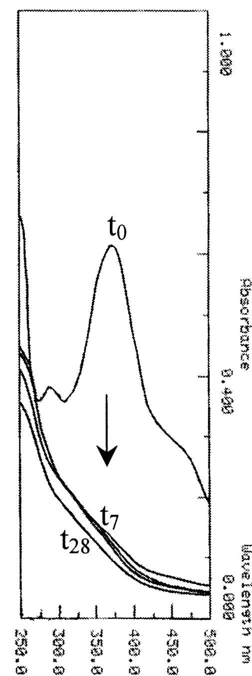


Fig. 2. Decolourisation of the LCM containing S*m*-s dye by *P. chrysosporium* (pre-adapted to S*m*-s dye). UV-vis spectra were recorded every 7 days for 28 days. The arrow indicates the direction of spectrum change.

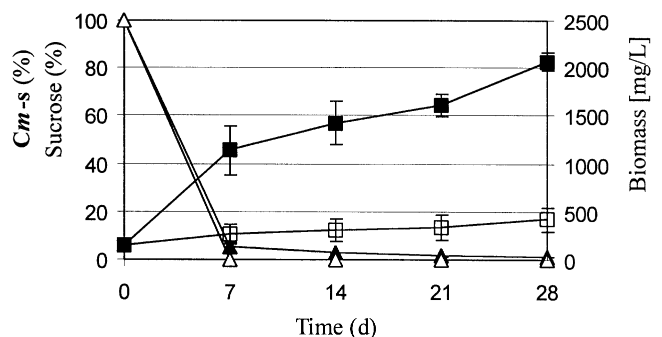


Fig. 3. Time courses of C*m*-s dye (▲), sucrose (△), assay biomass (■) and control biomass (□) concentrations determined during the decolourisation of the LCM by *P. chrysosporium* pre-adapted to the PAM media containing carboxylic dyes with the substituent group in the *meta*-position during the 28 days of incubation.

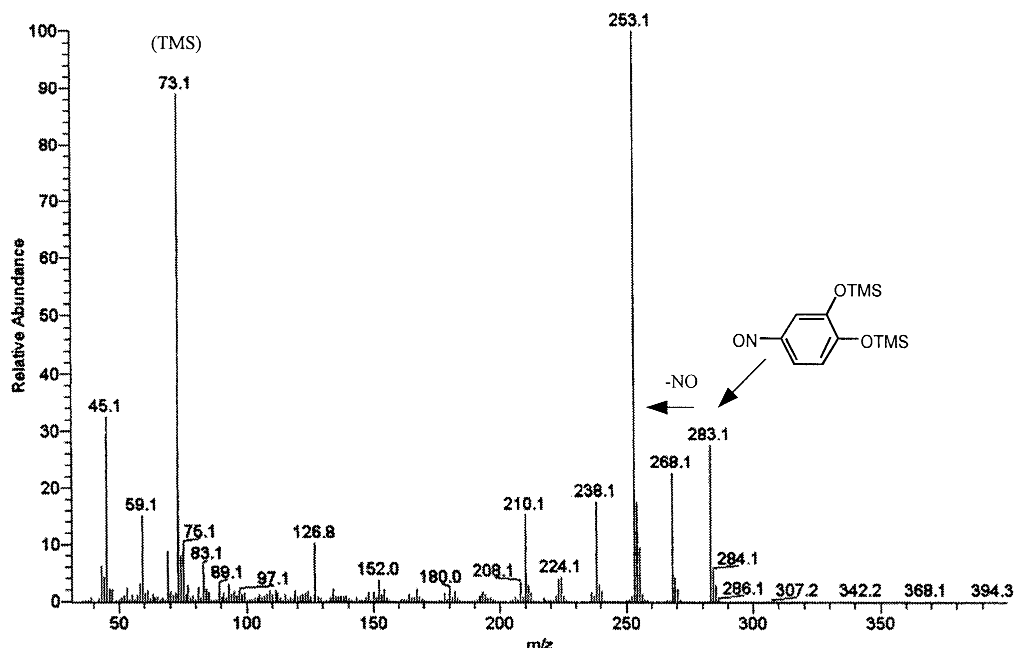


Fig. 4. Mass spectrum of TMS derivative of the nitroso catechol metabolite.

of decolourisation was observed; iii) continuous increase in biomass up until the end of the assay, always showing higher values of biomass concentration than the control without dye (Fig. 3). No significant adsorption of dyes to the biomass was observed using the heat-killed control, and a correlation between the biomass and the decolourisation was established by regression analysis reaching values of $r^2 = 0.99$.

Enzymatic assays performed in the culture supernatants allowed the detection of LiP activity at day 14 (5 U ± 2.1), GLOX residual activity at day 1 (0.04 U ± 0.029) and on the same day protease activity was 3 U ± 0.2, showing a maximum peak of 5 U ± 0.002 at day 14 and 0.3 U ± 0.13 at day 28. MnP and Lcc were never detected.

GC-MS analysis of a 7-day sample extract of *Cm-s* degradation, at which the higher percentage of decolourisation had already occurred, allowed the identification of a key catechol metabolite derivative with a molecular ion at m/z 283 (Fig. 4), having a retention time of 26.86 min, among other peaks corresponding to aromatic compounds which were not unambiguously identified by comparison of their mass spectra with the mass spectral library; some of them showed a base peak corresponding to syringil-TMS-derivative with a molecular ion at m/z 225. Some fatty acids and acylglicerides from the LCM were also identified.

β-ketoadipate resulting from catechol cleavage was detected by the aromatic ring cleavage test in the 14 day sample. A possible metabolic pathway was proposed (Fig. 5).

Notwithstanding this, the importance of the chemical structure of the dye in the LCM and in the PAM was in fact noted. In the LCM, mostly high and rapid percentages of decolourisation were obtained using carboxylic instead of sulphonic dyes (Fig. 6), dyes with substituent groups in the *meta*-position instead of the *para*-position (Fig. 6)

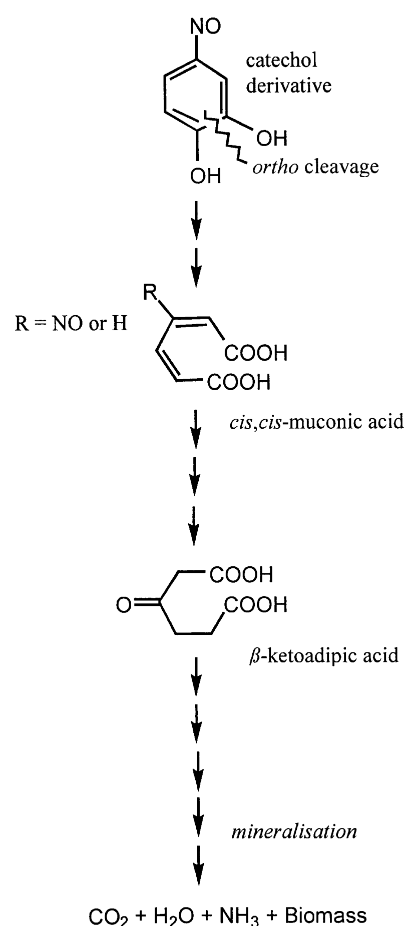


Fig. 5. Proposed metabolic pathway from the catechol derivative towards mineralisation.

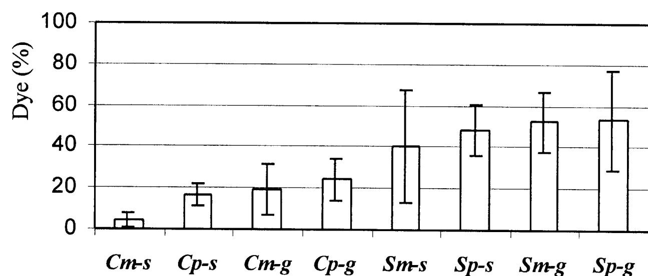


Fig. 6. Decolourisation averages of the LCM media containing each dye, after 28 days of incubation, with the fungus pre-adapted to the PAM medium containing each dye.

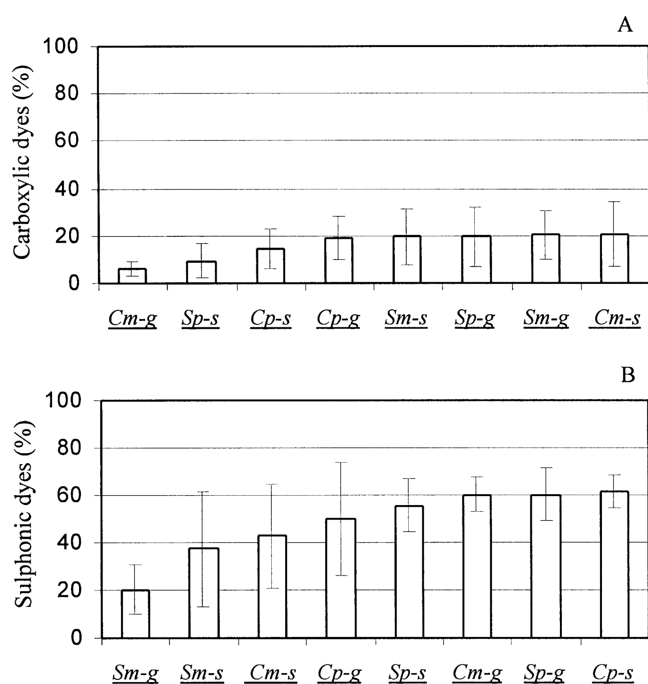


Fig. 7. Decolourisation averages of the LCM media containing the carboxylic (A) or the sulphonic (B) dyes, after 28 days of incubation, with the fungus pre-adapted to the PAM medium containing each dye.

and, dyes with syringol as a coupling component, instead of guaiacol (Fig. 6). LCM containing S*m*-s or C*m*-s, presented the highest percentages of biodegradation, respectively, of the sulphonic and carboxylic dyes.

On the other hand, the chemical structure of the dye present in the PAM was shown to be distinctly important to enhance the percentage of decolourisation of the LCM. Similarities between the chemical structure of the dye present in the PAM and in the LCM resulted in earlier and higher percentages of decolourisation of the latter. PAM containing C*m*-g or S*m*-g were the optimum media for enhancing the percentages of decolourisation, respectively, of the LCM containing carboxylic and sulphonic dyes (Fig. 7). PAM containing S*m*-g was the optimum medium for obtaining higher percentages of decolourisation with lower values of biomass.

## 4. Discussion

The results obtained showed that the decolourisation of the culture medium was related to the biodegradation of the dyes. Growth inhibition by the presence of the dye in the LCM did not occur, and compared with the biomass control without dye, the use of the dye as a carbon source was the cause of the biomass increase (Fig. 3). The detection by GC-MS analysis of aromatic compounds that showed a base peak corresponding to a syringil-TMS derivative clearly demonstrates their relationship with C*m*-s dye biodegradation. Furthermore, the identification of an important metabolite, a nitroso catechol-TMS-derivative (Fig. 4), resulting from oxidative cleavage of the azo bond, together with the enzymatic detection of β-ketoadipate, obtained from *ortho*-cleavage, suggested a metabolic pathway leading to mineralisation (Fig. 5). To confirm this hypothesis, a more detailed GC-MS study is needed at later stages of the assay.

*P. chrysosporium* was able to decolourise the LCM media containing the tested azo dyes, although to different extents. This can be justified by the potentialities of this fungus to utilize a wide variety of carbon sources due to its non-specific enzymatic system, although the structural differences in the dye present in the LCM and in the PAM markedly affect decolourisation. By doing a pre-adaptation of the fungus, the dye in the PAM can be considered an important cometabolic substrate to improve the potentialities of biodegradation of the dye in the LCM. This is supported by results from previous work that showed that in an early stage, sucrose consumption is required for the biodegradation of the dye in the LCM, and the PAM is better than other pregrowth media tested as a source of inoculum [23]. The importance of pre-adaptation may be due to the fact that the enzymes were already produced in the PAM where the fungus had grown in the same chemical composition of the LCM, since it was adapted to degrade the structures of the tested dyes.

The results obtained from enzymatic activity assays confirmed that laccase was not produced by *P. chrysosporium*. The detection of GLOX activity can point to the contribution of this extracellular enzyme, leading to the formation of $H_2O_2$ needed for catalysis by peroxidases. A minimum of peroxidases activity was required to obtain an effective decolourisation of the liquid culture media, although the presence of proteases could be the major cause for no detection of peroxidases activity in the culture supernatants.

In conclusion, to our knowledge, this is the first time that the performance of a pre-adaptation medium using the same fungus and the same selected group of synthesized bioaccessible reactive azo dyes has been studied, showing the importance of the chemical structure either of the dye to be degraded or of the dye to be used in the PAM, and the potentialities of pre-adaptation for enhancing the fungal capacities of biodegradation of these aromatic compounds.

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